

Development of fractionation procedures for comprehensive characterization of organic contaminants in surface waters using effect-directed analyses

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Doctoral thesis / Disertacija

2019

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Science / Sveučilište u Zagrebu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:217:083275>

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University of Zagreb
FACULTY OF SCIENCE

Sanja Bačić

**DEVELOPMENT OF FRACTIONATION
PROCEDURES FOR COMPREHENSIVE
CHARACTERIZATION OF ORGANIC
CONTAMINANTS IN SURFACE WATERS
USING EFFECT-DIRECTED ANALYSES**

DOCTORAL THESIS

Zagreb, 2018



Sveučilište u Zagrebu
PRIRODOSLOVNO-MATEMATIČKI FAKULTET

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**RAZVOJ FRAKCIONACIJSKIH
POSTUPAKA ZA SVEOBUHVAATNU
KARAKTERIZACIJU ORGANSKIH
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VODAMA PRIMJENOM ANALIZA
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Supervisor:
Dr. Marijan Ahel

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UČINCIMA**

DOKTORSKI RAD

Mentor:
Dr. sc. Marijan Ahel

Zagreb, 2018.

This PhD thesis was completed within the Marie Curie ITN-EDA-EMERGE project, under the supervision of Dr. Marijan Ahel, and was supported by the EU Seventh Framework Programme (FP7-PEOPLE-2011-ITN) under the grant agreement number 290100.

Acknowledgements

First of all, I would like to express special gratitude to my supervisor Dr. Marijan Ahel for giving me the chance to join his team, and for his continuous support and guidance throughout my PhD study.

Also, I would like to thank Dr. Senka Terzić, for introducing me with LC-QTOF technique, and for her support and advice regarding the instrument.

I am sincerely grateful to Dr. Tvrtko Smital for the knowledge and advice in ecotoxicology and most important for his support regarding bioassay conduction. I would like to give a special thanks to Dr. Petra Marić for performing the bioassays and providing valuable results that were of critical importance for this research.

I would like to thank all the scientists and fellow researchers involved in EDA EMERGE project for nice collaborative work. Special thanks to Dr. Sélim Ait-Aissa, Dr. Nicolas Creusot and Dr. Petra Spirhanzlova for performing additional bioassays.

I am also grateful to Dr. Werner Brack, Head of the Department of Effect-Directed Analysis at the Helmholtz Centre for Environmental Research in Leipzig, Germany, for giving me the opportunity to work in his laboratory during my one-month secondment, and for valuable knowledge shared with me during the project meetings. I'm very much thankful to Dr. Martin Krauss for his help with chemical analyses during my secondment and to Dr. Tobias Schulze for his support regarding the usage of the large volume SPE device.

I would like to thank all my colleagues from the Laboratory for Analytical Chemistry and Biogeochemistry of Organic Compounds for a warm welcome and pleasant working atmosphere. I enjoyed much working with you. Special thanks go to Iva, Ivona, Ivan, Vedranka and Milan for all good advice and help in the laboratory. I would like to extend gratitude to Nenad Muhin for his valuable help with the sampling and for his pleasant company during the long sampling hours on the Sava River.

Finally, I would like to thank my beloved parents Mira and Branislav, my sisters Andrea and Ena, and my brother Ivan, for unconditional love, support, care and encouragement during my whole life. I'm also so grateful to my husband Niko for his invaluable patience and support, understanding and encouragement during all these years. I would never have been able to do this without all of you, and there are no words that could express my gratefulness.

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Sveučilište u Zagrebu
Prirodoslovno-matematički fakultet
Kemijski odsjek

Doktorska disertacija

SAŽETAK

RAZVOJ FRAKCIONACIJSKIH POSTUPAKA ZA SVEOBUH VATNU KARAKTERIZACIJU ORGANSKIH ZAGAĐIVALA U POVRŠINSKIM VODAMA PRIMJENOM ANALIZA USMJERENIH UČINCIMA

Sanja Bačić

Bijenička cesta 54, 10000 Zagreb, Hrvatska

U ovom radu razvijeni su ekstrakcijski i frakcionacijski postupci za sveobuhvatnu karakterizaciju organskog onečišćenja u površinskim vodama primjenom analiza usmjerenih učincima (engl. Effect-Directed Analysis, EDA). Prilikom razvoja EDA protokola primijenjen je stupnjeviti pristup koji je uključio razvoj jednostavnog protokola, pogodnog za rutinske analize, te protokola za detaljnu višedimenzionalnu frakcionaciju s ciljem konačne identifikacije kritičnih spojeva. Za ekstrakciju organskih zagađivala upotrijebljena je kombinacija triju sorbensa komplementarnih svojstava, a za frakcionaciju su uspoređena tri različita pristupa. Sekvencijalna elucija zagađivala s miješanog sorbensa pokazala se najučinkovitijim pristupom jer uz dobra iskorištenja omogućuje i sustavno razdvajanje zagađivala u karakteristične frakcije prema naboju molekule. Razvijeni postupak primijenjen je za ekotoksikološku karakterizaciju zagađivala u rijeci Savi. Primjenom odabranih bioloških testova detektirani su značajni toksični učinci u rijeci Savi, a ciljane i pretražne kemijske analize ukazale prisutnost velikog broja zagađivala. Kombinacija rezultata bioloških i kemijskih analiza predstavlja važnu osnovu za prioritizaciju organskih zagađivala u bazenu rijeke Save.

(lxx + 167 stranica, 46 slika, 15 tablica, 227 literaturnih navoda, jezik izvornika: engleski)

Rad je pohranjen u Središnjoj kemijskoj knjižnici, Horvatovac 102a, Zagreb i Nacionalnoj i sveučilišnoj knjižnici, Hrvatske bratske zajednice 4, Zagreb.

Ključne riječi: biološki testovi / EDA / frakcionacijski postupci / kemijske analize / organska zagađivala / površinske vode / rijeka Sava

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Rad prihvaćen: 12. srpnja 2018.

Ocjenitelji:

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3. Dr.sc. Tvrtko Smital, zn. savj.



University of Zagreb
Faculty of Science
Department of Chemistry

Doctoral Thesis

ABSTRACT

DEVELOPMENT OF FRACTIONATION PROCEDURES FOR COMPREHENSIVE CHARACTERIZATION OF ORGANIC CONTAMINANTS IN SURFACE WATERS USING EFFECT-DIRECTED ANALYSES

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In this work, a novel extraction and fractionation protocol for comprehensive characterization of organic contamination in surface waters using effect-directed analyses (EDA) was developed. A tiered EDA approach was applied, which included a simple EDA protocol, suitable for routine applications, and a higher-tier protocol for a detailed multidimensional fractionation with a final aim to identify key contaminants. A mixed-bed sorbent, consisting of three complementary phases was applied for the extraction, while three different approaches were applied for the fractionation. Sequential elution was found to be the most efficient approach, which provided good recoveries of as well as successful fractionation various organic contaminants according to charge. The developed EDA procedure was successfully applied for the assessment of organic toxicants in the Sava River. Application of a battery of bioassays showed a significant toxicity of Sava River samples, while target and non-target analyses revealed the presence of numerous contaminant classes. The combination of biological and chemical analyses is an important basis for the prioritization of organic contaminants in the Sava River basin.

(lxx+ 167 pages, 46 figures, 15 tables, 227 references, original in English)

Thesis deposited in Central Chemical Library, Horvatovac 102A, Zagreb, Croatia and National and University Library, Hrvatske bratske zajednice 4, Zagreb, Croatia.

Keywords: bioassays / chemical analyses / EDA / fractionation procedures / organic contaminants / surface waters / Sava River

Supervisor: Dr. Marijan Ahel, Senior Scientist

Thesis accepted: 12th August 2018

Reviewers:

1. Dr. Nives Galić, Professor
2. Dr. Senka Terzić, Senior Scientist
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Sveučilište u Zagrebu
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Kemijski odsjek

Doktorska disertacija

PROŠIRENI SAŽETAK

Otpadne i površinske vode sadrže tisuće zagađivala različitih fizikalno-kemijskih i ekotoksikoloških svojstava. Stoga je procjena mogućih nepovoljnih učinaka koje bi ovi spojevi mogli izazvati u vodenom okolišu vrlo zahtjevan zadatak. Prevladavajući pristup, koji se primjenjuje u procjeni kemijskog stanja voda je određivanje relativno ograničenog broja ciljanih zagađivala pomoću specifičnih metoda optimiziranih za unaprijed odabrana prioritetna zagađivala [M. Carere, S. Polesello, C. Sollazzo and B. M. Gawlik, *TrAC-Trend. Anal. Chem.* **36** (2012) 12–14]. Brojna suvremena literatura pokazala je da ovakav pristup može dovesti do pogrešne procjene onečišćenja okoliša, te je za sveobuhvatnu procjenu kvalitete voda te prioritizaciju novih tipova zagađivala potrebna kompleksnija metodologija [W. Brack, H. J. Klamer, M. Lopez de Alda and D. Barcelo, *Environ. Sci. Pollut. Res. Int.* **14** (2007) 30–38; M. Fuerhacker, *Environ. Sci. Pollut. R.* **16** (2009) 92–97; P. C. von der Ohe, V. Dulio, J. Slobodnik, E. De Deckere, R. Kühne, R.-U. Ebert, A. Ginebreda, W. De Cooman, G. Schüürmann and W. Brack, *Sci. Total. Environ.* **409** (2011) 2064–2077]. Nasuprot ciljanim analizama, koje se primjenjuju u monitoringu prioritetnih zagađivala, razvijen je pristup koji se naziva analiza usmjerena učincima (prema engl. *Effect-Directed Analysis*, EDA). Taj pristup omogućava da se spregom specifičnih bioloških metoda za praćenje različitih učinaka i analitičko-kemijskih tehnika za identifikaciju pojedinačnih toksikanata povežu uočeni štetni učinci s kemijskim spojevima koji ih izazivaju. EDA-pristup se sastoji od tri odvojene faze: karakterizacije toksičnih učinaka, identifikacije toksičnosti te potvrde dobivenih nalaza. Da bi se taj pristup mogao provesti na uzorcima okoliša, koje u pravilu karakterizira kompleksan sastav prisutnih zagađivala, svi EDA protokoli uključuju detaljnu frakcionaciju ekstrakata, koja se može odvijati u jednom ili više koraka, te analizu dobivenih frakcija primjenom odgovarajućih kemijskih i bioloških metoda. Jednu od prvih studija primjenom EDA pristupa proveli su Schuetzle i Lewtas [D. Schuetzle and J. Lewtas, *Anal. Chem.* **58** (1986) 1060a–

1075a] za identifikaciju mutagenih spojeva u različitim matricama poput dima cigareta, pitke vode, ispušnih plinova dizel-motora te raznih proizvoda široke potrošnje. Kasnije je EDA pristup primijenjen i za identifikaciju toksičnih spojeva u tlu, sedimentu, otpadnoj, površinskoj te podzemnoj vodi. Krajem osamdesetih godina prošlog stoljeća Agencija za zaštitu okoliša Sjedinjenih Država (engl. *United States Environmental Protection Agency*, US EPA) standardizirala je tzv. TIE protokol (engl. *Toxicity Identification Evaluation*) za procjenu onečišćenja vodenog okoliša organskim i anorganskim zagađivalima [D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase I: Toxicity Characterization Procedures*. US Environmental Protection Agency, Environmental Research Laboratory, National Effluent Toxicity Assessment Center, 1988]. Ključni elementi za sveobuhvatan i uspješan EDA pristup su: sveobuhvatna ekstrakcija, dobro osmišljene frakcionacijske tehnike, pravilan odabir biotestova koji pokrivaju sve glavne tipove očekivanih toksičnih učinaka (engl. *endpoints*), te rutinski primjenjiva kemijska analiza.

Ekstrakcija zagađivala iz okolišnih uzoraka prvi je korak u EDA i ključan je za uspješnu identifikaciju bioloških efekata te pouzdanu procjenu rizika. Zbog relativno niskih koncentracija zagađivala u vodenom okolišu, vodene uzorke je potrebno koncentrirati odgovarajućim postupcima. U cilju smanjenja potrošnje organskih otapala ekstrakcija tekuće-tekuće (engl. *Liquid-Liquid Extraction*, LLE) u novije vrijeme zamijenjena je sa ekstrakcijom na čvrstoj fazi (engl. *Solid Phase Extraction*, SPE). Danas su na tržištu dostupni brojni SPE materijali koji imaju sposobnost selektivnog vezivanja spojeva koji se određuju. Za ekstrakciju lipofilnih spojeva iz tekućih okolišnih uzoraka najčešće se koriste punila temeljena na silikagelu modificiranom s oktilnim i oktadecilnim skupinama (C8 ili C18) te umreženi polimerni materijali kao što je polistiren-divinilbenzen [L. M. Hewitt and C. H. Marvin, *Mutat. Res.* **589** (2005) 208–232; F. Kummrow, C. M. Rech, C. A. Coimbrao, D. A. Roubicek and A. Umbuzeiro Gde, *Mutat. Res.* **541** (2003) 103–113]. Posljednjih desetak godina, veliku popularnost stekli su kopolimerni sorbensi mješovitog tipa koje karakterizira hidrofilno-lipofilna ravnoteža (engl. *Hydrophilic-Lipophilic Balance*, HLB). Takvi sorbensi omogućuju visoku učinkovitost ekstrakcije za spojeve vrlo širokog raspona polarnosti [T. Smital, S. Terzic, R. Zaja, I. Senta, B. Pivcevic, M. Popovic, I. Mikac, K. E. Tollefsen, K. V. Thomas and M. Ahel, *Ecotox. Environ. Safe.* **74** (2011) 844–851; M. Farré, G. Klöter, M. Petrovic, M. C. Alonso, M. J. L. de Alda and D. Barceló, *Anal. Chim. Acta* **456** (2002) 19–30]. Uvođenjem anionskih ili kationskih skupina na HLB polimere dodatno se može povećati efikasnost

ekstrakcije ioniziranih polarnih spojeva. Nedavno su razvijeni sorbensi na bazi ugljika poput aktivnog ugljena (engl. *graphitized carbon black*, GCB) koji su posebno pogodni za ekstrakciju polarnih spojeva iz tekućih uzoraka. Da bi se postigla maksimalna učinkovitost ekstrakcije za široki raspon organskih zagađivala iz okolišnih uzoraka, uključujući i anionske i kationske spojeve, kolonice za SPE se mogu puniti smjesom različitih sorbensa (engl. *mixed-bed multilayer SPE*). Tako su Huntscha i sur. [S. Huntscha, H. P. Singer, C. S. McArdell, C. E. Frank and J. Hollender, *J. Chromatogr. A.* **1268** (2012) 74–83] koristili kolonice punjene s četiri različita sorbensa: Oasis HLB, Strata XAW, Strata XCW i Isolute ENV+, kako bi kombiniranjem različitih mehanizama, uključujući hidrofobne interakcije i ionsku izmjenu, povećali iskorištenja za različite tipove ispitivanih organskih zagađivala. Za ekstrakciju zagađivala iz vodenih uzoraka, drugi autori su primijenili tzv. sekvencijalnu ekstrakciju uz upotrebu serijski vezanih sorpcijskih kolonica [T. Reemtsma, O. Fiehn and M. Jekel, *Fres. J. Anal. Chem.* **363** (1999) 771–776; M. Castillo and D. Barceló, *Anal. Chim. Acta* **426** (2001) 253–264].

Jedan od najkritičnijih koraka za uspješnu EDA studiju je pravilan odabir biotestova. Biotestovi koji se primjenjuju u EDA studijama trebaju pokrivati različita mjerila toksičnosti, kako bi obuhvatili što širi opseg potencijalnih toksičnih efekata pri čemu prednost imaju metode koje su prikladne za istovremenu analizu velikog broja uzoraka. Ciljano testiranje toksičnosti provodi se primjenom bioloških metoda *in vitro* ili *in vivo* na različitim nivoima biološke organizacije, pri čemu su od posebnog interesa učinci na vodene organizme, posebno ribe [C. Di Paolo, T.-B. Seiler, S. Keiter, M. Hu, M. Muz, W. Brack and H. Hollert, *Environ. Sci. Eur.* **27** (2015) 8]. Pored tradicionalnih mjerila toksičnosti koje se ispituju biotestovima poput preživljavanja, rasta i reprodukcije, u literaturi su opisani brojni biotestovi kojima se određuje hormonalna aktivnost (anti-/estrogenost, anti-/androgenost, anti-/tiroidna aktivnost, aktivnost glukokortikoida), neurotoksičnost, oksidativni stres ili genotoksičnost organskih spojeva prisutnih u okolišu [N. Creusot, H. Budzinski, P. Balaguer, S. Kinani, J.-M. Porcher and S. Aït-Aïssa, *Anal. Bioanal. Chem.* **405** (2013) 2553–2566.; S. C. Van Der Linden, M. B. Heringa, H.-Y. Man, E. Sonneveld, L. M. Puijker, A. Brouwer and B. Van Der Burg, *Environ. Sci. Technol.* **42** (2008) 5814–5820; R. E. Connon, J. Geist and I. Werner, *Sensors* **12** (2012) 12741–12771; V. Jálková, B. Jarošová, L. Blaha, J. Giesy, T. Ocelka, R. Grabic, J. Jurčíková, B. Vrana and K. Hilscherova, *Environ. Int.* **59** (2013) 372–383].

Nakon biološke analize ekstrakata okolišnih uzoraka, uzorci koji pokazuju značajnu toksičnu aktivnost se dalje frakcioniraju u jednom ili više koraka kako bi se lakše identificirali spojevi koji su odgovorni za uočeni toksični učinak. S obzirom da u većini studija EDA protokol za tekuće uzorke započinje ekstrakcijom na čvrstoj fazi, ovaj korak ujedno može omogućiti inicijalnu frakcionaciju [W. Brack, *Anal. Bioanal. Chem.* **377** (2003) 397–407]. Inicijalna frakcionacija može se postići i sekvencijalnom eluacijom oktadecilnih ili polistiren-divinilbenzenskih SPE kolonica [D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase I: Toxicity Characterization Procedures*. US Environmental Protection Agency, Environmental Research Laboratory, National Effluent Toxicity Assessment Center, 1988; K. Thomas, M. Hurst, P. Matthiessen, D. Sheahan and R. Williams, *Water Res.* **35** (2001) 2411–2416] te primjenom otapala rastuće hidrofobnosti. Međutim za frakcionaciju ukupnog ekstrakta te finu frakcionaciju dobivenih ekstrakata najčešće se koriste HPLC sustavi obrnutih faza, obično uz primjenu stacionarne faze C18. Kao pokretne faze, za frakcioniranje spojeva prema njihovim raspodjelnim koeficijentima (K_{ow}) najčešće se koriste linearni gradijenti metanola i vode [R. Urbatzka, A. Van Cauwenberge, S. Maggioni, L. Viganò, A. Mandich, E. Benfenati, I. Lutz and W. Kloas, *Chemosphere* **67** (2007) 1080–1087] ili acetonitrila i vode [I. Bobeldijk, A. Brandt, B. Wullings and T. Noij, *J. Chromatogr. A.* **918** (2001) 277–291]. Frakcionacija primjenom HPLC obrnutih faza može biti upotpunjena prethodnom primjenom kromatografije normalnih faza [S. A. Snyder, D. L. Villeneuve, E. M. Snyder and J. P. Giesy, *Environ. Sci. Technol.* **35** (2001) 3620–3625]. Kromatografija isključenjem (engl. *Size-Exclusion Chromatography*, SEC) ili gelpropusna kromatografija (engl. *Gel Permeation Chromatography*, GPC) kod kojih se separacija izvodi na temelju razlike u veličini/obliku molekula, je također jedna od često upotrebljivanih frakcionacijskih tehnika za uklanjanje elementarnog sumpora, dugolančanih alifatskih spojeva, humusnih tvari ili makromolekula poput lipida i proteina [W. Brack, *Effect-directed analysis of complex environmental contamination*, Springer Science & Business Media, 2011].

Kemijska analiza zagađivala prisutnih u okolišnim uzorcima predstavlja veliki analitički izazov, ne samo zbog različitih fizikalno-kemijskih svojstava spojeva već i zbog općenito niskih koncentracija analita, te složenosti matrice. Najčešće primjenjivane tehnike koje se koriste za kemijsku identifikaciju nepoznatih spojeva prisutnih u okolišnim uzorcima su vezani sustavi plinske kromatografije i spektrometrije masa (GC-MS), te tekućinske

kromatografije i spektrometrije masa (LC-MS) [I. Heisterkamp, J. Gandrass and W. Ruck, *Anal. Bioanal. Chem.* 378 (2004) 709–715; K. Thomas, K. Langford, K. Petersen, A. Smith and K. Tollefsen, *Environ. Sci. Technol.* **43** (2009) 8066–8071]. Dvodimenzionalna plinska kromatografija (GCxGC) također je našla primjenu u separaciji i identifikaciji hlapljivih organskih spojeva u složenim okolišnim uzorcima [X. Ouyang, P. Leonards, J. Legler, R. van der Oost, J. De Boer and M. Lamoree, *J. Chromatogr. A.* **1380** (2015) 139–145].

Cilj ovog rada je razvoj postupaka za sveobuhvatnu karakterizaciju organskog onečišćenja voda s posebnim naglaskom na poboljšanje protokola za ekstrakciju i frakcionaciju organskih zagađivala. U tu svrhu istraženi su postupci za ekstrakciju i frakcionaciju većeg broja reprezentativnih tipova organskih zagađivala primjenom različitih sorpcijskih mehanizama. Sveobuhvatna kemijska karakterizacija provedena je primjenom vezanih sustava LC-MS i GC-MS, a analiza realnih uzoraka je upotpunjena primjenom različitih biotestova u integriranom EDA protokolu. Razvijeni pristup primijenjen je za identifikaciju organskih zagađivala u rijeci Savi.

Za istraživanje učinkovitosti ekstrakcijskih i frakcionacijskih postupaka odabrana je modelna smjesa koja sadrži 96 organskih spojeva širokog raspona kemijskih struktura i kategorija zagađivala. Dio spojeva odabran je s liste prioritetnih zagađivala prema Okvirnoj direktivi o vodama (engl. *Water Framework Directive*, WFD), dok su drugi odabrani na temelju prethodnih studija na rijeci Savi [T. Smital, S. Terzic, R. Zaja, I. Senta, B. Pivcevic, M. Popovic, I. Mikac, K. E. Tollefsen, K. V. Thomas and M. Ahel, *Ecotox. Environ. Safe.* **74** (2011) 844–851; T. Smital, S. Terzić, J. Lončar, I. Senta, R. Žaja, M. Popović, I. Mikac, K.-E. Tollefsen, K. V. Thomas and M. Ahel, *Environ. Sci. Pollut. R.* **20** (2013) 1384–1395] na temelju njihove relativno visoke zastupljenosti i/ili mogućeg doprinosa ukupnim biološkim učincima. Odabirom je pokriven širok raspon različitih fizikalno-kemijskih svojstava spojeva, poput hlapljivosti, hidrofobnosti i topljivosti u vodi. Analiti u modelnoj smjesi uključuju klasične hidrofobne spojeve (npr. policiklički aromatski ugljikovodici, poliklorirani bifenili), spojeve srednje polarnosti (npr. pesticide) te polarne spojeve (npr. farmaceutske spojeve, ilegalne droge, tenzide), uključujući neutralne i ionizirane hidrofilne spojeve. Razvijena su i uspoređena tri različita protokola za inicijalnu frakcionaciju potencijalno prikladnih za tzv. pojednostavljeni EDA protokol (prema engl. *simplified EDA*): 1) frakcionacija ukupnog SPE ekstrakta na silikagelu (separacija analita na temelju polarnosti), 2) sekvencijalna elucija kolonica punjenih s tri različita sorbensa, neutralnim (kopolimer polistiren-divinilbenzena PS-

DVB; HR-X), anionskom smolom na bazi polistiren-divinilbenzena (HR-XAW) te kationskom smolom, također na bazi polistiren-divinilbenzena (HR-XCW) (separacija analita u 3 frakcije na temelju naboja, koristeći neutralne, kisele i bazične eluense) 3) sekvencijalna ekstrakcija primjenom serijski vezanih kolonica punjenih sa neutralnim sorbensom, anionskim i kationskim smolama (s ciljem separacije prisutnih organskih zagađivala na neutralnu, kiselu i bazičnu frakciju tijekom same ekstrakcije).

Frakcionacija na stupcu silikagela pokazala se učinkovitom za izolaciju nepolarnih spojeva pogodnih za GC-MS analize. Nepolarni spojevi poput policikličkih aromatskih ugljikovodika te polikloriranih bifenila detektirani su u prvoj (nepolarnoj) frakciji, potpuno odvojeno od srednje polarnih, te polarnih spojeva. Ukupni analitički povrati policikličkih aromatskih ugljikovodika s četiri i više aromatskih prstenova bili su u rasponu od 46 % do 116 %. Međutim, određeni analitički povrati za policikličke aromatske ugljikovodike manjih molekularnih masa, te za neke poliklorirane bifenile bili su dosta niži, uslijed gubitaka tijekom uparavanja do suha i izmjene otapala. Također, ovaj se postupak nije pokazao učinkovitim za frakcioniranje polarnih ioniziranih spojeva. Dobiveni niski analitički povrati polarnih spojeva ukazuju na ireverzibilnu adsorpciju polarnih spojeva na stupcu silikagela, prije nego na nepotpunu eluaciju. Drugi su autori također objavili rezultate koji pokazuju da su silikagelske nepokretne faze općenito nepovoljne za razdvajanje polarnih spojeva, prije svega zbog dobivanja niskih analitičkih povrata [S. K. Poole, T. A. Dean, J. W. Oudsema and C. F. Poole, *Anal. Chim. Acta* **236** (1990) 3–42; T. Reemtsma and M. Jekel, *J. Sep. Sci.* **17** (1994) 589–592]. Frakcionacija tijekom oba postupka ekstrakcije na čvrstoj fazi pokazala je jasne prednosti u separaciji polarnih spojeva, te dobivanju zadovoljavajućih analitičkih povrata. Srednje polarni spojevi poput pesticida detektirani su u prvoj (neutralnoj) frakciji, a dobiveni analitički povrati bili su u rasponu od 69 % do 110 %. Analitički povrati ioniziranih hidrofilnih spojeva poput nekih ilegalnih droga te farmaceutskih spojeva, dobiveni sekvencijalnim eluiranjem kolonica punjenih miješanim sorbensom i primjenom sekvencijalne ekstrakcije bili su slični (32 - 101 % i 32 - 108 % za ilegalne droge, 47 - 114 % i 32 - 110 % za farmaceutske spojeve). Međutim, primjena sekvencijalne eluacije kolonica punjenih miješanim sorbensom pokazala se znatno učinkovitijom u separaciji neutralnih i ioniziranih polarnih spojeva. Na primjer, primjenom sekvencijalne ekstrakcije s serijski vezanim kolonicama sve ilegalne droge detektirane su u prvoj, neutralnoj frakciji, dok je sekvencijalno eluiranje kolonica tijekom ekstrakcije na čvrstoj fazi omogućilo odjeljivanje

bazičnih droga u drugoj (bazičnoj) frakciji. Zanimljivo je da se analizirani kationski tenzidi nisu ponašali kao tipični kationi, već su bili podijeljeni tijekom fracioniranja između sve tri frakcije. Polarni neionski tenzidi poput polietilen glikola i polipropilen glikola detektirani su u prvoj (neutralnoj) frakciji, potvrđujući efikasnost separacije analita na temelju naboja. Očekivano, primjenom sekvencijalne elucije kolonica punjenih miješanim sorbensom kao i sekvencijalne ekstrakcije, hidrofobni spojevi poput policikličkih aromatskih ugljikovodika te polikloriranih bifenila detektirani su u prvoj frakciji. Međutim, kao i u slučaju silikagelske separacije analitički povrati policikličkih aromatskih ugljikovodika manjih molekulskih masa, bili su niži (< 30 %) uslijed gubitaka tijekom procesa ekstrakcije i uparavanja.

Pojednostavljeni EDA protokol primijenjen je za analizu uzoraka površinske vode sustavno prikupljene na odsječku rijeke Save na širem području Zagreba. U tu svrhu odabrane su četiri lokacije: Samoborski Otok, Podsused, Oborovo i Sisak Crnac. Odabirom lokacija obuhvaćeni su najvažniji poznati unosi komunalnih i industrijskih otpadnih voda. Uzorkovanje je provedeno korištenjem specijalnog uređaja koji omogućava istovremenu SPE ekstrakciju velikih uzoraka (50-100 L) *in situ*. Uređaj je opremljen s tri serijski vezane SPE kolone napunjene s tri različita sorbensa: neutralnim sorbensom polistiren-divinilbenzenskog tipa (HR-X), anionskom smolom na bazi polistiren-divinilbenzena (HR-XAW) te kationskom smolom, također na bazi polistiren-divinilbenzena (HR-XCW). Na taj način omogućena je maksimalna retencija širokog spektra spojeva prisutnih u vodi te sekvencijalna ekstrakcija i frakcionacija u neutralnu, kiselu i bazičnu frakciju. Ciljane analize prikupljenih uzoraka obuhvatile su velik broj kemijskih spojeva svrstanih u različite kategorije zagađivala (151), uključujući 55 farmaceutskih spojeva, 43 pesticida, 11 tenzida i transformacijskih produkata, policikličkih aromatskih ugljikovodika, usporivača gorenja, inhibitora korozije, sredstva za zaštitu od sunca i umjetnih zaslađivača, koji pokrivaju široki raspon fizikalno-kemijskih svojstava. Od ukupno 151 ciljanih spojeva, 63 spoja je detektirano najmanje na jednoj lokaciji. Očekivano, najmanji broj detektiranih spojeva, te najniže koncentracije izmjerene su u uzorku prikupljenom na referentnoj lokaciji Samoborski Otok. Najčešće detektirana kategorija spojeva u uzorcima rijeke Save bili su pesticidi. Međutim, izmjerene koncentracije pesticida u analiziranim ekstraktima rijeke Save bile su relativno niske. To se može objasniti činjenicom da je uzorkovanje bilo provedeno tijekom kolovoza i rujna, što nije glavna sezona primjene pesticida. Najviše koncentracije pesticida određene su za herbicid izoproturon (140 ng/L), te biocid triklosan (73,5 ng/L) u uzorku prikupljenom na lokaciji Oborovo. Među 18

farmaceutskih spojeva detektiranih iznad granice određivanja, najviše koncentracije izmjerene su na lokaciji Podsused za makrolidne antibiotike azitromicin (370 ng/L) i eritromicin (86 ng/L), dok je najviša koncentracija klaritromicina (48,5 ng/L) određena u uzorku prikupljenom na lokaciji Oborovo. Koncentracije nonilfenola detektiranom u svim uzorcima kretale su se u rasponu od 17 ng/L do 25 ng/L. Nonilfenoksiocena kiselina također je detektirana u svim analiziranim uzorcima s maksimalnom određenom koncentracijom od 12 ng/L (Samoborski Otok i Oborovo). Najzastupljeniji prirodni estrogen detektiran u uzorcima Save bio je estron (E1) sa najvišom izmjerenom koncentracijom u ekstraktu prikupljenom na lokaciji Oborovo (1,28 ng/L), dok su najviše koncentracije mjerene za prirodne glukokortikoidne spojeve kortizon i hidrokortizon bile 1,8 ng/L i 2,56 ng/L. Ostali spojevi detektirani u ekstraktima riječne vode pripadaju različitim kategorijama zagađivala poput usporivača gorenja, plastifikatora, sredstva za zaštitu od sunca, zaslađivača, repelenata itd. Dobivene frakcije istovremeno su testirane su biotestovima *in vivo* i *in vitro* kako bi se odredila moguća prisutnost toksičnih tvari. Baterijom biotestova pokriveni su glavni tipovi toksičnih učinaka uključujući: citotoksičnost (MTT test), fitotoksičnost (inhibicija rasta algi), embriotoksičnost (toksičnost za embrije riba *Danio rerio*), neurotoksičnost (inhibicija acetilkolinesteraze), mutagenost (*Ames* test), anti-/estrogenost (ER posredovana aktivnost), anti-/androgenost (AR- posredovana aktivnost), glukokortikoidna aktivnost (GR CALUX test) i anti-/tiroidna aktivnost (*in vivo* test tiroidne aktivnosti). Baterija biotestova omogućila je detekciju niza učinaka relevantnih za površinske vode. Najčešće uočeni toksični učinci u uzorcima bili su estrogenost, akutna toksičnost embrija riba zebrica te inhibicija rasta algi, dok je glukokortikoidna aktivnost izmjerena samo u uzorku prikupljenom na lokaciji Oborovo. Većina biološke aktivnosti zabilježena je u neutralnim ekstraktima, dok su u bazičnih ekstraktima učinci rijetko detektirani. U kiselim ekstraktima nije zabilježena biološka aktivnost. Najviša estrogena aktivnost izmjerena je u uzorku prikupljenom na lokaciji Oborovo. Do 35 % estrogene aktivnosti u uzorcima može se objasniti prisustvom triju spojeva uključujući bisfenol A, estron te nonilfenol, dok se 29 % glukokortikoidne aktivnosti može objasniti prisustvom prirodnog hormona hidrokortizona. Uzorci prikupljeni na lokacijama Samoborski Otok, Podsused i Oborovo bili su embriotoksični za ribe zebrice. Iznenađujuće, najveća embriotoksičnost izmjerena je u ekstraktu prikupljenom na referentnoj lokaciji Samoborski Otok gdje je ciljanim analizama detektirano samo 36 spojeva sa maksimalnom koncentracijom od 80 ng/L. Neale i Escher, 2014 [P. A. Neale and B. I. Escher,

Chemosphere **108** (2014) 281–288] su pokazali da ko-ekstrahirani otopljeni organski ugljik također može imati toksičan učinak, te utjecati na rezultate biotestova. Nonilfenol, nonilfenoksiocetna kiselina te oktilfenol (detektirani na lokaciji Oborovo), farmaceutski spojevi poput azitromicina (detektiran na lokaciji Samoborski Otok i Podsused), eritromicin (detektiran na lokaciji Podsused i Oborovo), te različiti pesticidi uključujući diazinon, fipronil i klorpirifos (detektirani na lokaciji Oborovo) također mogu djelomično doprinijeti toksičnosti ekstrakata. Umjerena toksičnost na alge izmjerena je u uzorcima prikupljenim na lokacijama Samoborski Otok, Podsused i Oborovo, sa najvišom inhibicijom rasta algi mjenom na lokaciji Podsused. Tang i Escher, 2014 [J. Y. Tang and B. I. Escher, *Environ. Toxicol. Chem.* **33** (2014) 1427–1436] su pokazali da su herbicidi glavni inhibitori rasta alga u otpadnim i površinskim vodama. Ciljane analize ekstrakata Save ukazale su također na prisustvo nekoliko herbicida uključujući izoproturon, atrazin, atrazin-desetil, terbutilazin, diuron te biocid triklosan.

Kako bi se izvršila detaljnija identifikacija spojeva koji u složenim okolišnim uzorcima dovode do specifičnog toksičnog učinka, provedena je detaljna EDA studija. Na temelju prethodnih rezultata odabrane su tri lokacije na rijeci Savi, uključujući Podsused, Oborovo i Hruščicu, te tri dodatna ispusta otpadne vode u rijeku Savu, uključujući efluent sa uređaja za obradu otpadnih voda grada Velike Gorice, Zaprešić, te kanal Hruščica. Ciljanim analizama obuhvaćen je prošireni broj zagađivala relevantnih za površinske vode. Od ukupno 226 analiziranih spojeva, 30 spojeva detektirano je na lokaciji Zaprešić, 69 spojeva na lokaciji Podsused, 110 spojeva na lokaciji kanal Hruščica, 72 spoja na lokaciji Hruščica, 106 spojeva u sekundarnom efluentu uređaja za obradu otpadnih voda u Velikoj Gorici, te 62 spoja na lokaciji Oborovo. Najčešće detektirana kategorija spojeva bili su farmaceutski spojevi, zatim različite klase pesticida, plastifikatori, hormoni, usporivači gorenja, perfluorirani spojevi, te drugi industrijski spojevi. Rezultati kemijskih analiza upotpunjeni su rezultatima procjene ekotoksičnog potencijala ukupnih ekstrakata primjenom baterije biotestova (MTT, AlgaeTox, EROD, ER-posredovana aktivnost, AR- posredovana aktivnost, ChgH-gfp medaka test). Estrogena aktivnost izmjerena je u svim ispitivanim uzorcima. Biološka aktivnost izražena u ekvivalentima estradiola (engl. *estradiol equivalents*, E2-EQs) bila je u rasponu od 0,06 ng/L do 36,02 ng/L. Očekivano, najveća aktivnost izmjerena je u uzorcima otpadne vode, posebno u uzorku prikupljenom na uređaju u Velikoj Gorici. Izmjerena estrogena aktivnost u efluentu bila je mnogo viša u usporedbi s drugim studijama o otpadnim vodama [W. Körner, U. Bolz,

W. Süßmuth, G. Hiller, W. Schuller, V. Hanf and H. Hagenmaier, *Chemosphere* **40** (2000) 1131–1142.; T. Smital, S. Terzic, R. Zaja, I. Senta, B. Pivcevic, M. Popovic, I. Mikac, K. E. Tollefsen, K. V. Thomas and M. Ahel, *Ecotox. Environ. Safe.* **74** (2011) 844–851]. Ove razlike mogu nastati uslijed razlika u sastavu neobrađenih otpadnih voda, ali i različite učinkovitosti uklanjanja zagađivala na uređajima za pročišćavanje otpadnih voda. Rezultati *in vivo* ChgH-gfp medaka testa pokazali su da je jedini estrogeni uzorak bio uzorak otpadne vode prikupljene na uređaju u Velikoj Gorici (21,6 ng/L E2-EQ). Androgena aktivnost nije izmjerena niti u jednom analiziranom uzorku (ukupnom ekstraktu). Rezultati MTT testa kojim se mjerila akutna toksičnost uzoraka riječne i otpadne vode pokazali su slab toksični učinak uzoraka otpadne vode prikupljene na uređaju za obradu otpadnih voda u Velikoj Gorici, te riječne vode prikupljene u Oborovu, dok je akutna toksičnost uzoraka prikupljenih na lokacijama Zaprešić, Podsused, kanal Hruščica i Hruščica bila ispod granica određivanja metode. Kronična citotoksičnost određivana primjenom AlgaeTox biotesta izmjerena je u svim analiziranim uzorcima, osim u uzorku prikupljenom u Zaprešiću što je posljedica izuzetno visoke razine razgradljive organske tvari u tom uzorku. Uzorak prikupljen na lokaciji Oborovo pokazao se najtoksičnijim za alge, čak toksičnijim od otpadne vode prikupljene u Velikoj Gorici. EROD test je ukazao na slab CYP1A indukcijski potencijal određen u ukupnim ekstraktima riječne vode, dok su očekivano povišene vrijednosti EROD indukcijskog potencijala izmjerene u otpadnim vodama. Izuzetak je uzorak prikupljen u Zaprešiću, kod kojeg je EROD indukcijski potencijal bio ispod granice određivanja.

Na temelju rezultata kemijskih i bioloških analiza ukupnih (nefracioniranih) ekstrakata riječne i otpadne vode, ukupni ekstrakt uzorka otpadne vode iz Velike Gorice te ekstrakt uzorka riječne vode prikupljene u Oborovu, odabrani su za finu frakcionaciju primjenom semi-preparativne tekućinske kromatografije visoke djelotvornosti (HPLC) uz primjenu kromatografije obrnutih faza. Finom frakcionacijom dobiveno je 37 frakcija koje su dalje analizirane vezanim sustavom LC-MS (pretražne analize) te odabranim biotestovima (MTT, AlgaeTox, EROD, ER-posredovana aktivnost, AR- posredovana aktivnost). Općenito, frakcionacija ukupnih ekstrakata otkrila je značajne inhibitorne efekte u ukupnim ekstraktima. Na primjer, suma E2-EQ vrijednosti mjerene u individualnim frakcijama uzorka Oborovo bila je viša nego u ukupnom ekstraktu. Također, rezultati mjerenja androgene aktivnosti frakcioniranih uzoraka otpadne vode Velike Gorice otkrili su nekoliko veoma aktivnih frakcija. Ovi rezultati ukazuju na važnost frakcionacije uzoraka radi prevencije dobivanja

lažno negativnih rezultata, što se često može dogoditi u složenim uzorcima poput otpadnih voda.

Pretražne analize biološki najaktivnijih frakcija uz upotrebu sustava tekućinske kromatografije vezane na spektrometar masa visoke rezolucije, ukazale su na dominantno prisustvo velikog broja homologa i oligomera različitih grupa tenzida i njihovih metabolita, uključujući anionske (linearne alkil benzenesulfonate, sulfofenil karboksilne kiseline, alkilsulfate, alkileter sulfata itd.) i neionske tenzide (polietilen glikole, alkohol polietoksilate, polietilenglikol dimetiletere itd.). Općenito, tenzidi su slabo do umjereno toksični za vodene organizme [G.G. Ying. *Environ. Int.* **32** (2006) 417–43], međutim s obzirom na njihove visoke koncentracije u riječnoj vodi, koje su često i do tisuću puta više od koncentracija klasičnih hidrofobnih zagađivala, postoji potreba za preispitivanjem ekološkog rizika za ovu kategoriju sveprisutnih okolišnih zagađivala. Također, skorije *in vitro* studije su pokazale da tenzidi mogu povećati toksičnost drugih zagađivala [C.A. Harris, P. Henttu, M.G. Parker and J.P. Sumpter. *Environ. Health Perspect.* **105** (1997) 802–811], najvjerojatnije zato jer mogu olakšati transport zagađivala kroz staničnu membranu [A. Hellenius and K. Simons, *Biochim. Biophys. Acta.* **415** (1975) 29–79] ili pak povećati njihovu bioraspodajivost [D.E. Kile and C.T. Chiou, *Environ. Sci. Technol.* **23** (1989) 832–838].

Iako je EDA pristup za procjenu zagađenja u okolišnim uzorcima već neko vrijeme usvojen u literaturi kao sustavan način za identifikaciju kritičnih organskih zagađivala, dosad publicirani protokoli su prikladni samo za uski raspon kemijskih spojeva te često ne pružaju uvid u polarnu frakciju. U ovom radu razvijeni su postupci za ekstrakciju i frakcionaciju organskih zagađivala u vodenim uzorcima koji mogu poslužiti za razvoj sveubuhvatnijih protokola koji su primjenjivi za sustavnu karakterizaciju uzoraka riječne vode. Pri tome posebna pozornost posvećena je razvoju pojednostavljenog protokola koji omogućava širu primjenu EDA pristupa. Primjenom razvijenih protokola na rijeku Savu dobiveni su novi uvidi o relativnoj važnosti pojedinih tipova zagađivala te data je osnova za regionalnu prioritizaciju organskih zagađivala i optimalno gospodarenje vodama.

§ 1. INTRODUCTION

Wastewater and surface water samples contain extremely complex mixtures of thousands of individual contaminants having different chemical properties and ecotoxicological characteristics. Despite increasing efforts to regulate their emission, the number of chemicals that are released into the environment is constantly increasing.¹ About 10^8 chemicals are indexed in the American Chemical Society's Chemical Abstracts System.² In 2005, about 9×10^6 chemicals were commercially available,³ while in typical environmental samples several thousands of compounds are detectable. Therefore, the assessment of hazardous chemical contamination in the aquatic environment is a challenging task.

Until the beginning of the 1990's, persistent non-polar hazardous compounds and heavy metals were in the focus of interest and awareness as priority pollutants and, consequently, they were subject to intensive monitoring programs and ecotoxicological studies. Moreover, a drastic reduction of their emissions has been achieved over the past decades due to the adoption of appropriate measures and elimination of the dominant pollution sources.⁴ However, owing to recent advances in environmental analytical chemistry, in particular the advent of liquid chromatography-mass spectrometry, the focus has been increasingly shifted to so-called "emerging contaminants" which have been recognized as a potential environmental problem. There is a widespread consensus that this kind of contamination requires legislative intervention. The term "emerging contaminants" is used to cover not only the newly developed compounds but also compounds newly discovered in the environment, often due to analytical developments, and compounds that have only recently been recognized for their ecotoxic properties and categorised as contaminants of environmental concern.⁵ They include a wide array of different compounds (as well as their metabolites and transformation products), such as human and veterinary pharmaceuticals, personal care products, pesticides, surfactants, industrial compounds, flame retardants, plasticizers, sweeteners, food additives, as well as engineered nano-materials. The main sources of emerging contaminants are direct discharge of industrial and domestic wastewaters, discharge of wastewater treatment plant (WWTP) effluents, agricultural drains to streams and rivers and overland flow after rainfall events. These contaminants do not need to be persistent in the environment to cause negative effect since their high

transformation/removal rates can be compensated by their continuous introduction into the environment.⁴ Therefore, one of the major challenges in the assessment of complex environmental mixtures is the identification of those chemicals that contribute significantly to observed effects. Numerous studies have shown that a reliable risk assessment cannot be achieved by simple monitoring a limited selection of pre-defined priority pollutants and indicated that a characterization should include a more systematic approach, which includes consideration of biological effects. In contrast to target analyses, which are applied in the monitoring of mandatory priority contaminants, effect-directed analysis (EDA) has been developed as a conceptual framework that integrates biological and chemical tools in order to link effects to causative substances. In this manner, EDA studies provide the insight into the relative significance of different contaminant categories, thus creating a basis for pollutant prioritization.

The aim of this thesis was the development of protocols for comprehensive characterization of organic pollutants in the aquatic environment, with a special emphasis on the improvement of extraction and fractionation procedures. Improved procedures for the extraction and fractionation of a wide range of representative water contaminants were developed by combining different sorption mechanisms. A comprehensive chemical characterization was achieved by combination of hyphenated techniques, including gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS), while chemical analyses were complemented with a battery of bioassays integrated in the well-defined EDA protocol. The developed EDA protocols were applied for the assessment of hazardous contaminants in the Sava River basin.

§ 2. LITERATURE REVIEW

2.1. Background

A large number of potentially toxic pollutants represent a major challenge for the efficient and reliable assessment of environmental risks associated with ever increasing pollution of aquatic systems with chemical substances having anthropogenic origin. However, only a small number of these chemicals is monitored and regulated⁶ under the assumption that these chemicals are responsible for the most significant risk to ecosystem and/or human health.³ In Europe, the protection of natural water resources is regulated by the Water Framework Directive (WFD)⁷ and the Groundwater Daughter Directive to WFD⁸ that are implemented in European member states legislations and international river basin management. The monitoring and regulation of the chemical status of surface and ground waters refer to the priority substances listed in WFD and amended by the GWD and the Environmental Quality Standards (EQS) Directive.^{6,9} The prevailing approach, applied in the assessment of the chemical status of a water body, is still the determination of relatively limited number of target contaminants using specific methods, optimized for the pre-selected priority chemicals of concern.¹⁰ Recently, there has been an accumulating evidence in the literature, indicating that such an approach can lead to erroneous assessment of the chemical status, which suggests that comprehensive assessment of water quality and pollutant prioritisation requires a more complex methodology.^{11,12,13} Even though non-target analysis of complex mixtures allows the detection and identification of a broad range of compounds, it is time consuming and not routinely applicable. The results are often difficult to evaluate, since toxicological data of the compounds identified are missing. Toxicity assessment using biotests is an established alternative for hazard assessment of complex environmental mixtures. Using biotests, *a priori* knowledge of the key contaminants is not necessary and interactive toxicity among the components is reflected by the results. However, biotesting alone does not provide information on the compounds causing the measured effects and is therefore not a sufficient basis for risk reduction measures such as remediation or emission control. Since comprehensive chemical monitoring of all environmental chemicals together with the enormous number of transformation products appears to be impossible, integrated approaches

are required to reduce the complexity of possible or actual environmental contamination, while limiting the chance of overlooking significant contributors to risks and effects.

To meet this challenge, in the early 1980s, in contrast to target analyses, which are applied in the monitoring of mandatory priority contaminants, researchers came up with a new approach called effect-directed analysis (EDA) that integrates biological and chemical tools in order to link effects to causative substances in complex mixtures. The basic assumption behind EDA is that although ecosystems and humans are exposed to complex mixtures of compounds, only few toxicants dominate adverse effects.¹ Early studies applying this new approach were reported by Schuetzle and Lewtas.¹⁴ Since then, EDA has continuously advanced and has frequently been applied to toxicant identification in air particulate matter, soils, sediments, effluents, surface water and groundwater. In the late 1980s, the US Environmental Protection Agency (EPA) developed a scientific concept and a regulatory approach called Toxicity Identification Evaluation (TIE) for characterization and identification of chemicals in toxic effluents causing effects to aquatic organisms.¹⁵ This approach has been reviewed from different perspectives, involving also studies performed under the numerous synonyms (e.g. bioassay-guided fractionation, activity-guided fractionation etc.) that have been historically used in the literature, and the major areas of application include drug discovery, toxicology, forensics, and environmental sciences, all evolving through a different history, with differing nomenclature and focus, but a common general approach and methodology.¹⁶

In environmental studies, both EDA and TIE have been applied, with the aim to identify bioactive chemicals in complex mixtures by a step-by-step toxicity assessment of environmental samples. TIE focuses on the characterization of unmodified water and sediment samples and involves whole organisms to detect toxicity and ecological relevance as a primary goal, while in EDA studies the main goal is structural identification of bioactive chemicals and the assessment of their contribution to overall endpoint-specific activity.¹⁷ EDA focuses on the analysis of organic chemicals, typically using solvent extracts of environmental compartments, exposed organisms or technical products tested with *in vitro* or small scale *in vivo* assays. In both protocols, it is assumed that there are specific drivers of adverse effects of complex mixtures, which might be individual compounds or mixtures of a small number of chemicals.¹⁷ TIE protocol comprises three separate phases including: I) Toxicity characterization by assignment of toxicity to general groups of toxicants such as

heavy metals, non-polar organics or ammonia, II) Identification of suspected toxicants and III) Confirmation of the suspected cause of toxicity.¹⁸ In EDA protocol, phase I measures the bioassay response of the entire extract or fraction, in the phase II the activity assessment and chemical identifications are performed in the fractions showing toxic response, and in the phase III the identified compound is tested for the confirmation step.¹⁹

Numerous studies increasingly rely on advanced EDA concepts by using multiple endpoint bioassay batteries in combination with fractionation techniques followed by confirmatory targeted chemical analysis. The application of EDA has proved to be a valuable approach in investigating the chemical nature of the compounds responsible for various toxic effects in environmental samples, and represents a most promising tool for the risk assessment of complex environmental matrices.

2.1.1. Principle of the effect-directed analysis

The aim of the EDA approach is the identification of chemical causes of biological effects, i.e. the establishment of cause-effect relationships. EDA procedure is based on biotesting of complex environmental mixtures in combination with a sequential reduction of mixture complexity by different fractionation protocols (Figure 1). The procedure is directed by the bioassays, aiming to remove compounds without significant contribution to sample toxicity and isolating and identifying predominant toxicants.

The first step of EDA is the extraction of toxicants from complex matrices, which is already a selective step, as it is focused on the extraction of organic compounds, excluding the heavy metals and inorganic anions from the further process. This step is followed by testing of the extracts with appropriate bioassays in order to screen the toxicity of the tested sample. In most cases, short-term cell-based *in vitro* or *in vivo* assays (human or animal cell lines, bacteria) or small organisms (e.g. Zebrafish embryos) are used to limit the amount of extract required.²⁰ If the tested samples show adverse effects in the selected bioassays, they are subjected to a detailed single- or multi-step fractionation to reduce the complexity of the extracts to well-defined fractions, containing a limited number of candidate compounds, which improves the possibility of unraveling their chemical identity. Furthermore, elimination of non-toxic compounds during the fractionation minimizes the appearance of synergistic, antagonistic or additive toxicity that often occur in such complex mixtures.²¹ After each

separation step the fractions are biotested for selection of active fractions for further investigation. When the complexity of the mixture is reduced to a group or few individual compounds, the fractions are subjected to chemical identification and quantification by complementary analytical techniques such as gas or liquid chromatography coupled with mass spectrometry (GC-MS, LC-MS). Finally, confirmation of identified key pollutants can be achieved by using the respective analytical standards after evaluating their chromatographic behavior (chemical confirmation) and toxic potencies in the bioassays (toxicological confirmation). This confirmation step evaluates how much of the initially observed toxicity in the sample can be attributed to the mixture of identified toxicants.²²

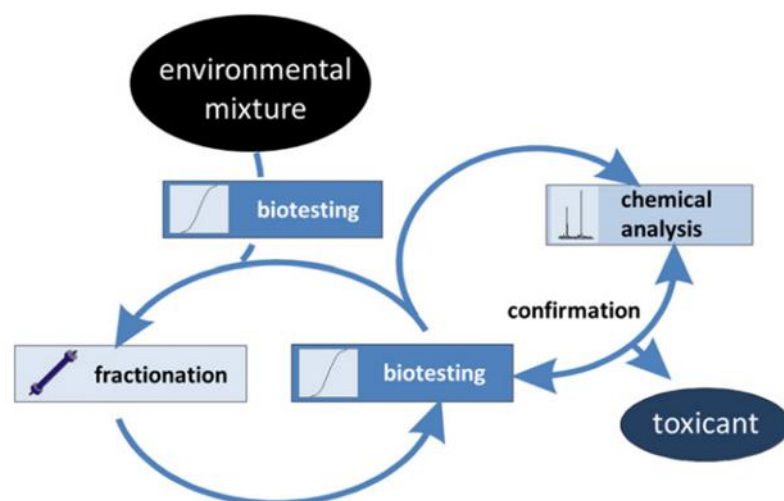


Figure 1. Scheme of effect-directed analysis of complex mixtures.¹⁶

Therefore, the key elements for a comprehensive and successful EDA procedure are: comprehensive extraction procedure, well-designed fractionation techniques, selection of a battery of high-throughput screening bioassays covering major endpoints, and routinely applicable chemical screening. In the following sections, the typical elements of EDA will be discussed separately.

2.2. Sampling strategies and extraction techniques in effect-directed analysis

2.2.1. Sampling strategies

Composition of environmental samples is rather complex and usually exhibits significant temporal and spatial variability.¹⁶ Different sampling strategies are therefore required to assure representativeness of the sample.

These strategies vary from a simple grab sampling at a specific time point to event-based sampling and time-integrated sampling. The grab sampling is the simplest and the cheapest sampling strategy, however it is also the least representative as it does not reflect temporal fluctuations and provides only a “snap shot” of pollution at a specific location. Therefore it should only be used when other sampling strategies cannot be applied due to time, costs or capacity restrictions.¹⁶

Time-integrated sampling is used for characterization of the average contamination over a longer period. It may be applied with either *in situ* or post-sampling enrichment. Time-integrated sampling with post-sampling enrichment may require the installation of automatic samplers that will enable time- or flow-weighted average over the duration of the sampling event. This sampling strategy is widely applied for collecting time-proportional or flow-proportional composite wastewater samples in wastewater treatment plants.²³ After sampling, samples are usually transferred to the laboratory for further enrichment using solid phase extraction (SPE) prior to fractionation and biological and chemical analysis. Alternatively, time-integrated sampling with *in situ* enrichment can be applied to prevent logistical, technical, economic and scientific issues related to the storage and transport of large volumes of water to the laboratory. However such approach requires more complex automated on-site sampling equipment in order to allow efficient and successful real time collection and extraction of large volumes of water. Alternatively, *in situ* enrichment may be achieved using passive sampling with a broad range of available devices. The available time-integrated sampling approaches can be carried out over days or weeks to yield highly representative samples of a given water body.²⁴

The main advantages of passive samplers are high capacity, time integrated response, and versatility for the monitoring of different chemical groups or types of water, autonomy, as

no external source of energy is required, and easy deployment.^{25,26} Passive samplers can detect contaminants at ng/L to mg/L levels and have been deployed for the monitoring of different groups of organic contaminants in environmental waters, such as flame retardants, polycyclic aromatic hydrocarbons (PAHs) and pharmaceuticals. There are several types of passive samplers used in environmental applications that provide specific advantages: semi-permeable membrane devices (SPMD),^{27,28} polar organic chemical integrative sampler (POCIS),^{29,30} and ceramic dosimeters.³¹ The uptake of the contaminants is usually achieved by means of sorption/binding onto a media which has specific retention properties. The properties that govern the uptake of chemicals by a passive sampler are usually polarity, water solubility and lipophilicity of target pollutants, and its performance depends on the type of water to be monitored and the sampler receiving phase.³²

Different approaches and devices for active sampling of large volume water samples have been developed.^{33,34} However, for analytical purposes many of the devices were best suited for low water volumes. An alternative time-integrated approach for water sampling applying *in situ* enrichment is on-site active large volume solid phase extraction (LVSPE), which was recently developed by Schulze et al.³⁵ The device is designed for sampling of 50 L - 1000 L of water. It enables collection and pre-concentration of large samples for effective assessment and chemical analyses, as well as sequential extraction of large volumes of water with different adsorbents in a compact cartridge system.

Event-based sampling is the approach based on the assumption that the exposure to environmental contaminants cannot be always sufficiently defined by long-term average concentrations alone but the periodical peak concentrations have to be taken into account.³⁶ Peak concentrations may occur during heavy rain events that cause run-off of pesticides and contaminated soil particles from agricultural land, as well as during the snow melt.^{37,38,39,40} Accidental spills of toxicants from industry⁴¹ could also cause ecotoxicologically significant peak events. Using grab sampling but also using time-integrated sampling with longer subsampling intervals, there is a high chance of overlooking short term peak concentrations, especially for compounds with an expected intermittent release.

2.2.2. *Enrichment of water samples using solid phase extraction*

Extraction of contaminants from environmental samples is the first stage of EDA and is a crucial step for successful detection of biological effects and accurate risk assessment. This step assures that the composition of contaminant compounds in the organic extract to be subjected to further fractionations corresponds to the original water sample.⁴² Moreover, due to the low environmental concentrations, the analysis of water samples will typically not result in observable effects unless the organic contaminants present in the sample are efficiently pre-concentrated during the extraction. In EDA, this step is frequently a compromise between removal of matrix compounds and pre-concentration of the compounds of interest that exhibit a broad range of physico-chemical properties.

Contaminated waters, such as industrial effluents, landfill leachates, ground and surface waters, may contain an enormous variety of organic pollutants which cannot be extracted by a single method.¹⁸ There are various methods available for the extraction of contaminants from aqueous samples, however, not all of them are suitable for EDA studies. Nowadays solid-phase extraction (SPE) is the most widely used technique for the concentration of environmental contaminants and the removal of interfering compounds from aqueous samples. In comparison to previously used liquid/liquid extraction (LLE) technique, SPE provides numerous advantages, such as low solvent consumption, low costs and reduction of processing time. Moreover, it is possible to automate the whole process. SPE has favorable properties for field sampling, eliminating the need to transport and store bulk samples for processing by the receiving laboratory.⁴³ While for grab sampling this enrichment step is performed in the laboratory, on-site active sampling (e.g. on-site LVSPE) integrate sampling and water extraction in one single step.¹⁶

In SPE, the analytes to be extracted are partitioned between a solid and a liquid phase, while the analytes must have a greater affinity for the solid phase than for the sample matrix. Compounds retained on the solid phase can be removed by eluting with a solvent with a greater affinity for the analytes. The most common retention mechanisms are based on van der Waals forces (non-polar interactions), hydrogen bonding, dipole-dipole forces (polar interactions), and cation-anion interactions (ionic interactions).

In order to cover compounds ranging from non-polar to polar and/or negatively or positively charged compounds at certain pH ranges, careful selection of the right SPE

procedure is pivotal.⁴⁴ However, most of the available extraction methods focus on specific ranges of lipophilicity. Until 1997, most EDA studies were focused on lipophilic compounds, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and some pesticides. Nowadays, a wide range of SPE materials suitable to cope with an increasing variability of organic compounds is available on the market. Octyl (C8) and octadecyl (C18) phases, as well as polystyrene-divinylbenzene XAD resins, have proven to be quite useful for extraction of lipophilic compounds from aqueous samples.^{45,46} Since many polar and non-polar polyaromatic compounds with three or more fused rings are mutagenic, a selective extraction method for these compounds from aqueous environments with respect to mutagenicity identification was developed using so-called blue rayon sorbent.⁴⁷ The sorbent consists of a blue pigment, copper phthalocyanine trisulfonate, which is covalently bonded to a fiber, rayon. This sorbent is commonly used in the field to monitor mutagens in surface waters.⁴⁸ Within the last 10 years, in order to extract compounds with wider range of hydrophobicity, additional phases, such as polymeric hydrophilic–lipophilic balance (e.g. Oasis HLB) and polystyrene-based polymers (e.g. Isolute ENV and Strata X),^{23,49} have been increasingly used. The introduction of anion (e.g. Oasis WAX, Strata XAW) or cation exchange sorbents (e.g. Oasis WCX, Strata XCW), as well as a mixed-mode, reversed-phase/strong cation/anion-exchange polymers (e.g. Oasis MCX, Oasis MAX), additionally increased the recovery of some categories of polar organic contaminants. Carbon-based sorbents, which include graphitized carbon black (GCB) and porous graphitic carbon (PGC), are also available. Many examples illustrate the various common features of graphitized carbons, such as the extraction of very polar analytes and multiresidue extractions.⁵⁰ Due to its structures, these sorbents have effectively unique properties in retention, as they behave as normal phase (NP), reversed-phase (RP) and ion exchanger sorbents. However, desorption problems have been encountered with GCB cartridges: pure MeOH, acetonitrile or methylene chloride were shown to be unable to desorb some pesticides as well as other organic pollutants, so that a mixture of methylene chloride – MeOH (80:20, v/v) was recommended. It was also pointed out that residual water had to be reduced to a minimum. Otherwise, low and irreproducible recoveries were obtained, because the water can hinder intimate contact between the desorption mixture and the GCB.⁵⁰

Several authors reported the use of multiple-step SPE procedures in order to achieve a versatile and broad extraction, since a single extraction under neutral or acidic conditions is

limited to hydrophobic or only weakly polar compounds. This results in more flexible methods that can easily be extended to new emerging polar contaminants, eliminating the need to develop multiple analytical methods for the same compound classes.⁵¹ In order to ensure an exhaustive extraction for a wide range of compounds, including ionized polar compounds, Huntscha et al.⁵¹, used four mixed sorbents packed in a column (Oasis HLB, Strata XAW, Strata XCW, Isolute ENV+), thus combining different mechanisms, including hydrophobic interactions, as well as anionic and cationic exchange, to increase the recovery and diversity of the extracted dissolved organic contaminants. Other authors used sequential SPE approach (columns connected in series) in order to enhance the prospects of successful toxicant extraction from aqueous samples.^{52,53}

Care must be taken to assure that EDA manipulations do not cause changes in toxicity of samples due to extraction or any further step that would not occur in the environment. The need to pre-concentrate water samples comes with some risks that should be carefully considered.¹⁶ First, during the enrichment of organic chemicals on solid phases there is a risk of toxicity in blank samples which may result from impurities eluted from sorbent. The impurities could be also enriched during the reduction of the solvents used for the elution of the organic compounds from the SPE material. The use of high-purity materials and extensive washing could reduce the blank problems. However, even high quality grade solvents that are suitable for target analyses, and contain no impurities or compounds that chromatographically coelute with the target compound, may contain compounds such as stabilizers and preservatives that are toxic or elevate the activity observed in the bioassays. Matrix components of the samples could be also pre-concentrated during the SPE and may interfere with biotesting by causing non-specific effects or reducing availability of sampled toxic compounds in the bioassay system and may also interfere with chemical analysis.⁵⁴ This may be solved by clean-up, for example by removing high molecular mass sample components using size exclusion chromatography (SEC) or extract dialysis through a semi-permeable membrane. However, every clean up step introduces additional risks of artifacts, compound losses or blank problems.

In general, the use of selective sorbents/resins for the extraction of samples has proven to be less problematic than use of solvents in terms of artifactual toxicity and losing toxicants of concern.¹⁶

2.3. Selection of bioassays

One of the most critical issues for a successful EDA procedure is the selection of bioassays which should include different end-points in order to cover the broadest range of the possible toxic effects. Bioassays used in EDA studies are small scale biological systems on a molecular, cellular (*in vitro*) or whole organism (*in vivo*) level which can detect biological effects in a concentration-dependent manner. Therefore, the selection of bioassays determines which toxicants are finally identified.

Carefully selected sets of biomarkers allow an assessment of exposure to and effects of toxic chemicals, as well as the health status of organisms. The promising concept of “adverse outcome pathways (AOP)” links mechanistic responses on the cellular level with whole organism, population, community and potentially ecosystem effects and services. For most toxic mechanisms, however, practical application of AOPs will require more information and the identification of key links between responses, as well as key indicators, at different levels of biological organization, ecosystem functioning and ecosystem services.⁵⁵ Major criteria for bioassay selection are reproducibility, sensitivity, the ability to provide quantitative results and the power to discriminate toxic and nontoxic fractions, but rapidity, high throughput (since often large number of fractions need to be tested) and low volume requirement should also be taken into account.¹⁸ Due to these practical limitations, short-term, small-scale bioassays are generally favored over long-term, large-scale bioassays. Table 1 provides a list of bioassays that are compatible with the EDA studies.

2.3.1. *In vitro* bioassays

In vitro bioassays as a screening tool to characterize contamination of a variety of environmental matrices have become increasingly popular in the field of environmental toxicology, due to their sensitivity, versatility to include different specific endpoints, high throughput potential and also for ethical reasons. When selecting a bioassay, metabolic capacity of cell lines needs to be considered, since metabolism can be a crucial modifier of toxicity, detoxifying many chemicals, but also activating some.¹⁶ Numerous sets of toxicological endpoints can be addressed with *in vitro* bioassays, however, still only a fraction of possible biological targets for toxicants are covered in test batteries for environmental

monitoring. Each of these *in vitro* bioassays for EDA has advantages and disadvantages, and thus suitable bioassays should be selected for specific purposes.

Non-specific cytotoxicity/cell viability, growth and/or proliferation can be evaluated in *in vitro* cell-based assays as indicators of effects on a cellular level. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromid] assay is a widely accepted rapid and sensitive *in vitro* method for the assessment of cytotoxicity, which determines the viability of cells, measuring the activity of mitochondrial dehydrogenases.^{23,56} The chronic toxicity of the samples could be determined using ISO validated freshwater algal growth inhibition test with the unicellular green alga.⁵⁷

Since bioluminescence of *Vibrio fischeri* is a standardized and frequently applied tool for testing individual compounds and environmental samples for cytotoxicity, it was also applied in EDA studies.⁵⁸ Short-term tests with *Vibrio fischeri* cover effects on the bacterial energy metabolism. They are well-known to detect log K_{ow} -dependent nonspecific effects, including narcosis and uncoupling, and some electrophilicity-based effects.¹⁸ The Microtox test is highly reproducible, rapid, easy to handle, requires low sample volumes, and may be advanced to a real high-throughput instruments. However, this bioassay has certain disadvantages. Many ecotoxicologically relevant specific effects (e.g. effects of herbicides, insecticides and antibiotics) and long-term effects are hardly detectable in this test system,⁵⁹ which is in clear contrast to the belief of some authors that this organism is quite universally sensitive.

The most widely investigated endpoint related to activation of metabolic enzymes is the aryl hydrocarbon receptor (AhR) – mediated activity used for identification of dioxin-like compounds.⁶⁰ Several EDA studies on AhR-mediated effects, performed on sediments from European river basins and coastal areas, have identified numerous persistent organic compounds (e.g. polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), dinaphthofurans naphthalenyl – benzothiophene) as key pollutants.^{61,62,63} The EROD assay quantifies the activity of 7-ethoxyresorufin-O-deethylase (EROD), a cytochrome P450 monooxygenase 1A (CYP1A) dependent enzyme, via fluorescence measurement. Although EROD induction is a nontoxic effect, it is a commonly used surrogate measure for the identification of hazardous aromatic compounds by EDA. Stable cell lines, such as rainbow trout liver (RTL-W1) cell lines,^{62,64} the PLHC-1 cell line⁶⁵ or rat liver cell line H4IIE⁶⁶ are usually used to assess the exposure to dioxin-like chemicals

that bind to the AhR in EROD bioassay. The Micro-EROD assay, performed using the wild type rat hepatoma cell line H4IIE,⁶⁷ follows the same principle and methodology as the EROD assay, only with slight modifications. With respect to rapidity, reproducibility, the ease of handling and low sample volume consumption for EDA, cell line-based test systems appear to be ideal biological detectors.¹⁸ Transactivation assays, such as the chemically activated luciferase expression (DR CALUX) or chemically activated fluorescence expression (CAFLUX) assays, which are based on recombinant cells, are also frequently used for the detection of AhR-agonists in environmental samples.^{68,69}

Many chemicals released into the environment have been shown to interfere with the endocrine systems of different organisms and known to act as endocrine disrupting chemicals (EDCs) through their binding to nuclear receptors (NRs) and the possible alteration of the biological functions mediated by these receptors. Numerous studies based on the stably transfected yeast or cell lines expressing different NRs were published, as well as studies on estrogen (ER) and androgen (AR) receptors (Table 1). These assays are used for detecting agonistic (estrogenic, androgenic) or antagonistic (antiestrogenic, antiandrogenic) activity by the addition of competing endogenous ligand. Many environmental chemicals can bind to other nuclear receptors that play a critical role in the regulation of the endocrine system and metabolism. For instance, several other xenobiotic-activated receptors, including glucocorticoid (GR), mineral corticoid (MR), progestagens (PR), thyroid (TR) or pregnane X receptors (PXR), were successfully applied in EDA studies.^{70,71} These receptors are known to be activated by a wide range of environmental pollutants, including pharmaceuticals, surfactants, phthalates and personal care products, and have been proposed as a xenosensors for the detection of a broad range of environmental chemicals having different physico-chemical properties.⁷⁰ Natural estrogens (17 β -estradiol and estrone) and synthetic derivatives, such as ethinylestradiol, have been recognized as major contributors to estrogenic activity in river sediment extracts or fractions. Recombinant cell culture or yeast based systems, such as Chemical Activated Luciferase Gene Expression (CALUX) and Yeast Estrogen Screen (YES), based on a reporter gene approach and respond to substances that bind to the human estrogen receptor, showed to be prominent test systems for detection of estrogenic activity.^{72,73,74} For identification of androgenic compounds, Yeast Androgen Screen (YAS) is often used.⁷⁵ All of these test systems were quite successful in identifying natural and synthetic hormones in water samples.¹⁸

Many environmental mutagens are polyaromatic compounds covering a wide range of polarity. Detection and identification of mutagens in complex environmental mixtures is often based on the Salmonella/microsome assay as developed by Ames et al.⁷⁶ Salmonella assays offer several advantages: (1) they are relatively inexpensive and simple to perform, (2) they only require small amounts of sample material, (3) several different strains have been developed to allow characterization of a wide range of mutagens, and (4) over 70% of Salmonella mutagens are carcinogenic.⁷⁷ Therefore, although described more than 40 years ago, this bioassay is still in use.^{78,79,80} The bioassay measures the ability of a sample to revert histidine mutations that were previously induced in the *Salmonella typhimurium* bacteria cells. Strains with different metabolic competences can be used, allowing the discrimination of compounds with different metabolic pathways.⁷⁷ Some studies investigating the genotoxic potential of chemicals used a combination of bioassays for the evaluation of effects. Kirkland et al.⁸¹ applied Ames assay and the *in vitro* micronucleus test in parallel. The micronucleus test identifies cytogenetic damage, which result in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.¹⁶ As a result, there is extensive information in the literature on classes of compounds that cause DNA damage and respective effects in different bioassays, which helps the clarification of the type of DNA damage caused by a sample and can aid in identifying the toxic compounds causing the effect.¹⁶ For the assessment of genotoxic potential of the samples, the Comet assay or single-cell gel electrophoresis that evaluates and quantifies single and double – DNA strand breaks in individual cells could be also applied.

2.3.2. *In vivo* bioassays

In vivo bioassays are traditionally used for the assessment of apical endpoints, such as mortality, growth or developmental disorders. Due to their more accurate estimation of (eco)toxicologically relevant effects as compared with *in vitro* measurements, *in vivo* bioassays are increasingly being utilized in EDA.⁸² Algal assay,^{83,84} Pulse Amplitude Modulation (PAM) assay,⁸⁵ Daphnia assay,⁸⁶ zebrafish embryo larval assay,^{87,88,89} and sediment contact test^{90,91} have been used as a tool for measurement of biological effects in environmental samples.

In algal bioassays used in EDA studies, growth inhibition of freshwater *Scenedesmus vacuolatus* or marine microalgae *Dunaliella tertiolecta*^{10,92} is measured, even though other algal species can also be used. The growth inhibition test modified to microplates, which takes 24 – 72 h, has relatively low sample consumption and thus can be used for high throughput screening and EDA purposes.¹⁶ The PAM fluorometry assay is a sensitive and rapid high-throughput assay used for direct inhibition measurements of photosynthesis in algal species.⁹² Acute effects on photosynthesis in the PAM assay are indicative for chronic effects, for example algae growth. The algal assay provides not only response to non-specific toxicity, but also to specific inhibition of photosynthesis, which actually dominated in many water samples.⁹³ *Daphnia magna*, the big water flea, is one of the most widely used organisms in standardized aquatic ecotoxicology tests,^{94,95} most often for testing acute effects of substances.⁹⁶ It is an organism that is particularly sensitive to acetylcholinesterase inhibitors (e.g. many insecticides) and thus an interesting *in vivo* alternative to *in vitro* enzyme inhibition. The OECD Guideline 202⁹⁵ suggests a test period of 24 – 48 h, the use of small beakers, five neonates per replicate, and a minimum number of four replicates per concentration.

Assays based on embryos and larvae of zebrafish (*Danio rerio*) have advantages including small size, ease of culture, high fecundity, rapid development, external fertilization and development, and transparency of the embryo.⁸⁹ Moreover, their small size makes it feasible to do assays with individual larvae in a well of 96-well plates and to fit very well to EDA requirements. Numerous endpoints have been developed, including lethality (coagulation of the embryo and/or undetected heartbeat) and teratogenicity (malformation of the head, tail, or heart, scoliosis, deformity of yolk, and growth retardation).⁸² The fish embryo toxicity test (FET), preferably using the zebrafish *Danio rerio*, is a very important organism level bioassay that has been integrated in biotest batteries in environmental monitoring programmes and the working group on bioassays of the NORMAN network.⁸⁹

The mudsnail *Potamopyrgus antipodarum* has been reported to be excellent test organism for the determination of endocrine effects on organisms after exposure via spiked sediments. *P. antipodarum* is known to be very sensitive to endocrine disrupting compounds in the laboratory, whereby reproduction increased after exposure to estrogens and decreased after exposure to androgens.⁹⁰ At the same time, its reproduction appeared to be more

sensitive than the induction of estrogenic gene expression in the Yeast Estrogen Screen (YES) assay.⁹⁷

Other promising perspectives in EDA are coming from the establishment of new *in vivo* embryo-larval assays based on either wild-type or transgenic lines in fish.^{98,99} These mechanism-based *in vivo* assays reflect a true physiological response in an intact organism (non-invasive measurement), while considering the bioavailability and pharmaco/toxicokinetics of the test substances.

Table 1. Examples of *in vitro* and *in vivo* bioassays that are compatible for effect-directed analysis (adapted from Hong et al.⁸²).

Bioassay name	Test organism	Endpoint	Sample	Dosing	Time	Major agonists	References
<i>In vitro</i> assays							
Ames assay	Bacteria (<i>Salmonella Typhimurium</i>)	Mutagenicity	Organic extracts of sediments	DMSO	48 h, 72 h	B(a)P, B(a)F, and Pery	Brack et al. ⁷⁸
			Organic extracts of sediments	DMSO	48 h	PAHs, sterols, and naphthoic acids	Higley et al. ⁸⁰
			Organic extracts of river water	DMSO	48 h	Amino- and/or nitro-compounds	Gallampris et al. ⁷⁹
MTT assay	<i>Poeciliopsis lucida</i> hepatocellular carcinoma (PLHC-1) cells	Cytotoxicity	Organic extract of the municipal wastewater effluents	<i>n</i> -propanol	72 h	Surfactants	Smital et al. ²³
Microtox assay	Bacteria (<i>Vibrio fischeri</i>)	Inhibition of bioluminescence	Organic extracts of landfill leachates	MeOH	15 min	Organic compounds (log K_{ow} of 3.5–3.7)	Lei and Aoyama ⁵⁸
CAFLUX assay	Mouse hepatoma cell (HIG1-flu)	AhR-activity	Organic extracts of worm, sediments and crude oils	EtOH	6 h, 24 h	Organic compounds (log K_{ow} of 5–8)	Vrabie et al. ⁶⁹
CALUX assay	Rat hepatoma cell (H4IIE-luc) and human breast cell (MVLN)	AhR- and ER activities	Organic extracts of sediments	<i>n</i> -HEX or ACN	72 h	PAHs for AhR-activity NPs for ER-activity	Khim et al. ⁷⁴

Table 1. – continued.

Bioassay name	Test organism	Endpoint	Sample	Dosing	Time	Major agonists	References
<i>In vitro</i> assays							
DR CALUX®	Rat hepatoma cells (H4IIE)	AhR agonism	Organic extracts of fly ash, feed and sediment	DMSO	48 h	PCBs, PCDDs, PCDTs	Behnisch et al. ⁶⁸
	Rainbow trout cell (RLT-W1)	CYP1A1 enzyme activity	Organic extracts of sediments	DMSO	24 h	PCDD/Fs, PCBs, PCNs	Brack et al. ^{61,100}
	H4IIE	CYP1A1 enzyme activity	Organic extracts of sediments	DMSO	48 h	PAHs, methyl-PAHs, alkyl-PAHs	Kaisarevic et al. ⁶⁶
EROD assay	<i>Poeciliopsis lucida</i> hepatocellular carcinoma (PLHC-1) cells	CYP1A1 enzyme activity	Organic extracts of river water and sediments	DMSO	nd	PAHs (sediments); polar compounds (river water)	Smital et al. ⁶⁵
	RLT-W1	CYP1A1 enzyme activity (EROD)	Organic extracts of suspended solids	DMSO	72 h	Non-prioritized PAHs	Wölz et al. ⁶⁴
YES	Yeast (<i>Saccharomyces Cerevisiae</i>)	Estrogenic activity	Organic extracts of produced water	EtOH	72 h	Short-chain alkylphenols (C1-C5)	Thomas et al. ⁷²
			Organic extracts of sediments	EtOH	22 h	E2, estrone, NP, chlorophene	Schmitt et al. ⁷³

Table 1. – continued.

Bioassay name	Test organism	Endpoint	Sample	Dosing	Time	Major agonists	References
<i>In vitro</i> assays							
YAS	Yeast (<i>Saccharomyces Cerevisiae</i>)	Androgenic activity	Organic extracts of water samples	MeOH	72 h	Testosterone, dihydrotestosterone, androsterone	Thomas et al. ⁷⁵
AChE assay	AChE isolated from the electric eel (<i>Electrophorus electricus</i> L.)	AChE inhibition	Organic extracts of different environmental samples	DMSO	30 min	Paraoxon, dichlorvos	Froment et al. ¹⁰¹
<i>In vivo</i> assays							
Algal assay	Green algae (<i>Scenedesmus vacuolatus</i>)	Inhibition of reproduction (growth)	Organic extracts of sediments	DMSO	24 h	PAHs	Grote et al. ⁸³
PAM assay	Marine microalgae (<i>Dunaliella tertiolecta</i>)	Effective photosystem II efficiency	Organic extracts of passive sampled water	MeOH	4.5 h	Atrazine, diuron, irgarol, isoproturon, terbutryn, terbutylazine	Booij et al. ⁹²
Daphnia assay	Daphnia (<i>Daphnia magna</i>)	Immobilization	Organic extracts of sediments	Acetone	24 h	Methyl parathion, tributyltin	Brack et al. ⁸⁶

Table 1. – continued.

Bioassay name	Test organism	Endpoint	Sample	Dosing	Time	Major agonists	References
<i>In vivo</i> assays							
FET assay	Zebrafish (<i>Danio rerio</i>)	Lethality, developmental malformation, and tetragenicity	Organic extracts of soils	DMSO	6 d	11H-BbF, 9-methylacridine, 4-azapyrene, retene	Legler et al. ⁸⁷
Sediment contact test	Mud snail (<i>Potamopyrgus antipodarum</i>)	Mortality, growth, and inhibition of reproduction	Wet field sediments	nd	28 and 56 d	NP and BPA	Schmitt et al. ^{90,91}
<i>In vivo</i> nuclear receptor-mediated reporter gene activation	Transgenic fish embryos, choriogenin h-gfp medaka line	Induction of GFP via aromatase in choriogenin h-gfp medaka	Reference compounds	DMSO	48 h	EE2, E2, testosterone, anastrozole, fadrozole, tamoxifen	Spirhanzlova et al. ⁹⁹

AChE - acetylcholine esterase; ACN - acetonitrile; AhR - aryl hydrocarbon receptor; AR - androgen receptor; BaF - benzo[a]fluoranthene; BaP - benzo[a]pyrene; 11H BbF – 11H-benzo[b]fluorene; BPA- bisphenol A; CALUX - chemical activated luciferase gene expression; DMSO - dimethyl sulfoxide; E2 - 17 β -estradiol; EE2 - 17 α -ethynylestradiol; ER - estrogen receptor; EROD - ethoxyresorufin-O-deethylase; EtOH - ethanol; FET - zebrafish embryo acute toxicity assay; GFP - green fluorescent protein; n-HEX - n-hexane; MeOH - methanol; NP - nonylphenol; PAHs - polycyclic aromatic hydrocarbons; PAM - pulse amplitude modulation; PBDEs polybrominated diphenyl ethers; PCBs - polychlorinated biphenyl; PCDD/Fs - polychlorinated dibenzo-pdioxins and dibenzofurans; PCDDs - polychlorinated dibenzodioxins; PCDTs - polychlorinated dibenzothiophenes; PCNs - polychlorinated naphthalene; Pery - perylene; nd - not described; YAS - yeast androgen screen.

2.4. Fractionation techniques in effect-directed analysis

After biological screening of raw extracts of environmental samples, samples exhibiting significant toxicity are subjected to fractionation in order to facilitate subsequent identification and quantification of compounds causing adverse effects. Fractionation helps reduce the complexity of toxic mixtures and removes non- or less toxic co-extracted components, which is an important prerequisite for a successful identification of toxicants.¹⁶ Thus, fractionation is one of the key pillars of EDA approach. Fractionation can be based on several physico-chemical properties of the analytes, including polarity, hydrophobicity, molecular size, planarity and the presence of specific functional groups, and it is typically based on preparative chromatography. The most frequently applied separation principles include adsorption (typically normal phase liquid chromatography NP-LC),¹⁰² partition (typically reversed-phase liquid chromatography RP-LC),¹⁰³ ion exchange chromatography (IEC),⁷⁹ size exclusion chromatography (SEC),¹⁰⁰ and affinity-based separation techniques.¹⁰⁴

The predominating fractionation technique in EDA is liquid chromatography (LC), which is typically applied as open column chromatography and high performance liquid chromatography (HPLC). For specific applications on volatile chemicals, preparative capillary GC may be an interesting alternative for high resolution separation.¹⁰⁵ Both preparative and analytical separation techniques in EDA should provide information that could be useful for further chemical identification and subsequent hazard assessment. The optimal fractionation procedure strongly depends on the problem to be addressed and may be developed with standard compounds relevant for toxicological endpoints and the matrix to be assessed.¹⁶ The major requirement for fractionation techniques is that different compounds or compound classes are actually recovered in different fractions with acceptable and reproducible recoveries, with the absence of blank toxicity. Selectivity and orthogonality of separation steps is also an overarching criterion for optimal design of a fractionation procedure. An important element associated with fractionation in EDA is obtaining a sufficient volume of fractions for biotesting, further fractionation, and chemical analysis.

Since EDA of aqueous samples in most cases starts with SPE, this step is also suggested as a possible approach for initial fractionation.¹⁸ There are two approaches for initial fractionation using SPE:

1) Sequential elution from C18 or XAD sorbents with solvents of increasing hydrophobicity. This approach has been applied in the TIE protocols, which focused mainly on lipophilic compounds.¹⁰⁶ For moderately hydrophilic toxicants, initial fractionation can also be achieved by sequential elution of polystyrene-divinylbenzene phases.¹⁰⁷

2) Sequential SPE with focus on hydrophilic compounds in aqueous samples. The procedure starts with SPE on an endcapped C18 phase at pH 7 for neutral compounds followed by SPE on polystyrene-divinylbenzene phase at pH 7 to extract predominantly aromatic compounds with slightly higher polarity. In order to extract more hydrophilic and acidic compounds, the SPE procedure is completed by repeating the extraction at pH 4.5 and pH 2.5 using the polymeric phase ethylvinylbenzene-divinylbenzene, which is tolerant to extreme pH values.⁵²

However, the full EDA studies require a more detailed multi-step fractionation scheme, which usually involves the preparative HPLC fractionation. Among different HPLC techniques, RP-HPLC is the predominating technique for preparative separation of complex mixtures extracted from aqueous samples, but also from soils, sediments, air particulate matter and other solid matrices.¹ Despite the multitude of different RP-phases on the market, RP HPLC fractionation in EDA studies employ mainly columns with C18 stationary phase. This is in agreement with the aims of the TIE protocols as suggested by the US-EPA,¹⁰⁶ since C18 columns enable fractionation of the components according to their lipophilicity (i.e. K_{ow} values). Linear gradients of methanol and water¹⁰⁸ or acetonitrile and water,¹⁰⁹ respectively, were preferably used to fractionate the components according to their K_{ow} values. Watanabe et al.,¹¹⁰ additionally applied phenyl-hexyl phases after the C18 fractionation to isolate mutagenic aromatic compounds from Japanese river waters, exploiting the aromatic selectivity of this phase. Other authors⁷⁹ applied three-step fractionation for the isolation and selection of polyaromatic mutagens from surface waters. Firstly, they applied SPE fractionation using mixed-cation exchange cartridge (MCX) and mixed-anion exchange cartridge (MAX) columns to separate the analytes into three fractions according to their charge (acidic, basic and neutral). For a finer fractionation, the authors first applied a C18 RP-HPLC column, followed by further fractionation of the most active acidic and neutral fractions using preparative phenyl-hexyl column. In order to estimate the contributions of the most potent estrogens to observed estrogenic effect, Beck et al.¹¹¹ tested five different RP-phases for separation of estrogenic compounds in marine surface waters. The optimal

separation was achieved using a Synergy™ Polar-RP column (Phenomenex), which was filled with an ether-linked phenyl phase with polar endcapping.

While single-step fractionation procedure may suffice for EDA of relatively simple mixtures, in the case of complex environmental samples, a combination of different fractionation methods is necessary to reduce the complexity of mixtures containing originally thousands of chemical compounds to few individual toxicants. EDA could benefit greatly from exploiting the multitude of commercially available stationary phases with different selectivities. The usual RP-HPLC fractionation can be significantly improved in combination with NP-HPLC¹¹² and vice versa.¹¹³ In contrast to C18 in RP-HPLC, there is no single predominant stationary phase in NP-HPLC fractionation. NP-HPLC enables separations according to a broad range of compound properties. Classical NP-phases such as alumina⁷⁸ or silica¹¹⁴ have been frequently used in EDA studies, while the applied protocols vary with respect to the amount of sorbent, internal diameter of column, elution solvent, volume of eluting solvent and flow rate.⁸² Recently, organic modified silica phases, such as aminopropyl silica,¹¹⁵ cyanopropyl silica,⁸⁸ and cyano-amino-bonded silica⁷² are gaining in popularity. Generally, all of these phases enable reasonable separation, with amino groups providing greater selectivity to acids and bases.

Several combinations of complementary HPLC fractionation techniques were reported in the literature. For the detection of selected estrogenic compounds in aqueous samples, Snyder et al.¹¹² have carried out a rough fractionation using NP HPLC, followed by biotesting and fine fractionation of major toxic sub-fractions using RP-HPLC. For a comprehensive assessment of the hazardous chemical contamination in the Sava River basin, authors applied two-tiered procedure, consisting of a simple pre-fractionation into three fractions on deactivated silica gel column, and subsequent fractionation using both NP-HPLC and RP-HPLC.¹¹⁶ Thomas et al.⁷² applied NP-HPLC fractionation procedure using a cyano-amino-bonded silica HPLC for isolation of estrogenic compounds from the North Sea off-shore produced water extracts.

EDA of sediment and air particulate matter-associated toxicants usually involves preparation of extracts, in solvents that are not miscible with water such as DCM.¹⁸ The solubility of contaminant mixtures, found in sediments, in polar solvents used for RP chromatography is significantly lower than in DCM, and there is the risk of precipitation of lipophilic components during solvent exchange when preparing the sample for RP-HPLC

fractionation. Therefore, NP-HPLC is usually a first choice for initial fractionation of extracts obtained from solid samples. In order to enhance the throughput, Lübcke-von Varel et al.,¹⁰² developed an automated on-line fractionation method using coupled and automatically connected columns, including cyanopropyl- and nitrophenylpropyl-bonded silica and porous graphitised carbon stationary phases. Exploiting the potential of each column, compounds were separated mainly according to their polarity, number of aromatic carbons and planarity. Excellent group-specific resolution, high reproducibility and good recoveries suggested that this method is suitable for the fractionation of a multitude of major sediment contaminants in one HPLC-run.

Separation according to molecular size by size-exclusion chromatography (SEC) or gel permeation chromatography (GPC) is also frequently applied in EDA studies, especially as a first separation step to remove elemental sulfur, long chain aliphatic compounds, humic substances, or biological macromolecules such as lipids and proteins.¹ After applying GPC, Qu et al.¹¹⁷ performed an NP-based primary fractionation using silica based sorbents, while the fractions having the highest potency were further processed in a secondary fractionation step by employing RP-HPLC to screen the samples for potential neurotoxic effects. Heisterkamp et al.¹¹⁸ applied SEC for fractionation to isolate estrogenic compounds from sewage treatment plant effluent extracts. SEC was also applied for toxicity-directed fractionation of tannery wastewater with regard to molecular weight, as well as for sediment extracts and airborne particles.¹ For identification of androgen-disrupting compounds in river sediments using EDA, Weiss et al.¹¹⁹ applied three fractionation steps by combining GPC, RP- and NP-HPLC. Simon et al.¹²⁰ also tested different multi-step clean-up procedures including dialysis, GPC and NP-HPLC for the removal of lipids having smaller molecular size, such as cholesterol and some fatty acids, from biological samples. Efforts were also made to reduce the number of subsequent clean-up steps, but both the GPC and the NP-LC steps proved to be essential to separate the lipids and the endogenous hormones from the xenobiotic compounds.²² In specific cases, SEC can also separate closely related target analytes with different molecular sizes, such as polychlorinated naphthalenes, biphenyls, dioxins, and furans.¹⁰⁰

Table 2. A summary of fractionation techniques applicable for effect-directed analysis (EDA) studies.

Equipment / column	Mobile phases / elution solvents	No. of fractions	Sample type	References
RP-HPLC C18, Luna (250x10 mm, 10 µm, 100 Å)	A: H ₂ O B: MeOH	10	River water, river sediment	Urbatzka et al. ¹⁰⁸
RP-HPLC C18, Symmetry (250x4,6 mm, 5 µm)	A: H ₂ O B: MeOH	8	Landfill leachates	Lei and Aoyama ⁵⁸
RP-HPLC C18, Vydac TP254 (250x4,6 mm, 5 µm)	A: H ₂ O B: MeOH	9	Fish bile	Houtman et al. ¹²¹
RP-HPLC C18, BDS Hypersil (250x10 mm, 5 µm)	A: H ₂ O B: MeOH	30	Wastewater	Grung et al. ¹⁰³
1. GPC (AccuPrep, Bio-Beads S-X3)	DCM	nd	River sediment	Qu et al. ¹¹⁷
2. SPE SiOH sorbent	F1: DCM/ <i>n</i> -HEX (1:9, v/v) F2: DCM/ <i>n</i> -HEX (2:8, v/v)	2		
3. RP-HPLC C18, ZORBAX Eclipse XDB (250x9,4 mm, 5 µm)	A: H ₂ O B: MeOH	30		
1. SPE C18 and PSDVB	F1-F4: 20, 60, 80 and 100% MeOH for C18 F5-F8: 20, 60, 80 and 100% MeOH for PSDVB	8	Oil sands process waters	Yu et al. ¹²²
2. RP-HPLC C18, ZORBAX Eclipse XDB C-8 (250x10 mm, 5 µm)	A: MeOH B: H ₂ O with 5% formic acid + 20 mM NH ₄ Ac (pH=7)	60		
1. NP-HPLC Silica, Luna (250 x 4.6 mm, 5 µm)	A: 30% DCM in <i>n</i> -HEX B: MeOH	3	Wastewater, river water	Snyder et al. ¹¹²
2. RP-HPLC C18, Prodigy (250 x 4.6 mm, 5 µm)	A: H ₂ O B: ACN	9		

Table 2. – continued.

Equipment / column	Mobile phases / elution solvents	No. of fractions	Sample type	References
1. SPE (SDB-1)	F1: <i>n</i> -HEX F2: MeOH	2	River water	Scheurell et al. ¹¹³
2. NP-HPLC (for F1) Nitrophenyl-silica column (125 x 4 mm, 5 µm)	A: DCM B: <i>n</i> -HEX	8		
3. RP HPLC (for F2) C18, Lichrosphere 100 (125 x 4 mm, 5 µm)	A: DCM B: <i>n</i> -HEX	8		
1. SPE (mixed-cation exchange cartridge, MCX) SPE (mixed-anion exchange cartridge, MAX)	F1: MeOH/toluene (50:50, v/v) F2: MeOH/ammonium hydroxide (90:10, v/v); toluene F3: MeOH/toluene (50:50, v/v) F4: MeOH /formic acid (90:10; v/v); toluene	4	River water	Gallampois et al. ⁷⁹
2. RP-HPLC C18, Zorbax Eclipse PAH (250 x 9.4 mm, 5 µm)	For F3: H ₂ O/MeOH For F4: MeOH/ NH ₄ Ac (50 mM, pH 4)	11		
3. RP-HPLC Phenyl-hexyl column, Zorbax Eclipse (250×21mm, 5 µm)	For F3: H ₂ O/MeOH For F4: MeOH/ NH ₄ Ac (50 mM, pH 4)	16		
NP-HPLC Cyano-amino bonded silica (250 × 10 mm, 5 µm)	A: <i>n</i> -HEX B: DCM C: 2-propanol	31	Produced water	Thomas et al. ⁷²
Open column chromatography: Silica gel deactivated with 15% H ₂ O	A: <i>n</i> -HEX B: DCM C: MeOH	3	Wastewater	Smital et al. ^{23,65}

Table 2. – continued.

Equipment / column	Mobile phases / elution solvents	No. of fractions	Sample type	References
1. Open column chromatography: Silica gel deactivated with 15% H ₂ O	F1: <i>n</i> -HEX F2: DCM F3: MeOH	3		
2. NP-HPLC (for F1 and F2) Aminosilica column (250 x 10 mm)	A: <i>n</i> -HEX B: <i>n</i> -HEX:2-propanol (10:90,v/v)	30+30	River water	NATO project ¹¹⁶
3. RP-HPLC (for F3) C18 (250 x 10 mm)	A: H ₂ O:ACN (80:20, v/v) B: ACN	30		
1. GPC PSDVB columns (600× 25 mm, 50 Å, 10 µm,)	F1: 16.5–24.0 min. fraction using DCM F2: 29.0–36.0 min. fraction using DCM	2		
2. RP-HPLC C18, Vydac 201TP510 (250 x 10 mm, 5 µm)	A: H ₂ O B: MeOH	5	River sediment	Weiss et al. ¹¹⁹
3. NP-HPLC µPorasil column (300 x 7.8 mm, 10 µm)	A: <i>n</i> -HEX B: DCM C: ACN	8		
1. SPE Oasis MCX (mixed-cation exchange cartridge)	F1: MeOH F2: 5% NH ₄ OH in MeOH	2		
2. NP-HPLC: µPorasil column (300 x 7.8 mm, 10 µm)	A: <i>n</i> -HEX B: DCM C: ACN	4	Blood plasma	Simon et al. ¹²⁰
1. GPC	DCM	nd		
2. RP-HPLC C18, Vydac 2Tp510 (250 x 10mm, 5 µm)	A: H ₂ O B: MeOH	20	Landfill soil	Legler et al. ⁸⁷
NP-HPLC Nucleosil 100-5 NO ₂ (250 mm × 21 mm, 5 µm) Nucleosil 100-5 CN (250 mm × 21 mm, 5 µm) Cosmosil PYE (250 mm × 10 mm, 5 µm) Hypersil PGC (50 mm × 10 mm, 7 µm)	A: <i>n</i> -HEX B: DCM	18	River sediment	Lübcke-von Varel et al. ¹⁰²

Table 2. – continued.

Equipment/column	Mobile phases /elution solvents	No. of fractions	Sample type	References
NP-HPLC Hypersil APS-2 (aminopropyl silica) (250 mm x 7 mm, 10 µm)	A: <i>n</i> -HEX B: DCM C: Ethyl acetate	3+4	Wetland sediments	Regueiro et al. ¹¹⁵
1. GPC Bio-Beads S-X3 gel column	DCM	-	Fluvial sediment	Fetter et al. ⁸⁸
2. NP-HPLC Cyanopropyl silica column (VP 125/21 Nucleosil 100-5 CN)	A: <i>n</i> -HEX B: DCM C: ACN	10		
1. Open column chromatography: Alumina deactivated with 4.5% H ₂ O	F1: <i>n</i> -HEX F2: <i>n</i> -HEX:DCM (95:5, v/v)	2	River sediment	Brack et al. ¹⁰⁰
2. NP-HPLC Nitrophenylpropyl silica (NO) Nucleosil 100-5 NO (250 mm × 21 mm, 5 µm)	A: <i>n</i> -HEX B: DCM	7		
3. RP-HPLC Nucleosil 100-5 C PAH (250 x 4 mm, 100 Å)	A: H ₂ O B: ACN	10		
4. Electron-donor-acceptor HPLC PYE (250 x 10 mm, 5 µm)	A: <i>n</i> -HEX B: DCM	11		
5. SEC PLgel (600 x 25 mm, 10 µm)	THF	3		

ACN - acetonitrile; DCM - dichloromethane; F - fraction; GPC - gel permeation chromatography; MeOH - methanol; nd - not defined; NP-HPLC - normal phase high performance liquid chromatography; *n*-HEX - *n*-hexane; NH₄Ac - ammonium acetate; PSDVB - polystyrene divinylbenzene; RP-HPLC - reverse phase high performance liquid chromatography; SDB- styrene-divinylbenzene copolymer; SEC- size exclusion chromatography; SPE - solid phase extraction; THF - tetrahydrofuran.

2.5. Toxicant identification in effect-directed analysis

An important step in EDA is toxicant identification and structure elucidation of the compounds causing the biological response of the bioassay. This may often involve a combination of target analysis of known toxicants, suspect screening (e.g. analysis of site-specific compounds or expected transformation products) and non-target screening through the structure elucidation of true unknowns.¹⁶ The instrumental methods applied for compound identification in EDA studies should ideally allow a detection of compounds below levels showing effects in the bioassay, as it is otherwise not possible to detect the causative substance. Also, ideally, the analytical methods should be able to cover all compounds potentially occurring in the analysed fraction. Several confirmation techniques can be used for structure elucidation, including spectroscopic and mass spectrometric techniques. Nuclear magnetic resonance (NMR) spectroscopy, which allows obtaining the most detailed structural information, requires usually a much larger amount of a compound, which precludes its use in most environmental studies.¹²³ Therefore, toxicant identification typically relies on gas chromatography coupled to mass spectrometry (GC-MS) and liquid chromatography coupled to mass spectrometry (LC-MS). Analysis of organic contaminants in environmental samples is a real analytical challenge, not only because of the diversity of chemical properties of these compounds, but also because of generally low concentrations (usually part per billion or part per trillion levels) and the complexity of environmental matrices. Both LC-MS and GC-MS techniques cover significant and overlapping fractions of the chemical space of environmental pollutants, but neither addresses all chemicals (Figure 2). The major properties of the compounds, such as hydrophobicity and volatility, should be considered when planning the analytical strategy, as these parameters have an impact on the applicability of GC-MS or LC-MS techniques. Therefore, the combined use of these two complementary techniques should be regarded as an optimal approach to detect, characterize and confirm contaminant composition in environmental samples.

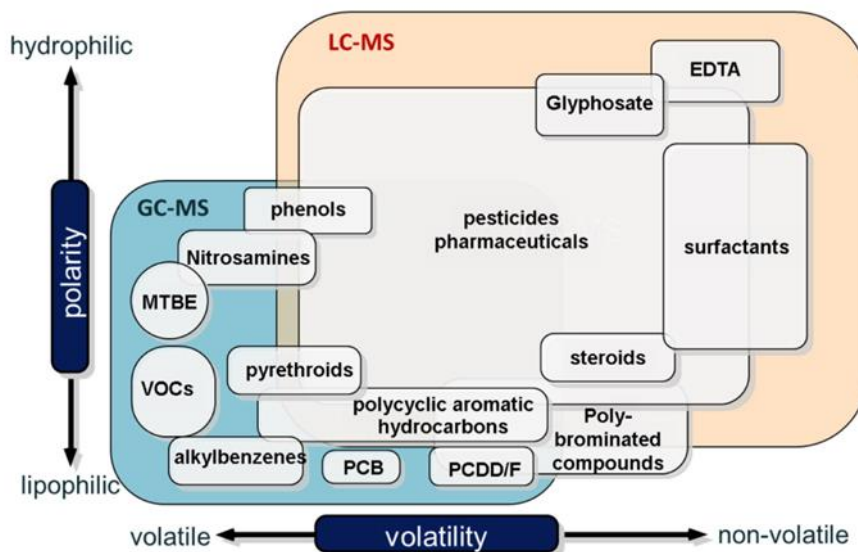


Figure 2. The application field of the two most important analytical techniques GC-MS and LC-MS, in the two dimensions of hydrophobicity and volatility. The figure is taken from Brack et al.,¹⁶ and follows the concept by Ternes et al.¹²⁴

2.5.1. Liquid chromatography coupled to mass spectrometry

During the last 20 years, LC-MS technologies have opened up new windows of opportunity for chemical identification in complex environmental samples, in particular for thermolabile and polar compounds. This is especially true for metabolites and transformation products, which are generally more polar than their parent molecules. As a result, polar organic micropollutants have moved increasingly into the focus of environmental scientists, regulatory agencies, and politicians.¹²³ To meet the challenges posed extreme complexity of mixtures of known and unknown compounds found in environmental samples and by low concentration levels of individual compounds in complex matrices, different LC-MS technologies have emerged. Recently, ultra-high-performance liquid chromatography (UHPLC) has been developed as an innovative and powerful separation technique based on the use of columns containing stationary phases with smaller particles size ($< 2 \mu\text{m}$) than in the conventional HPLC ($5 \mu\text{m}$). This has led to higher resolution and sensitivity and shorter analysis time. Also, in the last decade significant progress has been achieved in the field of high resolution mass spectrometry (HRMS) coupled with liquid chromatography (LC-HRMS).

The main ionization methods used for LC-MS are: electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Among the possible ionization techniques, ESI is by far the most widely used as it is applicable to molecules of wide range of polarities and molecular masses (~60–10,000). However, it is not appropriate for the ionization of molecules with low polarity. ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. Atmospheric pressure chemical ionization is a technique in which the analytical sample is subjected to a corona discharge. APCI is suitable for moderately polar substances, in the mass range of ~40–1,000 Da. APPI is a relatively new ionization technique in which samples are ionized using ultraviolet light. APPI achieves good ionization of low to moderate polarity compounds; however, this technique has a rather narrow mass range of ~20–500 Da.¹

The most common LC-MS configuration is the triple quadrupole (QqQ), followed by the quadrupole ion trap (QIT) for target-analysis in multi-residue methods. However, these instruments operate at unit resolution, and in the case of QqQ have a low sensitivity in full-scan mode, which limits their capabilities in the detection of unknowns. On the other hand, the quadrupole time-of-flight (Q-ToF), Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR) mass analyzers are generally used for structure elucidation of unknown compounds, due to their high mass accuracy.

The identification and quantification of organic contaminants at low concentrations requires both high sensitivity and selectivity against complex matrix backgrounds. For a wide range of compounds, selected reaction monitoring (SRM) of precursor-product ion transitions by using QqQ or QIT instruments fulfills these prerequisites. Usually, two ion transitions, in combination with their intensity ratio, are used for quantification and confirmation in order to avoid false positive results. Although this approach has proved adequate for the majority of polar compounds in environmental matrices, there are several limitations: 1) Under the constraints of at least two transitions, SRM methods are typically limited to about 100–150 target analytes, depending on chromatographic separation, as otherwise accuracy or sensitivity deteriorate, due to an insufficient temporal peak resolution or too short acquisition times for the individual MS/MS transitions, respectively; 2). For some analytes, only non-specific transitions, which are common also for matrix interferences, such as the neutral loss of H₂O or CO₂, might occur; 3) Some analyte ions, particularly those of low molecular

weight, show only one transition.¹²³ LC-HRMS offers promising solutions to these three limitations of SRM analysis, with possibility to detect hundreds of polar contaminants in targeted approaches without pre-selection of analytes due to its sensitivity and selectivity in full scan analysis. However, on QTOF instruments sensitivity is about 1 – 2 orders of magnitude lower than those of QqQ instruments in SRM mode and dynamic range is about 10-fold lower.¹²³ The LTQ Orbitrap instrument offers a better dynamic range and a sensitivity close to that of many QqQ instruments,¹²⁵ thus allowing for quantification and confirmation in a single analytical run. With regard to environmental monitoring programs, a major advantage of LC-HRMS is the possibility of retrospective analysis of full-scan data, which enables laboratories to search for “new” contaminants years after data recording.¹²³ Furthermore, LC-HRMS allows the detection of known compounds suspected of being present in environmental samples (suspect screening) without reference standards and screening for yet unknown non-target chemicals.¹²⁶

In suspect screening, chromatograms are analyzed for peaks of an exact mass derived from the known molecular formula of the suspect. In contrast to target analysis, the suspect screening approach does not rely on reference standards for quantification and confirmation. These reference standards are currently not available for a large number of potential environmental contaminants, in particular transformation products.¹²³ However, once a structure is available, the exact mass and isotope pattern can be calculated and thus the full scan information can be used to search for matching suspects. If a suspect is found, the structure still needs to be confirmed through MS/MS, retention prediction, and finally a reference standard, which is also needed for toxicity confirmation.¹⁶ In EDA studies, relevant suspects could be site-specific pollutants,¹²⁶ river-basin specific pollutants,^{13,127} known compounds with the measured effect (e.g. mutagenic or estrogenic substances). Possible suspects could also be transformation products, which show a close structural relationship to their parent compounds, known to be present either from literature studies or prediction systems.¹²⁸

If target analysis or suspect screening fails to identify peaks of interest potentially causing the effect in the EDA, non-target screening should also be performed. In recent years, non-targeted screening combined with EDA has been applied to various abiotic (e.g. sediment, soil, water, crude oil, and oil sands process water) and biotic environmental compartments (e.g. blood plasma of polar bear) using cutting edge instrumentation.

^{79,87,92,117,122} In true non-target screening, in which often no initial information on the analytes is available, automated peak detection and spectra deconvolution algorithms are applied, which typically reveal several thousand of peaks in an individual water sample.^{126,129} Subsequently, molecular formulas can be derived for the detected peaks based on accurate mass and isotope patterns. For each molecular formula, a large number of candidate structures exist, which have to be ranked or filtered to obtain a useful list of compounds for confirmation by reference standards.¹²⁶ To this end, a range of different approaches have been suggested to predict properties of candidates, such as MSⁿ fragmentation energies, product ion spectra, retention times or ion mobility drift times to compare predicted with experimental data.^{130,131,132} The application of both suspect and non-target screening suffers from the large effort of manual data evaluation.¹⁶ Therefore, systematic strategies with automated approaches are required to filter suspect compounds to be searched for and “relevant” peaks on which the identification efforts should focus.¹²⁶

2.5.2. Gas chromatography coupled to mass spectrometry

For many years, GC-MS has been the nearly exclusive technique for the identification of toxicants in EDA studies, due to its widespread availability, ease of use and possibility to obtain structural information without the need for a preparative isolation of the compounds.²⁰ GC-MS is a well-established technique of choice, especially for the analysis of persistent organic pollutants (i.e. priority contaminants), because it provides high selectivity and sensitivity for non-polar (or less polar) and volatile organic compounds. As many compounds present in water are not amendable to GC, nonselective derivatization (silylation, methylation etc.) was typically carried out prior to GC-MS analysis of nonvolatile, polar, or thermally labile fractions.¹³³ Derivatization is required to increase the volatility and thermal stability of these compounds. This involves derivatizing one or more polar groups of a compound to a less polar group. Derivatization can also be used to increase sensitivity, selectivity, or specificity of a given chromatographic separation. However, derivation step significantly increases the time needed for sample analysis, indicating that LC-MS technique is more suitable for identifying compounds with polar functional groups.

The most common MS systems for GC are (1) quadrupole, (2) ion trap – IT, (3) time-of-flight – ToF or (4) magnetic sector instruments. The first two techniques are often low-

resolution mass spectrometers. ToF mass spectrometers are available in low (mass unit), medium (mass resolution about 5,000) and high resolution (HR) version, while magnetic sector instruments are high-resolution mass spectrometers. GC-HR-ToF instruments, nowadays allow a full-scan acquisition with a sensitivity getting closer to that of selective ion monitoring (SIM) on quadrupole instruments or selected reaction monitoring (SRM) on triple quadrupole instruments.²⁰ GC coupled with magnetic sector MS has been available for a long time, however its application in EDA was limited to a few studies,¹³³ probably due to the high investment costs and challenging operation. Despite many advantages, not many EDA studies on aquatic resources have applied GC-HR-MS instruments, and GC coupled to low-resolution MS continues to play an important role today¹¹⁵. Nowadays, comprehensive two-dimensional GC (GCxGC) coupled to low-resolution rapid-scanning MS became an established technology for the separation and identification of volatile organic compounds in complex environmental samples.¹³⁴ Besides having higher resolving power than single column GC, GCxGC has the advantage that compounds are grouped in bands with the same chemical characteristics, which can be useful for the identification of unknown compounds.¹

Electron impact (EI) is the most widely used ionization technique in GC-MS which provides very reproducible structural information of the compound, often the molecular ion and fragment ions of the analytes. For low resolution EI spectra, extensive libraries of MS spectra are available (e.g. NIST, Wiley), mainly from quadrupole instruments, which are very useful for automatic library searches and identification of compounds.¹ Due to the availability of these libraries, individual laboratories do not have to develop their own in-house library, which can be very time consuming and costly. Thermo-labile compounds often give weak responses and no molecular ion when EI is applied. In this case, chemical ionization (CI) is a good alternative, with the appropriate ionization gas (e. g. methane, isobutene). In general, CI is gentler than EI ionization and little or no fragmentation of the molecular ion occurs. Still CI is less frequently used than EI ionization in environmental chemistry.

Unknown identification in GC-MS often involves manual interpretation of the spectra and thus a detailed knowledge of mass spectrometric fragmentation rules as well as experience, and is extremely time consuming for what may potentially be many peaks in EDA of complex samples.¹⁶ Nowadays, this process can be speeded up by several computer-assisted structure elucidation options.

§ 3. EXPERIMENTAL PART

3.1. Materials

3.1.1. Selection of analytes

For the investigation of different extraction/fractionation procedures 96 organic compounds were selected, which belong to various contaminant categories and encompass a wide range of chemical structures (Supplementary material (Table SI)). The list of used reference compounds is given in the Table 3. Some of the compounds were selected for the study as representatives of priority substances included in the Water Framework Directive (WFD), while others were chosen as important region-specific pollutants, based on previous study on the Sava River basin.^{23,65} Furthermore, the selected contaminants include different classes of classic and emerging water contaminants, with a special emphasis on covering a wide range of physico-chemical properties in terms of volatility, hydrophobicity and water solubility. A special emphasis was on polar compounds, including both neutrals as well as ionized organic compounds. Regarding usage patterns and predominant sources, the selected compounds can be divided in several contaminant categories, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and their transformation products (TPs), pharmaceuticals and their TPs, illicit drugs and their metabolites, plasticizers and flame retardants and surfactants.

Table 3. List of reference compounds used for the evaluation of extraction and fractionation procedures.

Compound name	Compound group / Usage pattern / Source
<i>Polycyclic aromatic hydrocarbons (PAHs)</i>	
Naphthalene	PAH, WFD priority, pyrolytic product
Acenaphthylene	PAH, pyrolytic product
Acenaphthene	PAH, pyrolytic product
Fluorene	PAH, pyrolytic product
Phenanthrene	PAH, pyrolytic product
Anthracene	PAH, WFD priority, pyrolytic product
Fluoranthene	PAH, pyrolytic product
Pyrene	PAH, pyrolytic product
Benz[a]anthracene	PAH, pyrolytic product
Chrysene	PAH, pyrolytic product
Benzo[b]fluoranthene	PAH, WFD priority, pyrolytic product
Benzo[k]fluoranthene	PAH, WFD priority, pyrolytic product
Benzo[a]pyrene	PAH, WFD priority, pyrolytic product
Indeno[1,2,3-cd]pyrene	PAH, WFD priority, pyrolytic product
Dibenz[a,h]anthracene	PAH, WFD priority, pyrolytic product
Benzo[g,h,i]perylene	PAH, WFD priority, pyrolytic product
<i>Polychlorinated biphenyls (PCBs)</i>	
2,4,4'-Trichlorobiphenyl (PCB 28)	polychlorinated biphenyl
2,2',5,5'-Tetrachlorobiphenyl (PCB 52)	polychlorinated biphenyl
2,2',4,5,5'-Pentachlorobiphenyl (PCB 101)	polychlorinated biphenyl
2,3',4,4',5-Pentachlorobiphenyl (PCB 118)	polychlorinated biphenyl
2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153)	polychlorinated biphenyl
2,2',3,4,4',5'-Hexachlorobiphenyl (PCB 138)	polychlorinated biphenyl
2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB 180)	polychlorinated biphenyl

Table 3. – continued.

Compound name	Compound group / Usage pattern / Source
<i>Pesticides and their TPs</i>	
Atrazine	pesticide, herbicide, WFD priority
Atrazine-desethyl	pesticide, herbicide, transformation product
Atrazine desisopropyl	pesticide, herbicide, transformation product
Simazine	pesticide, herbicide, WFD priority
Propazine	pesticide, herbicide
Sebuthylazine	pesticide, herbicide
Terbuthylazine	pesticide, herbicide
Diuron	pesticide, herbicide, WFD priority
Isoproturon	pesticide, herbicide, WFD priority
Chlorfenvinphos	pesticide, insecticide
Pentachlorophenol	pesticide, insecticide, herbicide
<i>Pharmaceuticals and their TPs</i>	
Zolpidem	psychiatric drug
Warfarin	anticoagulant
Torseamide	diuretic
Terbinafine	antifungal drug
Chlorthalidone	diuretic
Sulfathiazole	pharmaceutical, sulfonamide antibiotic
Sulfapyridine	pharmaceutical, sulfonamide antibiotic
Sulfamerazine	pharmaceutical, sulfonamide antibiotic
Sulfamethazine	pharmaceutical, sulfonamide antibiotic
Sulfamethoxazole	pharmaceutical, sulfonamide antibiotic
N-acetyl sulfamethoxazole	pharmaceutical, transformation product
Trimethoprim	pharmaceutical, antibiotic
Norfloxacin	pharmaceutical, fluoroquinolone antibiotic
Ciprofloxacin	pharmaceutical, fluoroquinolone antibiotic
Enrofloxacin	pharmaceutical, fluoroquinolone antibiotic
Azithromycin	pharmaceutical, macrolide antibiotic
Erythromycin – H ₂ O	pharmaceutical, macrolide antibiotic
Clarithromycin	pharmaceutical, macrolide antibiotic
Roxithromycin	pharmaceutical, macrolide antibiotic
Caffeine	stimulant in beverages, marker compound

Table 3. – continued.

Compound name	Compound group / Usage pattern / Source
<i>Illicit drugs and their metabolites</i>	
6-acetylmorphine	illicit drug
3,4-methylenedioxyamphetamine	illicit drug
Methamphetamine	illicit drug
Cocaine	illicit drug
Benzoylcegonine	illicit drug
Codeine	illicit drug
Methadone	illicit drug
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	illicit drug
Tetrahydrocannabinol	illicit drug
11-Hydroxy- Δ^9 -tetrahydrocannabinol	illicit drug
Δ^9 - Tetrahydrocannabinol carboxylic acid	illicit drug
<i>Plasticizers and flame retardants</i>	
Tributyl phosphate	flame retardant, plasticizer
Triphenyl phosphate	plasticizer, flame retardant
Bisphenol A	plasticizer, xenoestrogen
<i>Surfactants and their TPs</i>	
Benzyltrimethyl decylammonium chloride	cationic surfactant
Benzyltrimethyl dodecylammonium chloride	cationic surfactant
Benzyltrimethyl tetradecylammonium chloride	cationic surfactant
Benzyltrimethyl hexadecylammonium chloride	cationic surfactant
Polypropylene glycols	surfactants
Polyethylene glycols	surfactants
Alcohol polyethoxylates	surfactants
Nonylphenol polyethoxylates	surfactants
Nonylphenoxyacetic acid	surfactant transformation product, xenoestrogen
Nonylphenoxyethoxyacetic acid	surfactant transformation product, xenoestrogen
Octylphenoxyacetic acid	surfactant transformation product, xenoestrogen
Octylphenol	surfactant transformation product, WFD priority, xenoestrogen
Nonylphenol	surfactant transformation product, WFD priority, xenoestrogen

In addition, multiresidue methods were applied for the target analyses of 151 chemical compounds during the European demonstration programme (Table 10) and 226 compounds during the detailed EDA (Table SIII).

Besides reference compounds following chemicals were used:

- methanol, LC-MS analysed, J.T. Baker
- acetonitrile, J.T.Baker, Deventer, Netherlands
- ethylacetate, LiChrosolv®
- dichloromethane, Merck AG, Darmstadt, Germany
- *n*-hexane, p.a., Merck AG, Darmstadt, Germany
- mili Q water, Milipore Corporation, Billerica, USA
- formic acid, Fluka, Buchs, Switzerland
- 7N ammonia in methanol, Sigma-Aldrich
- argon 5.0, $\psi > 99.999\%$ Messer Croatia Plin, Zaprešić
- nitrogen 5.0, $\psi > 99.999\%$ Messer Croatia Plin, Zaprešić

3.1.2. Preparation of working reference standards

Analyte standards (Table 3) were supplied by different suppliers and were as a rule of p.a. purity ($> 99\%$), except for surfactants which were available as commercial complex mixtures. Stock solutions (1 mg/mL and 0.1 mg/mL) of analytes were prepared in appropriate organic solvents (methanol, acetone, acetonitrile, *n*-hexane). Mixed spike solutions (10 $\mu\text{g/mL}$) of all analytes were prepared in methanol, except spike solutions of PAHs and PCBs, which were prepared in *n*-hexane. Calibration curves were obtained by injecting standard solutions containing analytes in the concentration range from 1 to 300 ng/mL.

3.1.3. Materials

In all experiments, glassware was washed with chromsulfuric acid, rinsed with mili Q water and subsequently rinsed with methanol.

The extracts were collected in a 1.8 mL screw-cap glass vials and stored in the dark at 4 °C before the analysis. For the preparation of working reference standards for model experiments single channel micropipettes with replaceable tips were used (Eppendorf, Hamburg, Germany). For evaluation of SPE and fractionation protocols, columns filled with different SPE materials were used: neutral sorbent polystyrene-divinylbenzene co-polymer PS-DVB (Chromabond® HR-X, Macherey Nagel, Dueren, Germany), anionic resin based on the PS-DVB sorbent (Chromabond® HR-XAW, Macherey Nagel, Dueren, Germany), and cationic resin based on the PS-DVB sorbent (Chromabond® HR-XCW, Macherey Nagel, Dueren, Germany). In addition, polymeric sorbent Strata X and the following ion-exchange sorbents, based on modified silica, supplied by Phenomenex (Torrance, USA) were also employed: weak cation-exchange sorbent Strata WCX and weak anion-exchange sorbent Strata NH2 (WAX).

Empty polypropylene SPE tubes with polyethylene frits (20 µm pore size) frits were purchased from Supelco, Sigma-Aldrich, Taufkirchen, Germany. Silica gel 60 (0.063 - 0.200 mm) used for the evaluation of fractionation protocols was purchased from Merck, Darmstadt, Germany.

3.1.4. Instruments

For the evaluation of extraction/fractionation protocols following instruments were used:

- analytical balance ALC 210.4 (Acculab, Bradford, SAD)
- ultrasonic bath Sonis 10 (Iskra PIO.d.d., Šentjernej, Slovenia)
- SPE vacuum manifold system (Supelco, Bellefonte, SAD) equipped with adapters for drying the columns under the nitrogen gas stream
- turbovap evaporator (Turbovap LV, Zymark Corporation)
- ultra-high-pressure liquid chromatograph (LC) (Waters Acquity, Waters Corp., Milford, MA, USA) coupled to quadrupole/time-of-flight mass spectrometer, QTOF Premier (Waters Micromass, Manchester, UK)
- Thermo Electron HPLC system equipped with an autosampler (Surveyor, Thermo Electron, USA) and HPLC pump (MSPump, Thermo Electron) and interfaced to a triple

quadrupole mass spectrometer (MS/MS) (Quantum AM, Thermo Electron, San Jose, SAD)

- gas chromatograph (GC) (Agilent 6890 N) coupled with Agilent 5975 Inert XL mass selective detector (MSD)
- semi-preparative high-performance liquid chromatography (HPLC), Varian ProStar
- large volume sampling and solid-phase extraction device (LVSPE), (UFZ, Leipzig, Germany; Maxx GmbH, Rangendingen, Germany)

3.2. Methods

3.2.1. Description of the extraction/fractionation protocols

Three different approaches for the tier I fractionation that are suitable for simplified EDA protocols were developed and compared. These include:

1) sequential elution of the mixed bed multilayer SPE column using neutral, acidic and basic eluents (sequential elution of sorbed contaminants according to their charge)

2) sequential extraction using a serially coupled SPE columns filled with neutral resin, anion exchanger and cation exchanger, respectively (sequential extraction yielding the corresponding neutral, acid and basic fractions), and

3) silica gel fractionation of the total SPE extract (separation of contaminants according to polarity).

For the evaluation of SPE and fractionation protocols, several parameters were studied such as sorbent selection, type of elution solvent as well as volume of elution solvent.

3.2.1.1. Fractionation protocol 1 (FP1): Sequential elution of the mixed bed multilayer SPE column

The mixed-bed multilayer SPE column was prepared in-house by filling an empty PP tube (6 mL) with 200 mg of neutral sorbent, hydrophobic polystyrene-divinylbenzene copolymer (HR-X), as the first material in the enrichment flow direction, 100 mg of the anion resin (HR-XAW) as the second material, and 100 mg of cation resin as the third material (HR-XCW)

(Figure 3). Similar procedure was proposed by Huntcha et al.,⁵¹ for multiresidue analysis of 88 polar organic micropollutants in ground, surface and wastewater. In order to assess extraction recoveries of selected contaminants, 500 mL of Mili Q water samples (n=3) were fortified with 100 ng/L of standard mixture containing target analytes (Table 3). Procedural blanks (n=3) were also carried out to correct recoveries for possible contamination of the extraction materials. No adjustment of sample pH was performed. Mixed-bed multilayer SPE columns were preconditioned with 10 mL of solvent mixture MeOH/DCM (50:50, v/v), followed by 10 mL of Mili Q water. After sample percolation, mixed-bed SPE columns were washed with 6 mL of Mili Q water and dried for 30 min using nitrogen gas stream. Under the final working conditions, the columns were eluted sequentially with 3 eluents, including 10 mL of MeOH/DCM (50:50, v/v), followed by 10 mL of MeOH containing 2% of ammonia (NH₃) in MeOH and 10 mL of 1.7% formic acid in MeOH. The extracts (fractions) were reduced in volume to approximately 1 mL using a Turbovap evaporator, divided into two identical aliquots, transferred to a 1.8 mL screw cap vial and then carefully evaporated to the dryness using nitrogen gas stream. The residue was dissolved in 0.5 mL of Mili Q water/MeOH (50:50, v/v) for further LC-MS analysis (LC-QTOF) and in 0.5 mL of DCM for GC-MS analysis.

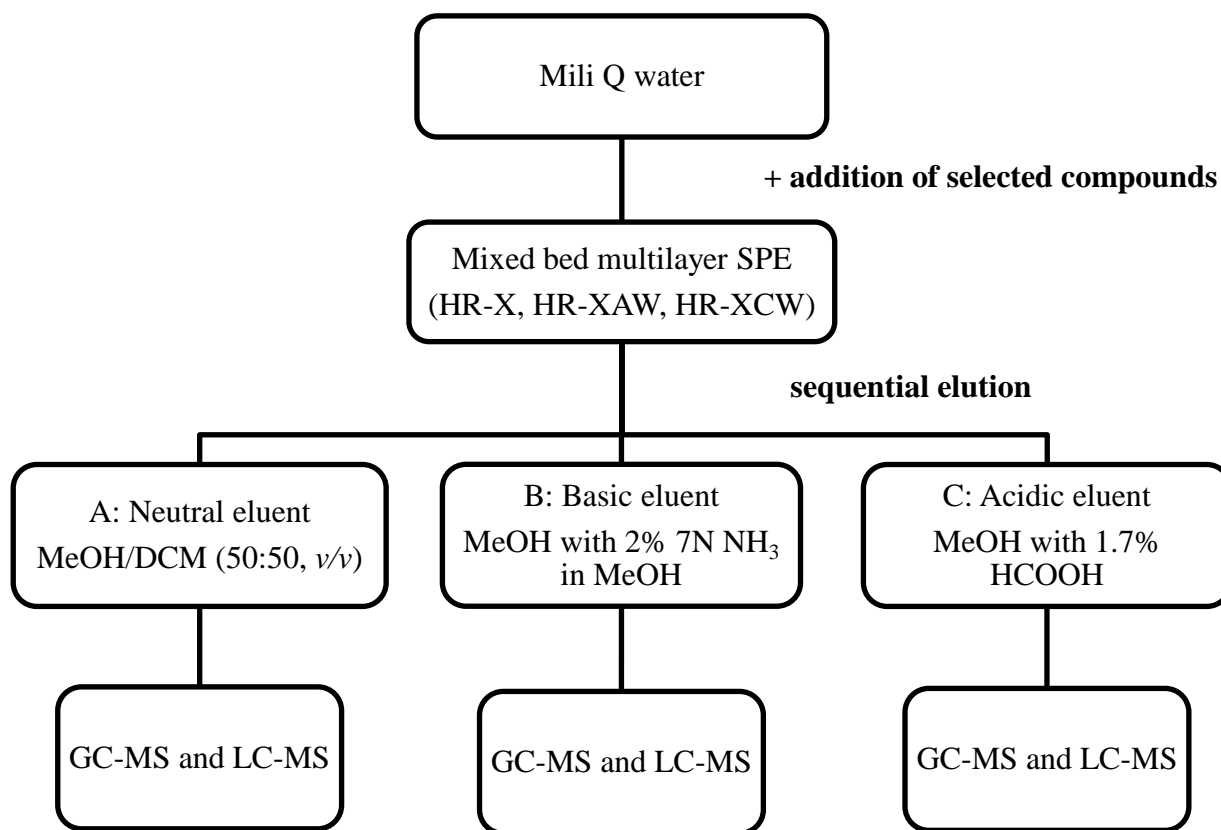


Figure 3. Protocol for the sequential elution of the mixed bed multilayer SPE column.

3.2.1.2. Fractionation protocol 2 (FP2): Sequential extraction using serially coupled SPE columns

A modified SPE protocol employing sequential extraction was performed using three separate SPE columns prepared in house by filling an empty PP tubes (6 mL). The first column was filled with 200 mg of neutral resin (HR-X), the second with 100 mg of anion resin (HR-XAW) and the third with 100 mg of cation resin (HR-XCW). The columns were connected in series (Figure 4). The conditioning and elution of the columns was performed separately for each column. HR-X cartridge was pre-conditioned with 10 mL of solvent mixture MeOH/DCM (50:50, v/v), followed by 10 mL of Mili Q water, while HR-XAW and HR-XCW columns were pre-conditioned with 10 mL of MeOH followed by 10 mL of Mili Q water. Water samples were fortified (n=2) prior to the extraction with 100 ng/L of standard mixture containing target analytes (Table 3). In parallel, procedural blanks (n=2) were

performed. No adjustment of sample pH was performed. After sample percolation, cartridges were separately washed with 6 mL of Mili Q water and dried for 30 min using nitrogen gas stream. Under the final working conditions, the column filled with HR-X sorbent was eluted with 10 mL of solvent mixture MeOH/DCM (50:50, v/v), HR-XAW column was eluted with 10 mL of MeOH with 2% NH₃ in MeOH, while HR-XCW column was eluted with 10 mL of MeOH with 1.7% formic acid. As previously described, all extracts/fractions were reduced in volume to approximately 1 mL using a Turbovap evaporator, divided into two identical aliquots, transferred to a 1.8 mL screw cap vial and then carefully evaporated to the dryness using nitrogen gas stream. The residue was dissolved in 0.5 mL of Mili Q water/MeOH (50:50, v/v) for further LC-MS (LC-QTOF) analysis and in 0.5 mL of dichloromethane for GC-MS analysis.

Alternatively, a similar protocol using silica-based SPE materials as ion-exchange sorbents was tested. In these experiments, ion-exchange sorbents, based on modified silica gels (weak cation-exchange, Strata-WCX; weak anion-exchange, Strata WAX) were coupled in series with the neutral polymeric sorbent Strata X in the following order: WCX, WAX and Strata X. The conditioning and elution of the columns were performed separately for each column. Before use, each column was pre-conditioned with 10 mL of MeOH. Two different series of experiments were performed: a) fractionation of the selected target contaminants using the multi-sorbent system described above, after their direct addition to the top of the first SPE column (WCX) in a methanol solution, followed by sequential percolation of 10 mL MeOH through WCX, WAX and Strata X cartridges (consequently, this experiment was supposed to show the fractionation potential of the individual sorbents for individual contaminants and does not include extraction efficiency from water samples) and b) extraction and fractionation of the selected contaminants by sequential percolation of model water samples, prepared by spiking 500 mL ultrapure water at the concentration of 100 ng/L, through the sorbents. All experiments were performed in triplicate. In parallel, procedural blanks (n=3) were performed.

After sample percolation, the cartridges were separately washed with 6 mL of Mili Q water and dried for 30 min using nitrogen gas stream. Under the final working conditions, the WCX cartridge was eluted with 10 mL of MeOH containing 1% formic acid, followed by 10 mL of MeOH containing 2% of 7N NH₃. The Strata NH₂ cartridge was first eluted with 10 mL of MeOH containing 2% 7N NH₃ in MeOH, followed by 10 mL of MeOH containing 1%

formic acid. Finally, the Strata X cartridge was eluted with 10 mL of ethylacetate, followed by 10 mL of MeOH, 10 mL of acidified MeOH (1% formic acid) and 10 mL basic MeOH (2% 7N NH₃). As previously described, all extracts/fractions were collected, reduced in volume to approximately 1 mL using a Turbovap evaporator, divided into two identical aliquots, transferred to a 1.8-mL screw cap vial and then carefully evaporated to the dryness using nitrogen gas stream. The residue was dissolved in 0.5 mL of Mili Q water/MeOH (50:50, v/v) for further LC-MS (LC-QTOF) analysis and in 0.5 mL of dichloromethane for GC-MS analysis.

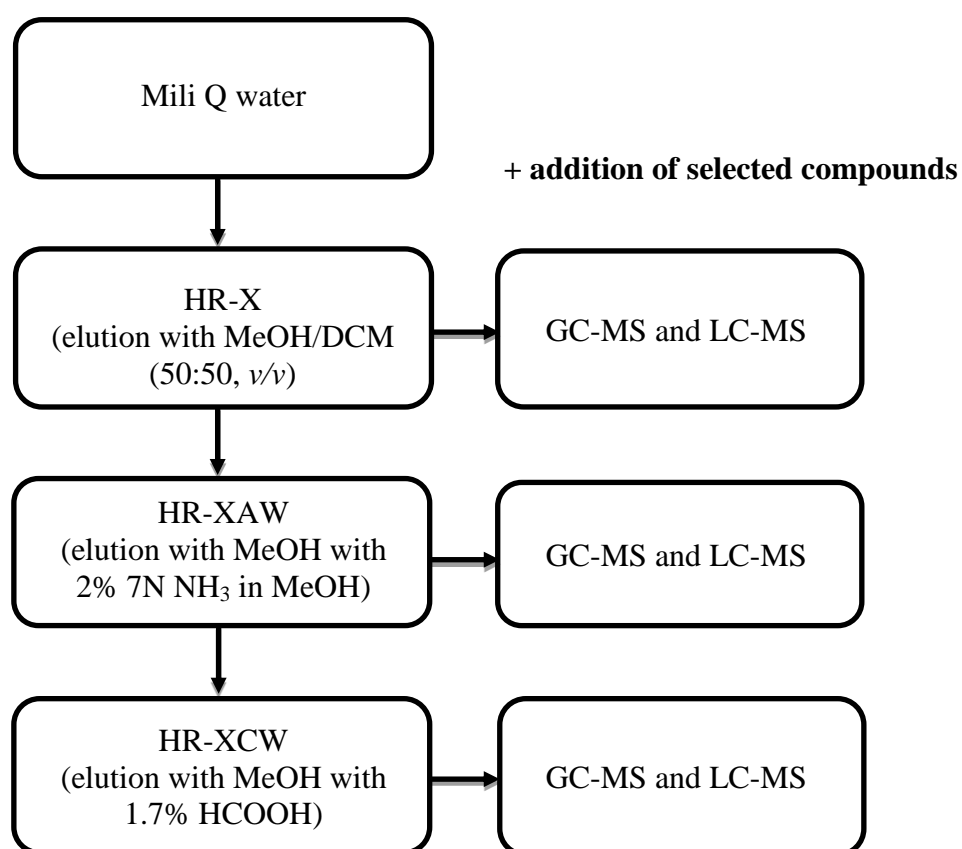


Figure 4. Protocol for the sequential extraction using serially coupled SPE columns.

3.2.1.3. Fractionation protocol 3 (FP3): Silica gel fractionation of the total SPE extract

Mili Q water (500 mL) was fortified with 100 ng/L of individual contaminants and passed through the mixed-bed multilayer SPE column. The procedure for column preparation, conditioning and elution is described above. After elution, the combined total extracts were reduced in volume to approximately 1 mL using a Turbovap evaporator, transferred to a 1.8 mL screw cap vial and then carefully evaporated to the dryness using a nitrogen gas stream.

The fractionation of the total extract was performed by applying normal-phase chromatography using silica gel as stationary phase (particle size 0.063-0.200 mm) deactivated with 15% of Mili Q water (w/w) (Figure 5). Prior to filling with the silica gel, the column was washed with MeOH, *n*-hexane and DCM respectively, and dried using nitrogen gas stream. Deactivated silica gel (5 mL) was carefully applied onto the chromatography column filled with *n*-hexane avoiding the formation of air bubbles. The evaporated extract, re-dissolved in *n*-hexane (1-2 mL) was applied to the top of the silica gel column (5 cm 1.0 cm i.d.). Three different solvents were used for elution of target compounds: *n*-hexane, DCM and MeOH according to Smital et al.²³ The non-polar compounds were eluted with 25 mL of *n*-hexane. The remaining residue in 1.8 mL vial was re-dissolved in 1–2 mL of DCM and transferred to the silica gel column, and subsequently the medium-polar compounds were eluted with 25 mL of DCM. The third (polar) fraction was obtained by repeating the same procedure with 25 mL of MeOH as residue solvent and eluent. Each of the fractions were reduced in volume using a Turbovap evaporator and transferred into 1.8 mL vials, and then carefully evaporated to the dryness using nitrogen gas stream. The residue was dissolved in 0.5 mL of water/methanol (50:50, v/v) for further LC-MS analysis (LC-QTOF) and in 0.5 mL of dichloromethane for GC-MS analysis.

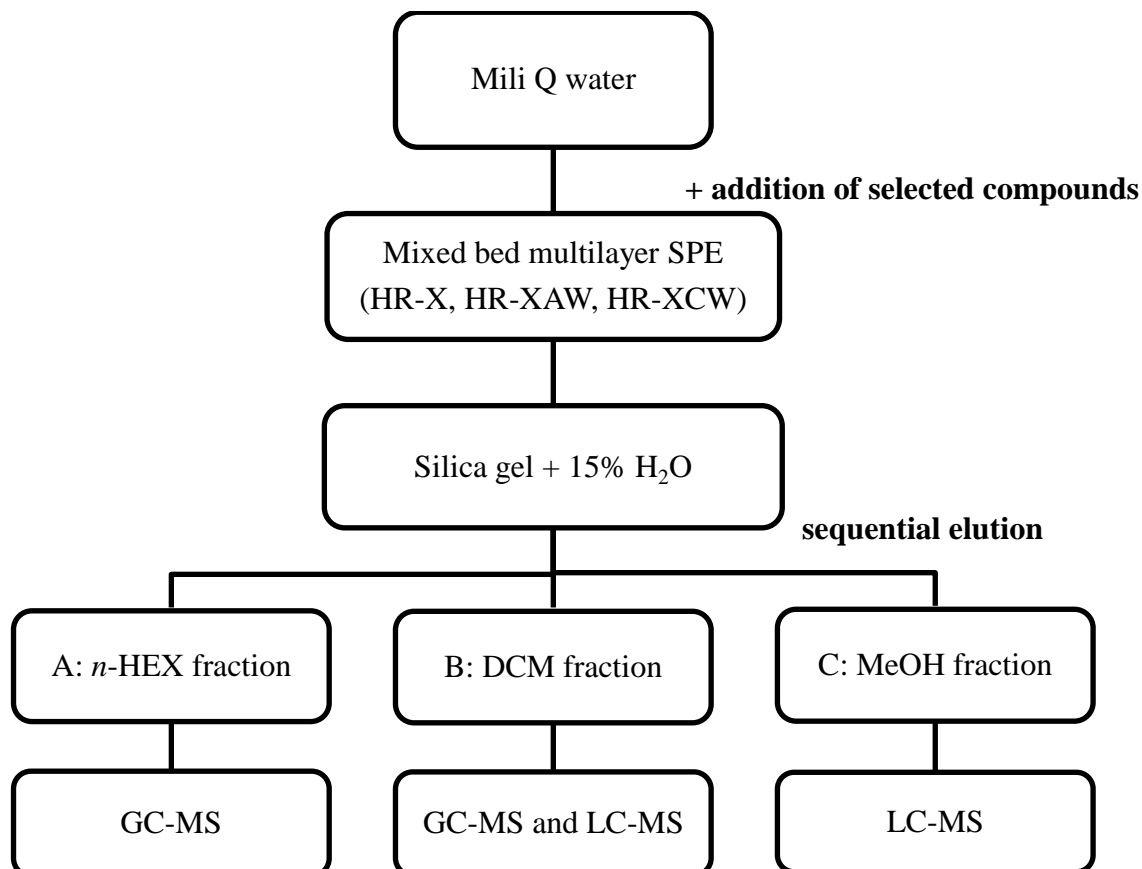


Figure 5. Protocol for the silica gel fractionation of the total mixed bed SPE extract.

3.2.2. Field experiment on the Sava River using simplified effect-directed analysis

In the context of implementation of EU WFD there is a growing demand for operational and investigative monitoring in different river basins across the Europe. As a consequence, both the scientific community and water practitioners recognised the importance of tiered approach for the assessment of hazardous organic contaminants. This comprises development of a simplified and cost-effective EDA protocol, making it feasible to cope with the requisite high sample throughput in basin-wide monitoring programs.

As a part of the EU-funded EDA-EMERGE project, a joint European demonstration programme (EDP) was conducted during 2013, with the aim of monitoring river waters throughout Europe and demonstrating the benefits of EDA in comparison to traditional monitoring programs which focuses on chemical analysis only. Four pairs of EDA-EMERGE partners showed the applicability of the methods which are being developed within the

framework of this project. Using the simplified protocol, which contained all necessary steps from planning of the sampling campaign to the reporting and interpretation of output data, with close collaboration of partner institutions, EDP was conducted at four sampling sites on the Sava River section situated in the wider area of the city of Zagreb. A simplified EDA protocol featuring a novel on site large-volume solid phase extraction device with limited fractionation, a selected set of bioassays and chemical analysis was applied for characterization of the Sava River basin.

3.2.2.1. Field sampling

Sampling was carried out from August to September 2013 using both grab sampling and the large volume sampling device (LVSPE). In addition, 2 L grab samples were collected and immediately shipped (at 4 °C) to laboratory for an *in vivo* thyroid activity assay. LVSPE sampling enabled extraction of 50 L of river water samples including primary on site fractionation into three fractions based on the affinity of sorbents to distinct compound groups.

The sampling using LVSPE was performed according to the method described by Schulze et al.³⁵ The LVSPE device was equipped with 3 serially coupled columns filled with neutral sorbent (HR-X), anionic resin (HR-XAW) and cationic resin (HR-XCW), respectively. Briefly, water sample entering the device was filtered via a 0.63 µm glassfiber (Sartopure® GF+ MidiCap, Sartorius) and then pressurized through three sorbent cartridges mounted in a sequence. The first cartridge filled with 8 g of neutral sorbent PS-DVB copolymer to capture neutral and semi-polar compounds was followed by the second cartridge, filled with 3.5 g of anionic resin based on the PS-DVB sorbent, to capture acidic compounds, and the third column filled with 3.5 g of cationic resin also based on the PS-DVB sorbent, to capture basic compounds that are cationic at water pH ranging from 6 to 8. In this way, an exhaustive retention of compounds having a wide range of polarities could be expected along with an initial fractionation on neutral, acidic and basic fraction, respectively.

Prior to the field sampling, all apparatus parts were cleaned with MeOH. The conditioning and elution of the cartridges was performed separately for each column (Table 4). After conditioning and sampling, the columns were stored and transported at 4 °C, while the openings were covered with aluminium foil to avoid contamination and drying of the wet

sorbent. After sampling, the cartridges were separately dried using a nitrogen gas stream to purge residual water and subsequently subjected to freeze drying for around 8 h. The elution of the cartridges was carried out according to Table 4.

Table 4. Protocol for the preparation, conditioning and extraction using LVSPE device.

	LVSPE
Solid phases	HR-X (8 g) HR-XAW (3.5 g) HR-XCW (3.5 g)
Conditioning	HR-X - 200 mL MeOH/EtAc (50:50, v/v) - 100 ml Mili Q water HR-XAW - 100 mL MeOH - 100 ml Mili Q water HR-XCW - 100 mL MeOH - 100 ml Mili Q water
Extraction	HR-X - 200 mL MeOH/EtAc (50:50, v/v) HR-XAW - 100 mL MeOH with 2% 7N ammonia in MeOH HR-XCW - 100 mL MeOH with 1.7% formic acid

MeOH - methanol, EtAc - ethyl acetate, HR-X - neutral sorbent polystyrene-divinylbenzene co-polymer; HR-XAW - anionic resin based on the polystyrene-divinylbenzene co-polymer; HR-XCW - cationic resin based on the polystyrene-divinylbenzene co-polymer; during sampling the sorbents were assembled in the order HR-X, HR-XAW and HR-XCW.

The extracts were divided into 13 aliquots according to the volume requirements of each bioassay and chemical analyses. The aliquots were evaporated to dryness using nitrogen stream. Dried extracts were stored at -18 °C until reconstitution in a solvent, followed by biological and chemical analyses. Two blank samples were prepared for the LVSPE sampling method. Extraction cartridges filled with clean sorbents were conditioned and extracted accordingly to obtain fabrication blanks. Laboratory blank was prepared by percolation of 2 L of demineralized LC-grade distilled water through the LVSPE device for 100 cycles, which is equivalent to sampling of 50 L. Both blanks were otherwise treated identically to the field

samples. In the field, the weather conditions as well as the main physico-chemical parameters of the sampled water (temperature, pH, dissolved oxygen, conductivity) were recorded. For chemical analyses an equivalent of 1 L of river water was required.

3.2.3. Field experiment on the Sava River using detailed EDA protocol

In order to further investigate which compounds are responsible for the detected toxic effects, a detailed EDA protocol was applied for the characterization of organic contaminants in the Sava River. The field experiment using the detailed protocol was similar to the simplified EDA but included an additional HPLC fractionation step followed by both biological and chemical analyses of the obtained fractions.

3.2.3.1. Field sampling for the detailed EDA protocol

Sampling was carried out during May and June 2015, using the large volume sampling device described above. For the full-scale EDA, 100 L of river water samples and 50 L for the wastewater samples were processed using LVSPE device. In order to avoid saturation and/or clogging of the sorbents, two sets of cartridges (HR-X, HR-XAW, HR-XCW) were used for river water samples. In addition to the field sampling, fabrication blank was prepared in the laboratory by extracting 1 L of tap water. The protocol for LVSPE device preparation, conditioning, sampling and extraction is described in detail above and summarized in the Table 4.

After extraction, the extracts were combined, reduced in volume to 200 mL using rotary evaporator (Buchi, Switzerland), and subsequently divided into three aliquots for further analyses. The first aliquot (60 mL), used for the initial characterisation of the total extract, was subsequently divided into six subaliquots for biological and chemical analyses. The second aliquot (100 mL) was used for the detailed HPLC fractionation, while the third aliquot (40 mL) was kept as back-up sample. The aliquots were evaporated to dryness using nitrogen gas stream. Dried extracts were stored at -18 °C until reconstitution in a solvent.

3.2.3.2. HPLC fractionation of the EDA samples

The total extracts were subjected to a detailed fractionation using semi-preparative high-performance liquid chromatography (HPLC) using a Varian ProStar instrument with a Model 410 autosampler, Model 330 photodiode array detector and Model 704 fraction collector. The extracts were separated by preparative reverse-phase chromatography using an octadecyl silica (C18) column (250 x 10 mm). A binary gradient at a mobile phase flow of 5 mL/min was applied. The mobile phase A consisted of a mixture of water and acetonitrile (90:10, v/v) and the mobile phase B was pure acetonitrile. The following gradient elution was applied: 0-10 min: 100% A; 10-15 min: the percentage of B linearly increased from 0 to 10%; 15-30 min: the percentage of B linearly increased from 10 to 100%; 30-40 min: 100% A. One mL of the extract was applied to the HPLC column. The total run time for HPLC separation was 40 min and the fractions were collected in time intervals of 1 min. For the collection of the fractions 12 mL glass tubes were used. In total, 37 fractions were collected. All subfractions were centrifuged (Sigma 3 - 16, Fisher Bioblock Scientific, United Kingdom) at 1000 x g for 1-2 min to spin down. The fractions were evaporated to dryness, redissolved in 10 mL of MeOH and divided into six aliquots for chemical and biological analyses.

3.2.4. Chemical analyses

3.2.4.1. LC-MS analyses

UHPLC-QTOF analyses. The analyses were performed using ultra-performance liquid chromatography (UPLC, Waters Corp., Milford, MA, USA) coupled to quadrupole-time-of-flight mass spectrometry (QTOF Premier, Waters Corp., Milford, MA, USA) equipped with an electrospray ionization source. The chromatographic separation was performed using 2.1 x 50 mm Acquity UPLC column filled with 1.7 μ m BEH C18 stationary phase (Waters Corp., Milford, MA, USA) at a flow rate of 0.4 mL/min. The column was maintained at room temperature. The injection volume was 10 μ L. In the positive ionization mode (PI) mobile phase consisted of eluent A containing 0.1% (v/v) formic acid in water, and eluent B consisting of 0.1% (v/v) formic acid in acetonitrile. The gradient elution started at 5% B for 1 min after which the percentage of B increased linearly in several steps up to 95% B in 19 min

and was kept at those conditions for another 2 min. The duration of the acquisition time together with the column conditioning to reach initial conditions was 23 min. In negative ionization mode (NI), eluents A and B were water and acetonitrile, respectively, without addition of formic acid, with the same elution gradient used in the PI mode. The parameters for the mass spectrometry on a QTOF Premier instrument were set as follows: the desolvation gas flow - 600 L/h, at the temperature of 280 °C, the cone gas flow - 30 L/h, source temperature - 120 °C. The capillary voltages in the PI and NI mode were 3500 V and 3000 V, respectively, while the cone voltage in both modes was set to 30 V. The mass spectrometer using a Z-spray-electrospray interface was operated in a V mode (range m/z 100-1000), applying collision energy of 4 eV. All spectra were recorded using extended dynamic range (DRE) option in order to correct for possible peak saturations and the data were collected in the centroid mode with a scan time of 0.08 s and interscan time of 0.02 s. In order to ensure maximum accuracy and reproducibility of the system, all acquisitions were carried out using an independent reference spray via the lock spray interface. Leucine enkephalin was applied as a lock mass both in PI (m/z 556.2771) and NI mode (m/z 554.2615). Instrument control, data collection, accurate mass and elemental composition of the precursor ions were calculated using the MassLynx software.

LC-ESI-MS/MS method for the determination of alkylphenolic compounds was adapted from Petrovic et al.¹³⁵ The final extracts were reconstituted in MeOH. The analysis was performed on a Thermo Electron HPLC system equipped with an autosampler (Surveyor, Thermo Electron, USA) and HPLC pump (MS Pump, Thermo Electron) interfaced to a triple quadrupole mass spectrometer (Quantum AM, Thermo Electron) equipped with an electrospray ionization source. Chromatographic separation of the analytes was done using Synergy Fusion 4- μm RP 80-Å column (Phenomenex, 3 x 150 mm) at a flow rate of 0.4 mL/min. The eluents used for the separation included water and MeOH. The injection volume was 10 μL . Multiple reaction monitoring (MRM) in negative ionization polarity (NI) was applied for the detection. Parameters of the ion source were as follows: spray voltage 3000 V, capillary temperature - 350°C, desolvation gas pressure - 40 arbitrary units, auxiliary gas pressure - 10 arbitrary units. Other source parameters were automatically tuned for maximal intensity of particular analyte and internal standard.

LC-(+/-)ESI-Orbitrap-HRMS was used for target screening of polar compounds using the method described in Hug et al.¹³⁶ Extracts were reconstituted in nanopure water:

acetonitrile (90:10, v/v). An Agilent 1200 LC coupled to a Thermo LTQ Orbitrap XL (Thermo Scientific) was used in both positive and negative electrospray ionization mode. The reversed-phase liquid chromatography separation was performed on a Kinetex Core shell C18 column column (3.0 ×100 mm; 2.6 μm particle size; Phenomenex) using a water-MeOH gradient (both with 0.1% formic acid) at a flow rate of 0.2 mL/min. The column was maintained at 22 °C. The injection volume was 10 μL. Full scan spectra were acquired in profile mode in a range of 100 to 1000 m/z at a nominal resolving power of 100,000 (FWHM) referenced to m/z 400. A mass accuracy < 7 ppm was assured over the whole mass range by external mass calibration using a calibration solution for the range from 138 to 1721 m/z. Data obtained was processed using Xcalibur software.

LC-(+/-)MS/MS method was used for the analysis of important endocrine disruptors employing an Agilent 1260 LC system coupled to a QTrap 6500 system (ABSciex) as described in Konig et al.¹³⁷ Extracts were reconstituted in nanopure water: acetonitrile (90:10, v/v). For the analysis of compounds in negative ionization mode a Kinetex C18 column (100 x 3.0 mm, 2.6 μm particle size, Phenomenex) was used. Gradient elution was done using 1 mM ammonium fluoride (A) and methanol (B) at a flow rate of 0.35 mL/min at 30°C. The injection volume was 10 μL. Spray voltage was -3.6 kV, temperature 380°C, nebulizer gas 60 psig, heater gas 60 psig, curtain gas 50 psig, and entrance potential -10.0 V. For the analysis of compounds in positive ionization mode the same LC column was used with 0.1% formic acid (A) and methanol containing 0.1% formic acid (B) at a flow rate of 0.35 mL/min at 30°C. The injection volume was 5 μL. The ion source settings were: spray voltage 3.9 kV, temperature 380°C, nebulizer gas 60 psig, heater gas 60 psig, curtain gas 50 psig, and entrance potential 10.0 V. The MS was operated in scheduled MRM mode. Data obtained was processed using Multiquant software 3.0 (ABSciex).

LC-(+/-)ESI-Orbitrap-HRMS/MS was used for determination of large number of various polar micropollutants according to the method described by Schymanski et al.¹³⁸

LC-(+)ESI-Orbitrap-HRMS/MS was used for the determination of glucocorticoids according to Schriks et al.¹³⁹

LC-QqQ-MS/MS was used for the determination of diglyme, triglyme, tetraglyme and aclonifen according to Tousova et al.¹⁴⁰

3.2.4.2. GC-MS analyses

GC-MS analyses were performed using an Agilent GC-MS system (Agilent 6890 N gas chromatograph interfaced with a single quadrupole mass detector 5975 Inert XL MSD). For compound separation 30 m x 0.25 mm x 0.25 µm DB-5MS column (Agilent Technologies) was used. After splitless injection of 1 µL of the extract using a PTV injector and a 7683B autoinjector module, the compounds were separated using temperature programming from 50 °C to 290 °C at a rate of 5 °C/min. Helium was used as carrier gas. The samples were acquired in selected ion mode (SIM).

GC-EI-MS was used for the determination of several legacy pesticides, WFD priority compounds and industrial compounds according to Tousova et al.¹⁴⁰

3.2.5. Biological analyses

3.2.5.1. Toxicological characterization of the samples

A set of 12 bioassays was applied to screen for both non-specific and specific toxicity of the water samples, including both *in vitro* and *in vivo* methods. It includes: ER-mediated activity (MELN cells) assay, AR-mediated activity (MDA-kb2 cells) assay, GR - CALUX® assay, algal growth inhibition assay, zebrafish embryo acute toxicity assay (FET), Ames test, *in vivo* thyroid activity, acetylcholine esterase (AChE) inhibition assay, MTT test, AlgaeTox test, EROD test and *in vivo* ChgH-gfp medaka test. All bioassay analyses were carried out by the specialized partner ecotoxicological laboratories (Table 5). A detailed description of the bioassay methods and test conditions is given in the Supplementary material (Anex I).

Table 5. List of partner ecotoxicological laboratories and bioassays.

Bioassay name	Bioassay type	Laboratory / Country
ER-mediated activity – MELN cells	<i>In vitro</i>	Institut National de l'Environnement Industriel et des Risques (INERIS) / France
AR-mediated activity – MDA-kb2 cells	<i>In vitro</i>	Institut National de l'Environnement Industriel et des Risques (INERIS) / France
GR - CALUX® assay	<i>In vitro</i>	Swiss Federal Institute of Aquatic Science & Technology (EAWAG) / Switzerland
Zebrafish embryo acute toxicity assay –FET assay	<i>In vivo</i>	RWTH Aachen University / Germany
AMES assay	<i>In vitro</i>	RWTH Aachen University / Germany
Acetylcholine esterase (AChE) inhibition assay	Enzymatic reaction	Norwegian Institute for Water Research (NIVA) / Norway
EROD activity	<i>In vitro</i>	Rudjer Boskovic Institute (IRB), Laboratory for Molecular Ecotoxicology / Croatia
MTT cytotoxicity test	<i>In vitro</i>	Rudjer Boskovic Institute (IRB), Laboratory for Molecular Ecotoxicology / Croatia
AlgaeTox assay	<i>In vivo</i>	Rudjer Boskovic Institute (IRB), Laboratory for Molecular Ecotoxicology / Croatia
Algal growth inhibition assay	<i>In vivo</i>	Environmental institute (EI) / Slovakia
ChgH-gfp medaka test	<i>In vivo</i>	Watchfrog / France
In vivo thyroid activity	<i>In vivo</i>	Watchfrog / France

3.2.5.2. Linking *in vitro* effects and detected compounds

For receptor mediated *in vitro* assays, mass balance calculations were conducted using relative effect potencies (REPs) of known agonists according to Kinani et al.¹⁴¹ to quantify the contribution of the detected target compounds to the observed biological activity. In brief, chemical equivalents (chem-EQ) were calculated by multiplying the measured concentration of a known agonist with its REP, as reported in earlier studies (Table 12 and Table 15). The

sum of chem-EQs of all agonists at each sampling site was then divided by the measured biological equivalent and the resulting ratio indicated how much of the biological effect could be explained by detected chemicals.

3.3. Selection and characterization of sampling sites for the field study in the Sava River

The Sava River (945 km) is the biggest tributary to the Danube River and represents natural north-western boundary of the Balkan Peninsula connecting and flowing through four countries: Slovenia, Croatia, Bosnia and Herzegovina, and Serbia. The area of the Sava River basin for these four countries is 97.713 km² which is about 40% of their land and source for 80% of total available water. Therefore, the monitoring activities and water quality assessments of the Sava River basin water is a strategic priority for all riparian countries.¹¹⁶

Due to the well-defined gradient of pollution, starting at low to-moderately polluted sites before the city of Zagreb (800.000 inhabitants, heavily industrialized) up to sites characterized by the pollution load from the Zagreb and Sisak city areas, the 120 km section of the Sava River was selected for the study (Figure 6).

A total of four sampling sites were selected for the EDP study: Samoborski Otok, Podsused, Oborovo and Sisak Crnac site. Overview of the sampling sites and major anthropogenic pressures is given in Table 6.

Table 6. Overview of the EDP sampling sites and major anthropogenic pressures.

	<i>Site/Location</i>	<i>Coordinates</i>	<i>Site characterization and major pressures</i>
<i>Sava RB</i>	Samoborski Otok (SO)	45.843083N 15.729167E	10 km upstream of the city of Zagreb, upstream reference location to the downstream sites affected by various pollution sources in the area of the city of Zagreb; a checkpoint for transboundary pollution from Slovenia ⁸³
	Podsused (PO)	45.793583N 15.852783E	Situated in the western part of the city of Zagreb; about 5 km downstream from the discharge point of the wastewaters from the WWTP of the city of Zaprešić, including significant contribution of effluents from pharmaceutical industry; marked input of macrolide antimicrobials ¹⁴¹
	Oborovo (OB)	45.686450N 16.246900E	Several km downstream of the main wastewater outlets of WWTPs of the cities of Zagreb (800,000 inh.) and Velika Gorica (60,000 inh.); The wastewaters of both cities are of the mixed type, including significant contribution of industrial WWs; both WWs receive full mechanical and biological treatment before the discharge
	Sisak Crnac (SC)	45.445267N 16.419267E	2 km downstream from the city of Sisak (50,000 inhabitants), affected by urban WW, iron works and oil refinery; notable petroleum hydrocarbon pollution in water and sediments ⁸³

WW - wastewater; WWTP – wastewater treatment plant.

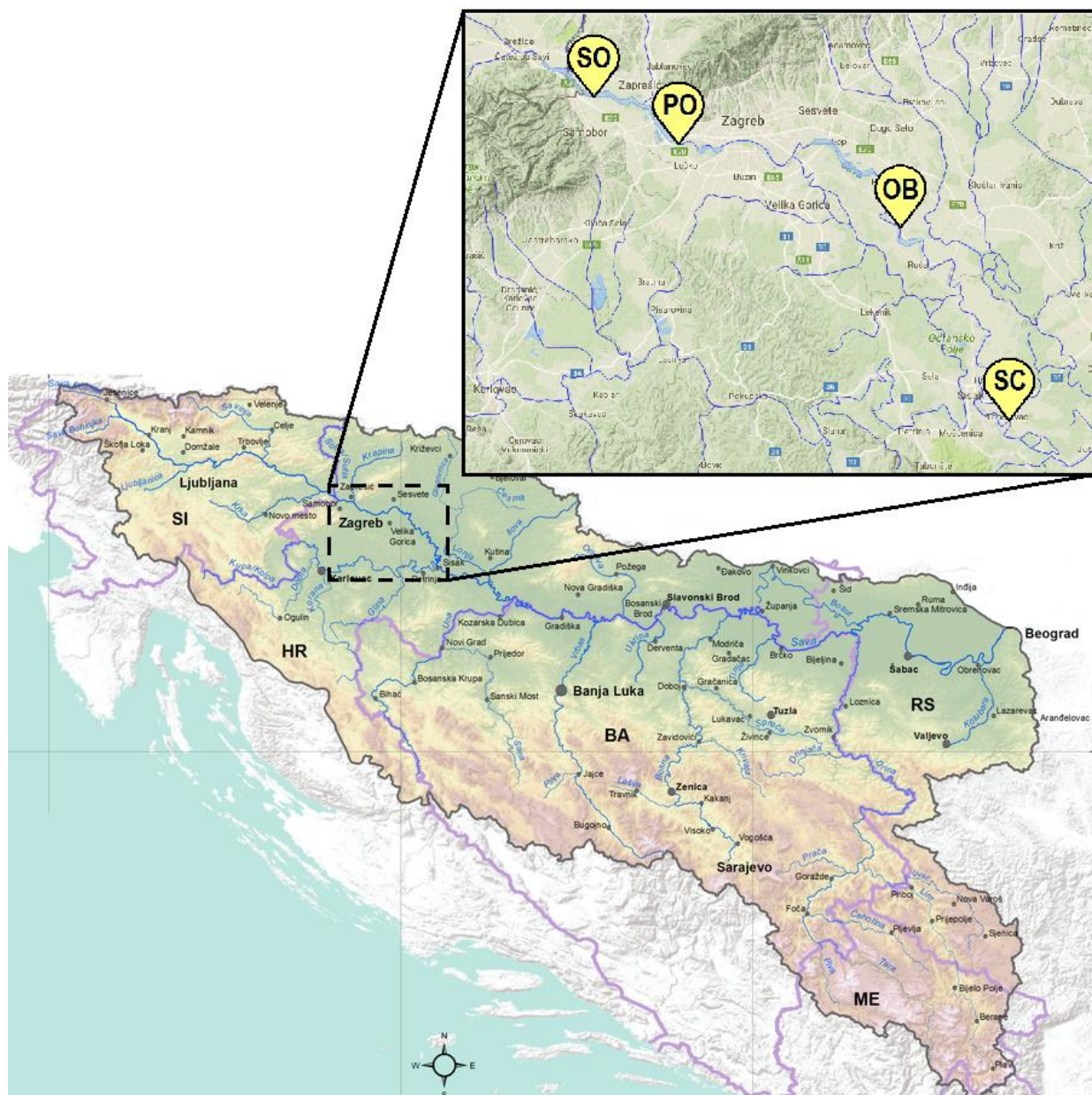


Figure 6. Overview map of the EDP sampling sites along the Sava River basin.

Based on the previous insights obtained during the EDP study, the 2 characteristic locations on the Sava River, Podused and Oborovo were selected as a possible goal for a full-scale EDA analysis, and one additional location on the Sava River (Sava Hruščica).

In addition, in order to link the situation in the river with the major inputs, three dominant wastewater inputs into the investigated Sava River section (Zaprešić, Hruščica canal, and Velika Gorica,) were also included (Figure 7). Description of the selected sites for

the application of the detailed EDA protocols for analysis of organic contaminants in the Sava River is given in the Table 7.

Table 7. Description of the sampling sites of wastewater effluents and river water for detailed EDA.

<i>Site</i>	<i>Coordinates</i>	<i>Site characterization and major pressures</i>
Zaprešić (ZA) Wastewater effluent	45.830333N 15.815361E	The discharge point of the pharmaceutical industry into the Sava River, near the city of Zaprešić; only mechanically treated
Sava-Podsused (PO) River water	45.793583N 15.852783E	Situated in the western part of the city of Zagreb; about 5 km downstream from the discharge point of the wastewaters from the WWTP of the city of Zaprešić, including significant contribution of effluents from pharmaceutical industry; marked input of macrolide antimicrobials ¹⁴¹
Hruščica canal (HC) Wastewater effluent	45.784764N 16.133496E	The main outlet of WWTP effluent of the city of Zagreb (about 3 m ³ /s; biologically treated) and some portion of untreated WW from the eastern suburbs of the city of Zagreb
Sava-Hruščica (H) WWTP	45.779919N 16.138961E	Situated 700 m downstream of the Hruščica canal discharge point
Velika Gorica (VG) Wastewater effluent	45.71553N 16.09208E	Secondary effluent from the WWTP of the city of Velika Gorica (60,000 inh.) – influent WW is of mixed type, municipal and industrial with possible contribution from the Zagreb international airport; biologically treated, poor efficiency
Oborovo (OB) River water	45.686450N 16.246900E	Several km downstream of the main wastewater outlets of WWTPs of the cities of Zagreb (800,000 inh.) and Velika Gorica (60,000 inh.); The wastewaters of both cities are of the mixed type, including significant contribution of industrial WWs; both WWs receive full mechanical and biological treatment before the discharge

WW - waste water; WWTP - wastewater treatment plant.

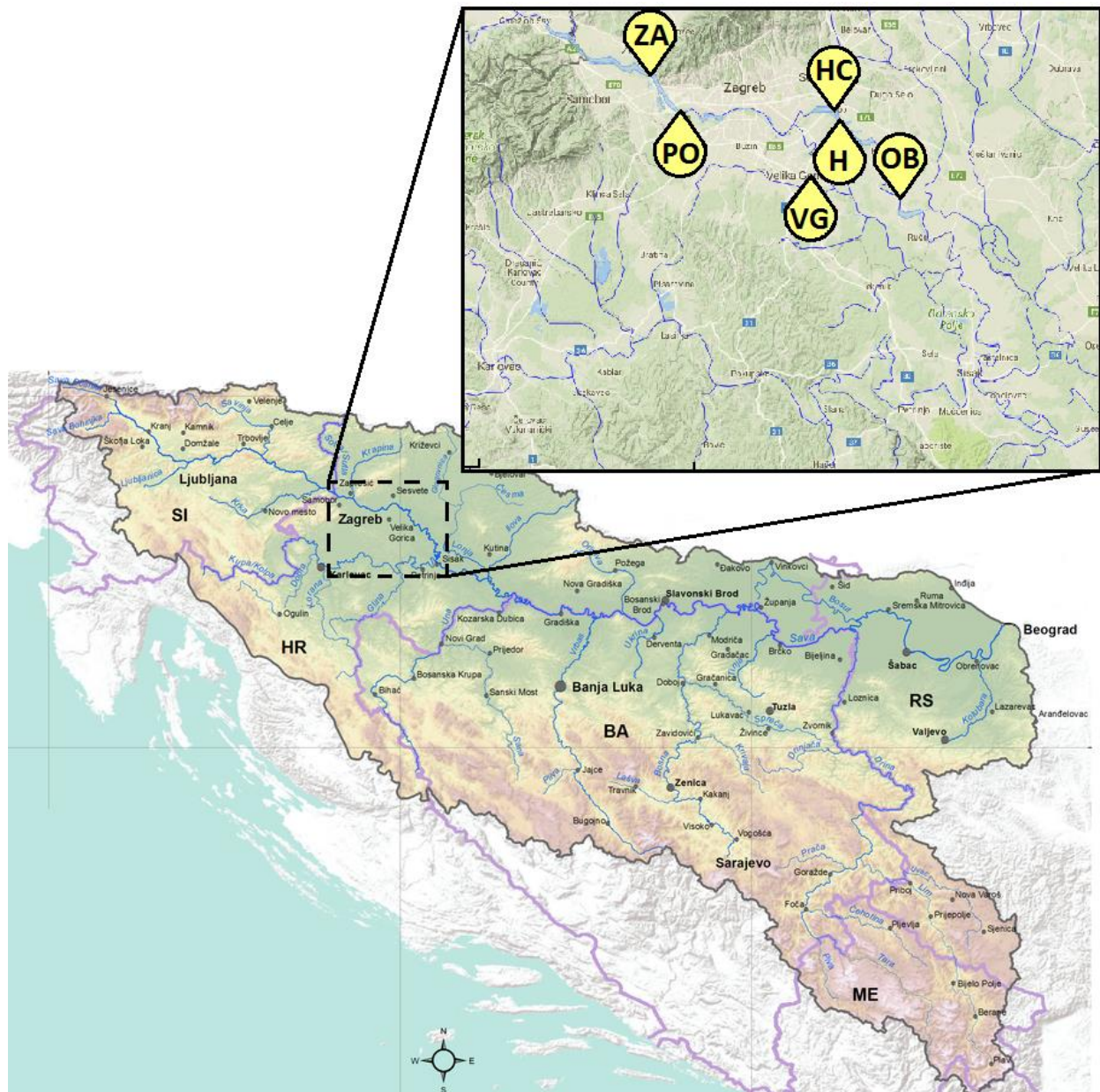


Figure 7. Overview map of the detailed EDA sampling sites.

§ 4. RESULTS AND DISCUSSION

4.1. Evaluation of comprehensive extraction/fractionation protocols

The key prerequisite for a comprehensive and successful EDA procedure is the combination of an exhaustive extraction with a well-designed fractionation procedure. The full-scale EDA however is a very demanding procedure since it involves extensive fractionation of the extracts, leading often to a large number of sub-fractions, which need to be analysed by sophisticated chemical methods. Consequently, this complex and laborious approach is not suitable for a systematic application in large monitoring studies. Significant progress in the assessment of priority pollutants could be achieved by introducing simplified EDA protocols, which comprise effect characterization and chemical screening based on a smaller number of well-defined fractions.

Three different approaches for the tier I fractionation that are suitable for simplified EDA protocols were developed and compared in this study. The comparison is based on the study of a broad spectrum of organic contaminants, including classic contaminants as well as several classes of polar emerging contaminants (Table 3 and Table SI). A special emphasis was on the assessment of recoveries of individual contaminants, which is essential for the reliable interpretation of the effects observed in the original sample. Obviously, the biggest challenge for the optimization of extraction/fractionation protocols was a wide range of physico-chemical properties in terms of volatility, hydrophobicity and water solubility of the target compounds. As a consequence, the chosen experimental conditions could not be set to reach the maximum performance in terms of sensitivity and selectivity for each individual compound class, but rather represent a compromise for simultaneous analysis of different compound classes present in complex mixtures.

4.1.1. Comparison of two fractionation protocols - sequential elution vs. sequential extraction

In order to investigate and optimize the recoveries and fractionation efficiency of mixed-bed multilayer SPE followed by a sequential elution (fractionation protocol 1, FP1) and sequential SPE using three serially coupled columns (fractionation protocol 2, FP2), model water samples, containing 96 different contaminants (Table 3) were analysed in triplicate.

In this study, three different SPE materials (HR-X, HR-XAW, and HR-XCW) were exploited in order to achieve high recoveries and an efficient separation of selected compounds into three fractions, according to their charge. Hydrophobic polystyrene-divinylbenzene copolymer (HR-X), with a very high surface area ($1000 \text{ m}^2/\text{g}$) was chosen to cover the unspecific molecular interactions of neutral and non-polar to medium polar compounds. Hydrophobicity is the driving force for the enrichment of compounds on reversed-phase materials such as poly(styrene-divinylbenzene) polymers. Weakly basic secondary and tertiary ammonium polymeric anion exchanger, HR-XAW, was selected to target anionic compounds at pH 7. Weak carboxylic acid modified polymeric cation exchanger, HR-XCW, was chosen to capture cationic compounds at pH 7. These two sorbents containing ion-exchange groups were used to increase the enrichment of ionic hydrophilic compounds that are not efficiently recovered by the HR-X material. It should also be stressed that the performance of SPE protocols are highly dependent on the complex sorption/desorption competitive mechanisms on the sorbent surfaces which also involve the characteristics of elution solvents (composition, pH, and ionic strength). The solvents used in this work were selected according to literature,⁵¹ and optimized in the preliminary experiments as described in Experimental section.

The results of these model experiments for different classes of selected contaminants are presented in Figures 8-18.

Ten compounds representing different chemical classes of pesticides were tested, including triazines and their transformation products, organophosphorus, organochlorine and phenylurea pesticides (Figure 8). When sequential extraction was applied, these medium polar compounds were recovered at relatively high recoveries (69 - 110%) exclusively in the first (neutral) fraction. Even the relatively polar and acidic pesticide pentachlorophenol was found only in the eluate from the HR-X column. The recoveries using mixed-bed sorbent (70% to 110%) were comparable with the results of sequential extraction. Moreover, most of the

investigated pesticides were eluted in the first (neutral) fraction. Only the acidic organochlorine pesticide, pentachlorophenol was recovered in the second fraction (73%) employing basic solvent (MeOH with 2% NH₃ in MeOH) or the elution.

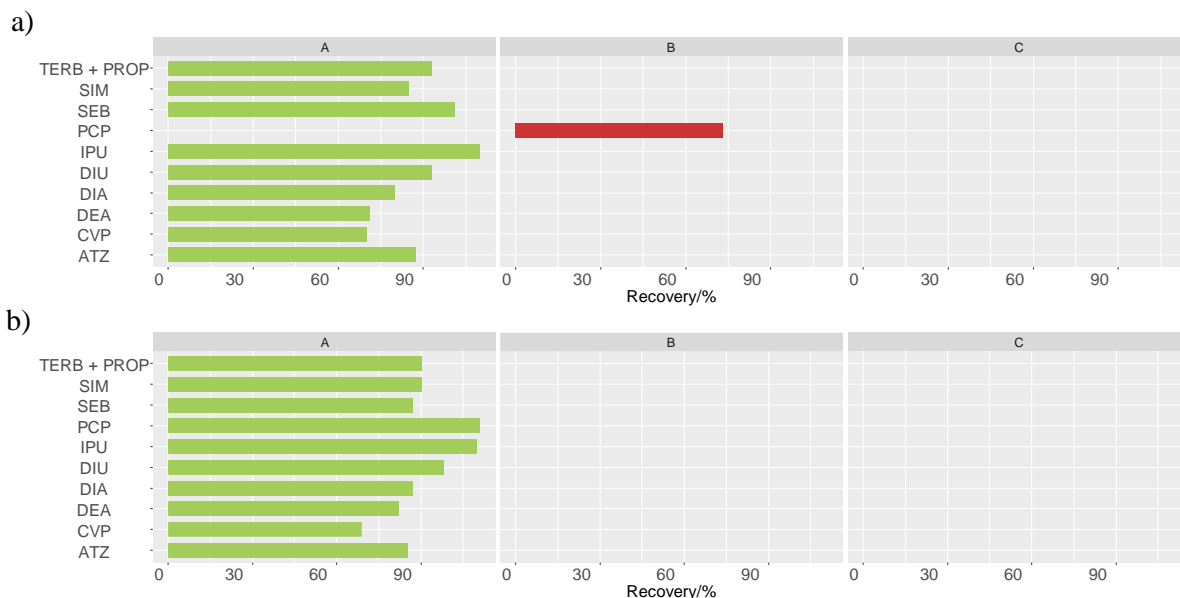


Figure 8. Comparison of two fractionation protocols for the assessment of pesticides in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). TERB+PROP - terbuthylazine and propazine; SIM - simazine; SEB - sebuthylazine; PCP - pentachlorophenol; IPU - isoproturon; DIU - diuron; DIA - atrazine desisopropyl; DEA - atrazin-desethyl; CVP - chlorphenvinphos; ATZ - atrazine.

The total recoveries of illicit drugs, which are predominately highly polar basic compounds, using sequential elution and sequential extraction procedures, were rather similar (32 - 101% and 32 - 108%, respectively). However, it was shown that the sequential elution provided a good separation between neutral and basic illicit drugs (Figure 9), while sequential extraction showed a rather poor potential for fractionation. All studied illicit drugs were recovered in the first fraction along with neutral compounds. It is interesting to note that sequential elution of mixed bed SPE columns allowed separation of slightly acidic cannabinoids, eluted with neutral solvent (fraction A), from the basic drugs, eluted in the second, basic fraction (fraction B). This shows a good performance of the used mixed-bed SPE method for contaminant fractionation. The only exception was benzoylecgonine, the major metabolite of cocaine,

which was recovered in the first fraction when both fractionation protocols were applied, with satisfactory recoveries of 81% for FP1 and 90% for FP2. Similar behavior of basic illicit drugs on mixed bed SPE cartridges was described by González-Mariño et al.¹⁴² and Senta et al.¹⁴³ who applied sequential elution of the Oasis MCX cartridges to increase the selectivity of the illicit drug analysis in wastewater samples.

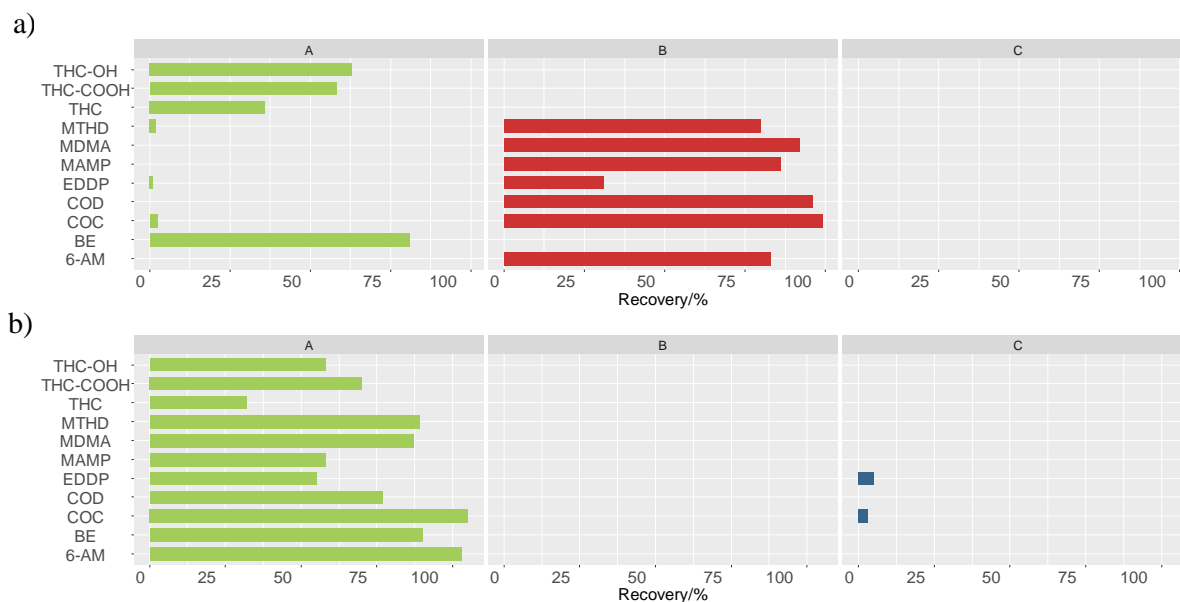


Figure 9. Comparison of two fractionation protocols for the assessment of illicit drugs in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). THC-OH - 11-Hydroxy- Δ^9 -tetrahydrocannabinol; THC-COOH - Δ^9 -tetrahydrocannabinol carboxylic acid; THC - tetrahydrocannabinol; MTHD - methadone; MDMA - 3,4-methylenedioxymethamphetamine; MAMP - methamphetamine; EDDP - 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; COD- codeine; COC- cocaine; BE- benzoylecgonine; 6-AM- 6-acetylmorphine.

In general, both fractionation protocols were found feasible for the analysis of 20 selected pharmaceuticals, covering various therapeutic groups with total recoveries in the range of 47-114% for mixed-bed multilayer SPE and of 32 - 110% for sequential extraction. Similarly to the illicit drugs, sequential elution provided a better separation between neutral and ionized polar compounds (Figures 10-11).

The total recoveries of sulfonamide antibiotics using sequential elution and sequential extraction procedures were satisfactory (78 - 101% and 64 - 98%, respectively). In both

applied procedures, all investigated sulfonamides were eluted in the first (neutral) fraction. Only N-acetyl sulfamethoxazole, a transformation product of sulfamethoxazole, was partly eluted in the first, neutral fraction (69%), while additional recovery in fraction B contributed with 32% to the total extraction recovery. Recoveries obtained for trimethoprim were also satisfactory for both applied procedures. When sequential extraction was applied, trimethoprim was eluted in the first fraction with an average recovery of 83%, while using sequential elution this compound was eluted in the second (basic) fraction with an average recovery of 93%.

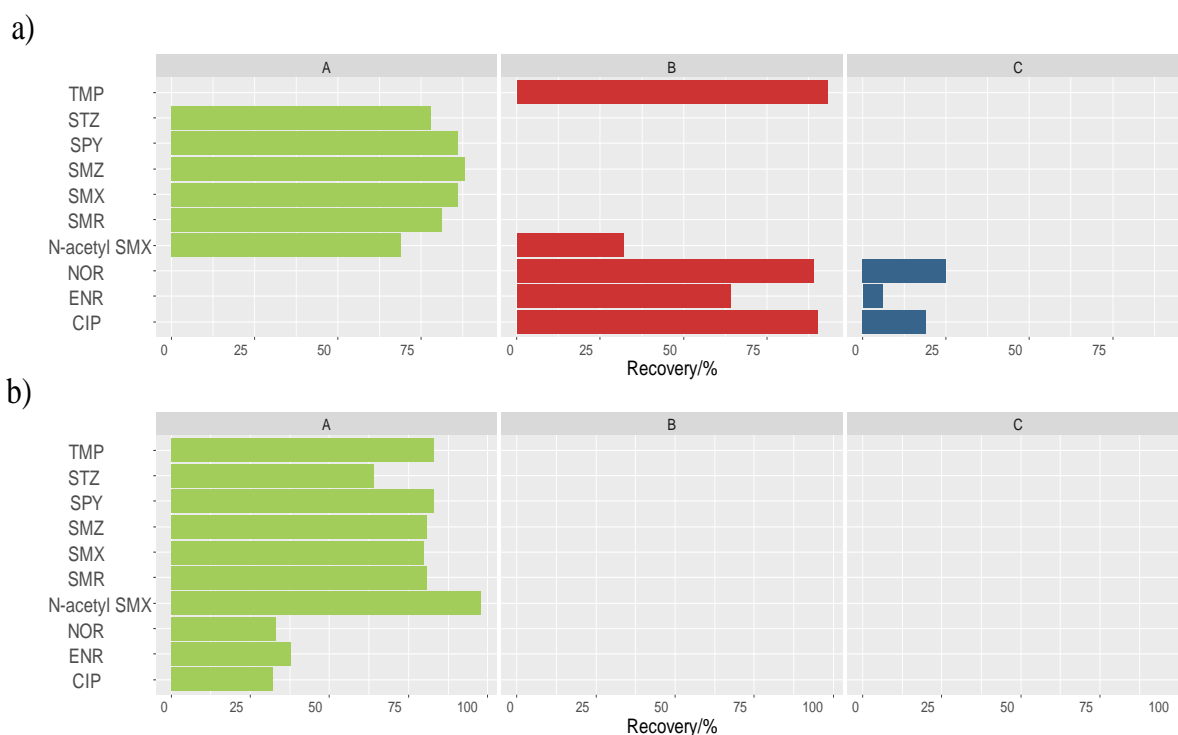


Figure 10. Comparison of two fractionation protocols for the assessment of sulfonamide, fluoroquinolone antibiotics and trimethoprim in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). TMP - trimethoprim; STZ - sulfathiazole; SPY - sulfapyridine; SMZ - sulfamethazine; SMX - sulfamethoxazole; SMR - sulfamerazine; N-acetyl-SMX - n-acetyl sulfamethoxazole; NOR - norfloxacin; ENR - enrofloxacin; CIP - ciprofloxacin.

None of the target fluoroquinolone antibiotics (norfloxacin, enrofloxacin, ciprofloxacin) could be quantitatively eluted with the neutral solvent neither from the neutral sorbent nor from the

mixed-bed sorbent. When sequential extraction was applied, fluoroquinolones were poorly eluted with MeOH/DCM (50:50, v/v) in the first fraction (< 38%). In contrast, a more complex situation was observed with mixed-bed multilayer cartridges. For the quantitative elution of fluoroquinolones from the mixed bed multilayer cartridges it was necessary to apply basified and acidified MeOH as the eluting solvents, which suggests the importance of multiple ionic interactions for the complex amphoteric molecules such as fluoroquinolones.

The total recoveries for the investigated macrolide antibiotics (Figure 11) were similar for both applied protocols being in the range of 47 - 82% and 65 - 84% for FP1 and FP2, respectively. However, significant differences were observed regarding the distribution of macrolide compounds between different eluates (fractions).

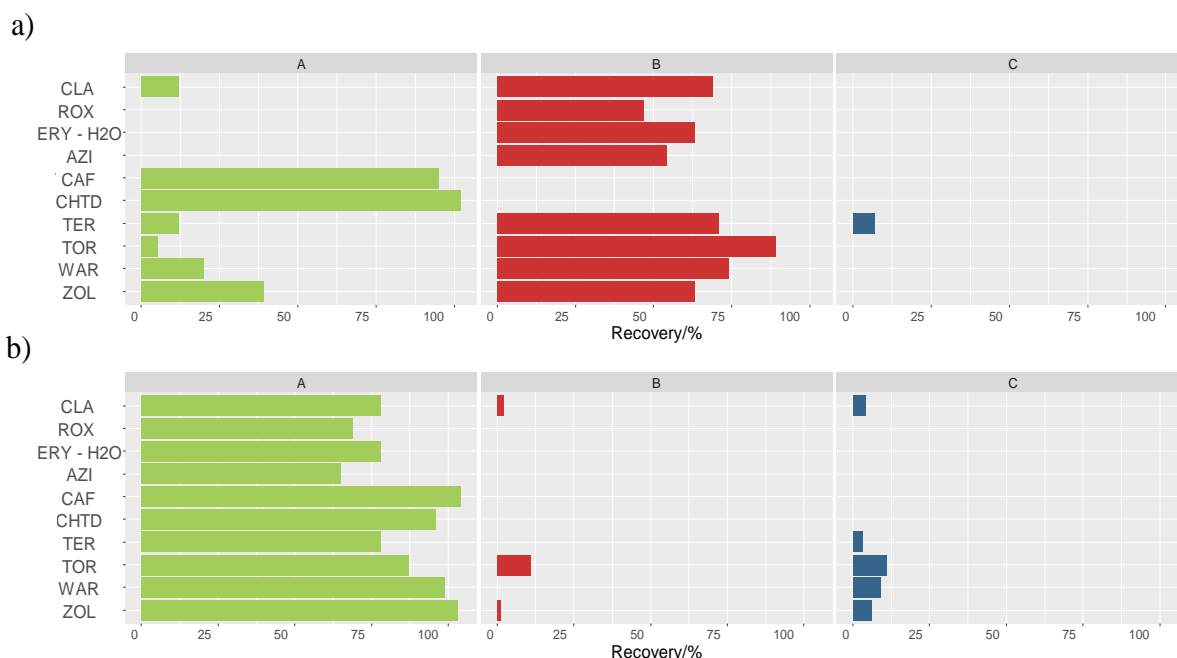


Figure 11. Comparison of two fractionation protocols for the assessment of macrolide antibiotics and other pharmaceuticals in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). CLA - clarithromycin; ROX - roxithromycin; ERY-H₂O - erythromycin - H₂O; AZI - azithromycin; CAF - caffeine; CHTD - chlorthalidone; TER - terbinafine; TOR - torsemide; WAR - warfarin; ZOL - zolpidem.

Sequential elution procedure allowed separation of slightly basic macrolide antibiotics from the neutral compounds in the second fraction (only clarithromycin was partly eluted in the

first fraction) (Figure 11). This indicates that the mechanism for interaction of the macrolides with the mixed bed SPE sorbents is different from the neutral compounds, suggesting that it is based on electrostatic rather than hydrophobic interactions. Chlorthalidone was fully eluted with the neutral solvent when both procedures were applied with recoveries of 102% for FP1 and 96% for FP2. Other tested pharmaceuticals (zolpidem, warfarin, torsemide and terbinafine) were mostly recovered in the first, neutral fraction, when sequential extraction was applied with recoveries in the range of 78 - 103% (with additional recoveries in fraction B and C < 11%). When FP1 was applied, these compounds were mostly eluted in the second fraction (63 - 89%).

Caffeine was eluted using neutral solvent when both procedures were applied with recoveries of 95% for FP1 and 104% for FP2.

Surfactants represent an excellent category of contaminants to demonstrate the capability of the tested analytical protocols to extract and fractionate neutral, acidic and basic amphiphilic compounds. On the other hand, previous study in the Sava River,⁶⁵ showed that surfactants were not only the most abundant compounds in the Sava River but were also among the contaminant classes posing the highest ecotoxicological risk. Therefore, this investigation included several representatives of non-ionic, cationic and anionic surfactants. The results of the recovery/fractionation experiments are shown in Figures 12-15. The results were, however, rather controversial, indicating some important limitations of the applied polymeric sorbents. It is interesting to note that benzalkonium cationic surfactants did not behave as typical cations and were split during sequential elution between the all three fractions (Figure 12). While only very small percentage was eluted in the neutral fraction, a significant percentage was found in the acidic fraction (fraction 3). This could be explained by the fact that acidic elution deactivates the cation exchange material through protonation of the carboxylic acid ligands, which disrupts the ionic interaction between the sorbent and cationic analytes, while basic elution leads to deprotonation of the anion exchange material and the cationic analytes that are retained by the cation exchange material.⁵¹ However, the total recoveries for the investigated cationic surfactants were fairly similar for both applied protocols being in the range of 54 - 94% and 26 - 111% for FP1 and FP2, respectively. As expected, the non-ionic surfactants of nonylphenol polyethoxylate (NPnEO) and alcohol polyethoxylate (AEO) type were found mainly in the neutral fraction, however with rather modest yields (< 33%). AEOs were split between all three fractions when both protocols were

applied, with total recoveries of 44 - 68% for FP1 and 60 - 69% for FP2. When using sequential extraction, additional recovery was detected in the third fraction (31 - 33%), which probably indicates some combined interaction of the polyethoxylate chains and lipophilic alkyl and alkylbenzene moieties with the polymeric sorbent.

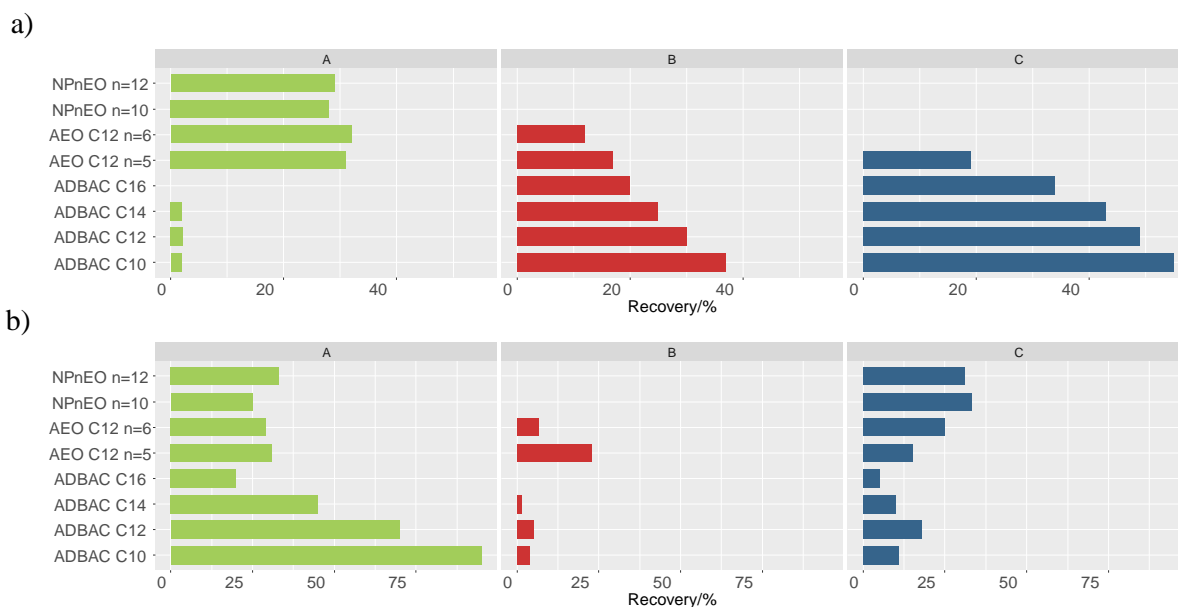


Figure 12. Comparison of two fractionation protocols for the assessment of cationic and non-ionic surfactants in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). NPnEO - nonylphenol polyethoxylate; AEO - alcohol polyethoxylate; ADBAC - alkyldimethylbenzylammonium chloride.

Polyethylene glycols (PEGs) and polypropylene glycols (PPGs) are closely related to non-ionic surfactants. The main structural difference is that PEGs and PPGs lack a distinct lipophilic alkyl moiety. As a consequence, they probably mimic the interactions of the polyethoxylate chain of nonionic surfactants. When sequential elution was applied, PPGs and PEGs were detected exclusively in the first fraction confirming the efficiency of separation according to charge (Figures 13-14). However, the recoveries were highly variable. While uniformly high recoveries were detected for different PEG oligomers (81 - 112%), recoveries obtained for the PPGs varied in a wide range of 29 - 96%, decreasing with the number of propoxy groups. A similar trend was observed for PPGs when sequential extraction was applied. The recoveries measured in the neutral fraction were in the range of 28 - 91%.

However, additional yields of PPGs were obtained in the third fraction and their percentage generally increased for the higher oligomers (10 - 29%). For comparison, recoveries of PEGs measured in the neutral fraction were in the range of 87 - 105%, while second and third fractions, contributed to the total recoveries with 3 - 29% and 2 - 27%, respectively. This confirms the assumption made for nonionic surfactants that polyethoxy chains play an important role in the adsorption/desorption behavior of these compounds.

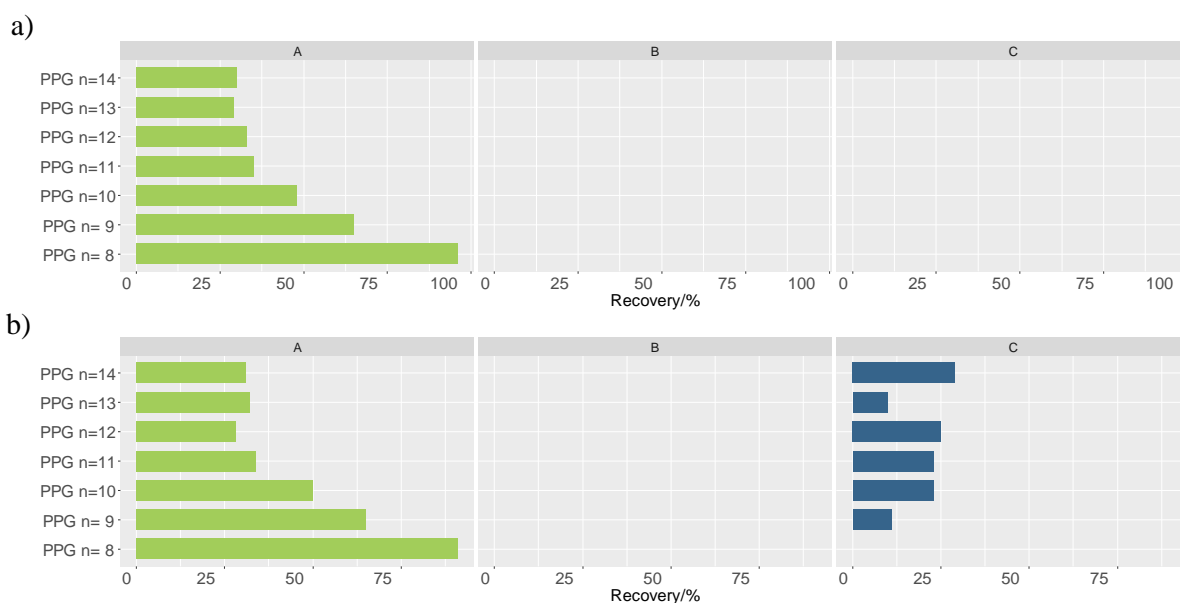


Figure 13. Comparison of two fractionation protocols for the assessment of polypropylene glycols (PPGs) in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns).

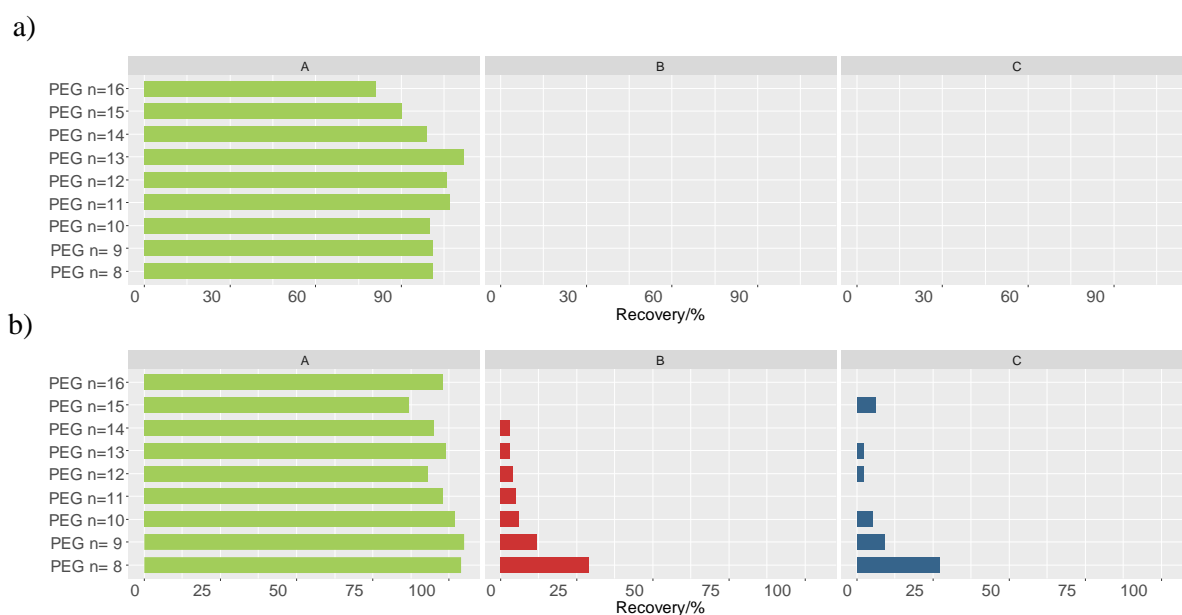


Figure 14. Comparison of two fractionation protocols for the assessment of polyethylene glycols (PEGs) in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns).

Fractionation protocols 1 and 2 were also applied on five surfactant-derived compounds: nonylphenol (NP), octylphenol (OP), nonylphenoxyacetic acid (NP1EC), nonylphenoxyethoxyacetic acid (NP2EC) and octylphenoxyacetic acid (OP1EC) (Figure 15), which are environmentally highly relevant contaminants due to their endocrine disrupting properties.¹⁴⁴ When FP1 was applied, these compounds were eluted using basic solvent and detected in the second fraction with recoveries in the range of 77 - 122%. NP and NP2EC were additionally recovered in the third fraction (16% and 14%, respectively). When the FP2 protocol was applied, all surfactant-derived compounds were detected in the first fraction along with the neutrals. Lower recoveries were obtained for NP and NP2EC (< 30%), while recoveries for other tested compounds were in the range of 62 - 82%.

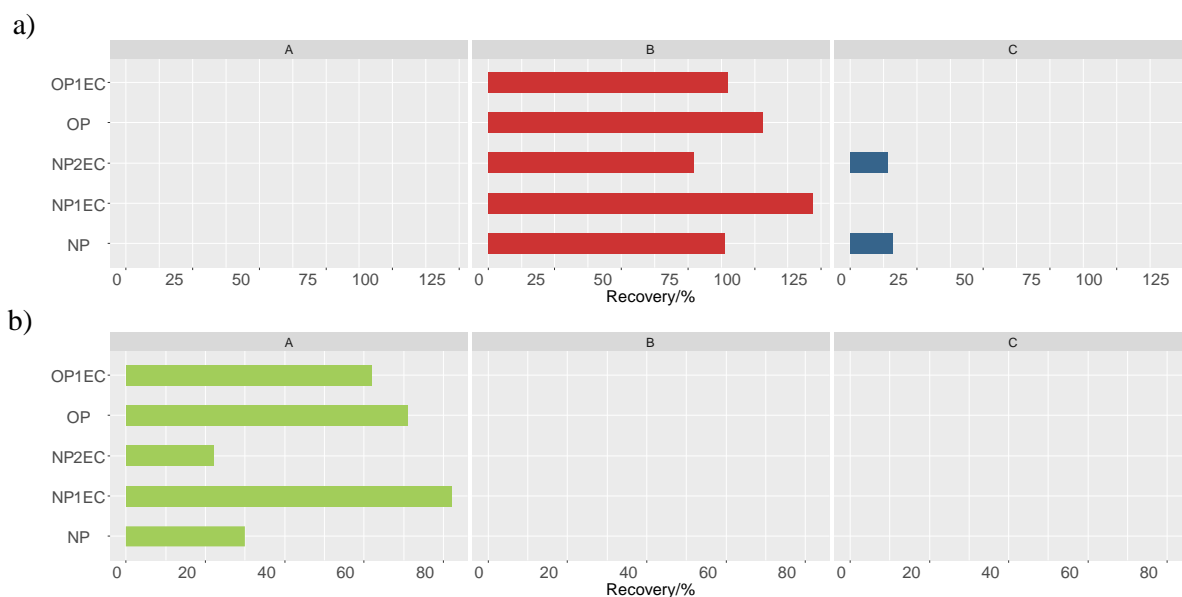


Figure 15. Comparison of two fractionation protocols for the assessment of surfactant-derived compounds in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). OP1EC - octylphenoxyacetic acid; OP - octylphenol; NP2EC - nonylphenoxyethoxyacetic acid; NP1EC - nonylphenoxyacetic acid; NP - nonylphenol.

The recoveries of three analysed plasticizers and flame retardants (tributyl phosphate (TBP), triphenyl phosphate (TPP) and endocrine disruptor bisphenol A (BPA)) were rather similar for both sequential elution and sequential extraction procedures (69 - 104% and 78 - 103%, respectively). As expected, these compounds were recovered in the first fraction, along with the neutral compounds when both protocols were applied (Figure 16).

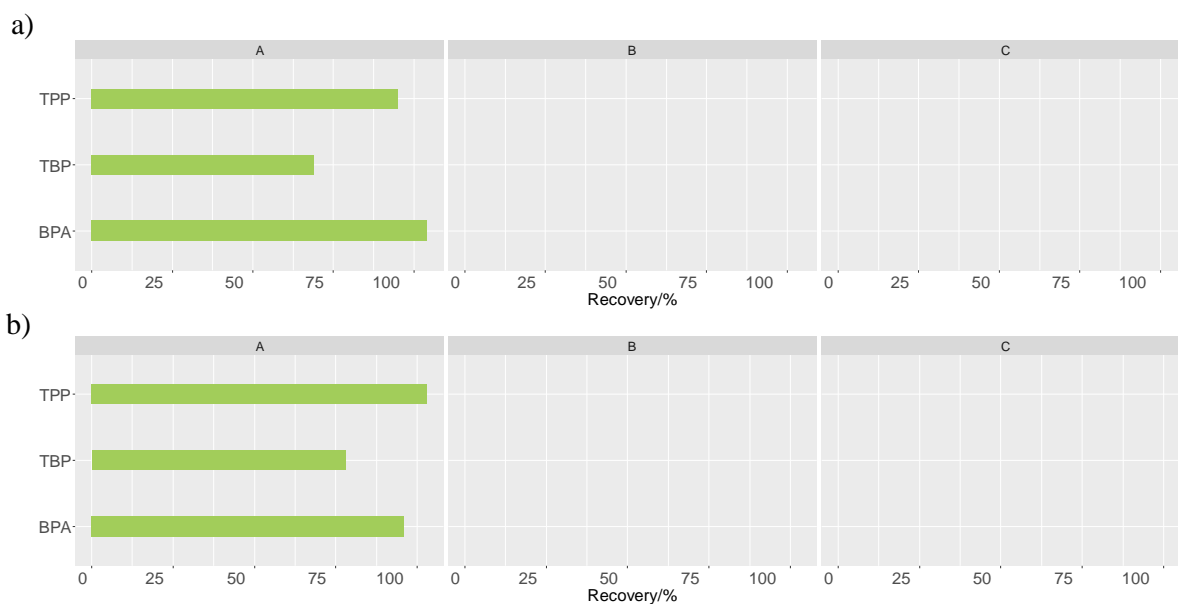


Figure 16. Comparison of two fractionation protocols for the assessment of plasticizers and flame retardants in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). TPP - triphenyl phosphate; TBP - tributyl phosphate; BPA - bisphenol A.

This investigation was primarily focused on emerging polar (hydrophilic) contaminants. However, some classical hydrophobic contaminants such as PAHs and PCBs were also included in the two protocols tested in order to examine quantitative aspects and the feasibility of the comprehensive analytical procedure including both types of contaminants. It should be noted that analysis of hydrophobic contaminants using the applied protocols requires adaptation of solvent exchange procedures to prepare the extracts for GC-MS analyses. The results of PAHs and PCBs analyses are shown in Figures 17 and 18. Expectedly, highly hydrophobic compounds such as PCBs and 16 PAHs listed for priority control by United States Environmental Protection Agency (US EPA), were detected exclusively in the first fraction, when both fractionation protocols were applied (Figures 17-18).

Recoveries of 7 investigated PCBs were in the range of 48 - 92% and 55 - 109% for mixed bed multilayer SPE and sequential extraction, respectively. However low recoveries for three low molecular weight PAHs (< 30%) were obtained due to evaporation losses during the extract work up, including evaporation to dryness and solvent exchange. When sequential

elution was applied higher recoveries (> 120%) were obtained for four PAHs (fluoranthene, pyrene, benzo[a]anthracene and benzo[k]fluoranthene). Similarly, when sequential SPE was applied recoveries higher than 120% were obtained for fluoranthene, pyrene, benzo[a]anthracene and chrysene. Booij et al.⁸⁵ also reported low recoveries for 9 analysed PCBs and 18 PAHs being < 20% for both Oasis HLB and Oasis MCX cartridges.

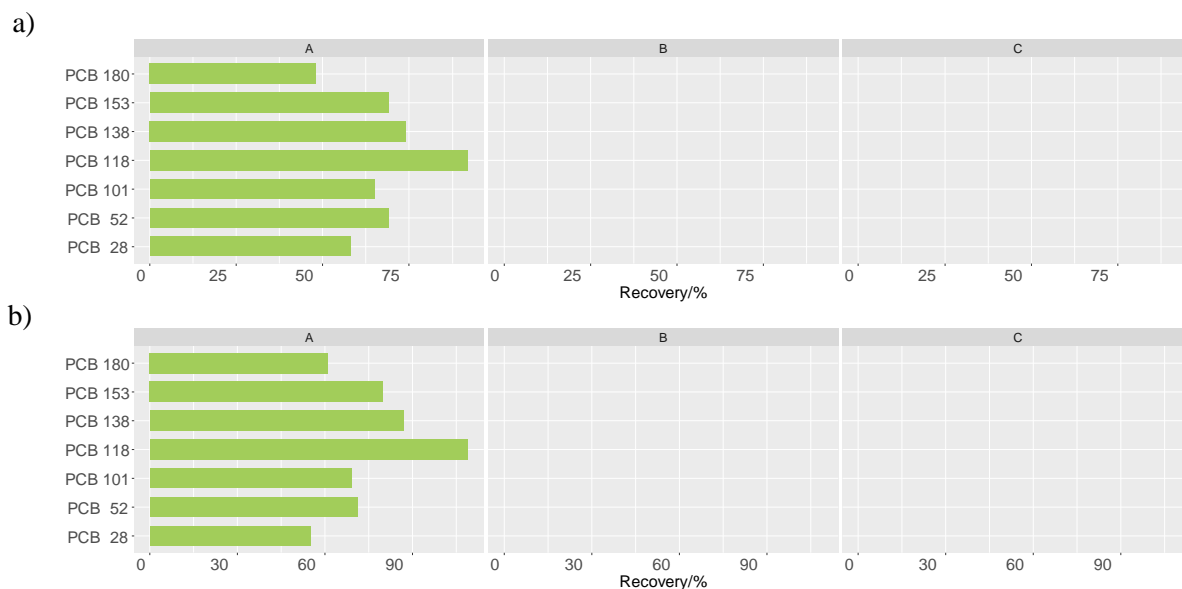


Figure 17. Comparison of two fractionation protocols for the assessment of polychlorinated biphenyls (PCBs) in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns).

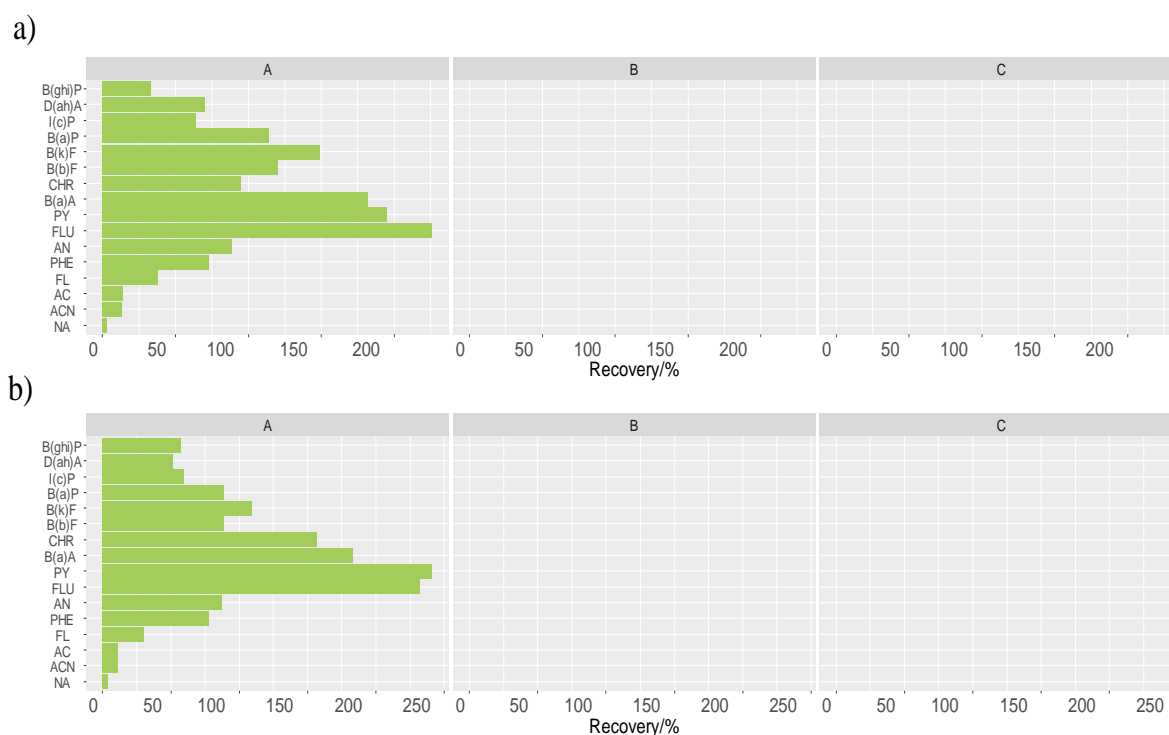


Figure 18. Comparison of two fractionation protocols for the assessment of polycyclic aromatic hydrocarbons (PAHs) in aqueous samples: a) FP1 - sequential elution of the mixed bed SPE column (A-neutral, B-basic, C-acidic fraction); b) FP2 - sequential extraction using a serially coupled SPE columns (A-HRX, B-HR-XAW, C-HR-XCW columns). B(ghi)P - benzo[g,h,i]perylene, D(a,h)A - dibenz[a,h]anthracene; I(c)P - indeno[1,2,3-cd]pyrene; B(a)P - benzo[a]pyrene; B(k)F - benzo[k]fluoranthene; B(b)F - benzo[b]fluoranthene; CHR - chrysene; B(a)A - benzo[a]anthracene; PY - pyrene; FLU - fluoranthene; AN - anthracene; PHE - phenanthrene; FL - fluorene; AC - acenaphthene; ACN - acenaphthylene; NA - naphthalene.

Since the experiments carried out with the polymeric sorbents showed that the separation of contaminants according to charge was often prevented by a strong interaction of some contaminants with polymeric structure of the sorbent, we tested an additional sequential extraction and fractionation protocol, employing ion-exchange sorbents, based on modified silica gels (Strata-WCX and Strata WAX) coupled in series with the neutral polymeric sorbent Strata X. As described in the experimental section, the SPE cartridges were connected in series in the following order: WCX → WAX → Strata X.

The results showing the fractionation behavior of the selected target contaminants on such multi-sorbent system, including the experiments without extraction (direct application of standard contaminant mixture to the top of the first column) and the experiments that involved extraction of target compounds from the model water samples, are presented in Table 8. Compared to the mixed-bed polymeric sorbents, the silica-based ion-exchange sorbents provided an improved separation of organic contaminants according to charge, when applying sequential extraction protocol. For simple neutral molecules such as pesticides, caffeine, PEG, TBP, TPP, BPA, and, expectedly, hydrophobic contaminants such as PAHs and PCBs, the recoveries and fractionation are rather similar for both sorbent types. However, a significant improvement was achieved regarding fractionation according to charge for some chemical types which exhibited a mixed behavior on polymeric sorbents. Using silica-based sorbents it was possible to get all organic anions having carboxylic and/or phenolic groups into the anionic fraction with high yields, except for THC-OH (pKa 3.7) and BPA (pKa 9.6), which were eluted in the neutral fraction. A rather complex behavior using silica-based ion-exchangers was observed for sulfonamides, which were eluted either in the acidic (WAX) or in the neutral (Strata X) fraction, depending on the pKa values of individual sulfonamides. In contrast, using polymeric sorbents, all sulfonamides ended up in the neutral (MeOH) fraction, indicating that their sorption/desorption was primarily governed by non-ionic (lipophilic) interactions.

It is interesting to note that most of the pharmaceutical containing a basic nitrogen atom (zolpidem, torsemide, terbinafine, macrolide antibiotics azithromycin, clarithromycin, erythromycin and roxithromycin, trimethoprim and fluoroquinolones norfloxacin, ciprofloxacin and enrofloxacin) were eluted in the cationic fraction (WCX), while warfarin and sulfonamide-like chlorthalidone were eluted in the anionic and neutral fraction, respectively. In addition, a number of illicit drugs are also basic compounds and they were also distributed entirely in the cationic fraction. In contrast, the cannabinoid compounds were found either in the neutral (THC and THC-OH) or in the anionic fraction (THC-COOH). This difference can be explained by a comparatively weaker ionic interaction of the THC phenolic group (pKa 10.6) compared to carboxylic group of THC-COOH (pKa 4.1). In contrast, using polymeric sorbents all cannabinoids were found in the neutral fraction.

Table 8. Fractionation behavior of selected model contaminants on serially coupled SPE cartridges employing silica-based ion-exchange sorbents.

Compound	Experiment 1 (without extraction)			Experiment 2 (incl. extraction)		
	Recovery % (X±RSD)			Recovery % (X±RSD)		
	Strata WCX	Strata WAX	Strata X	Strata WCX	Strata WAX	Strata X
<i>Pesticides</i>						
Diuron	-	-	101 (6)	-	-	91 (0)
Isoproturon	-	-	101 (3)	-	-	98 (4)
Chlorphenvinphos	-	-	103 (6)	-	-	80 (1)
Pentachlorophenol	-	88 (3)	-	3 (0)	46 (4)	4 (15)
<i>Pharmaceuticals and their TPs</i>						
Zolpidem	103 (2)	-	-	94 (1)	-	-
Warfarin	-	30 (4)	-	-	21 (22)	-
Torsemide	91 (3)	-	-	12 (2)	-	68 (2)
Terbinafine	118 (2)	-	-	89 (1)	-	-
Chlorthalidone	-	-	67 (19)	-	-	12 (51)
Caffeine	-	-	88 (5)	-	-	99 (3)
Azithromycin	90 (17)	-	-	113 (10)	-	-
Erythromycin - H ₂ O	163 (18)	-	-	183 (4)	-	-
Roxithromycin	76 (23)	-	-	65 (9)	-	-
Clarithromycin	96 (19)	-	-	86 (15)	-	-
Sulfathiazole	-	85 (0)	-	-	-	42 (16)
Sulfapyridine	-	-	61 (5)	-	-	44 (10)
Sulfamerazine	-	86 (3)	-	-	-	42 (8)
Sulfamethazine	-	-	67 (2)	-	-	45 (9)
Sulfamethoxazole	-	102 (4)	-	-	-	58 (1)
N - acetyl sulfamethoxazole	-	96 (6)	-	-	-	71 (1)
Trimethoprim	93 (2)	-	6 (3)	83 (1)	-	-
Ciprofloxacin	44 (43)	-	-	267 (2)	-	-
Enrofloxacin	58 (41)	-	-	391 (0)	-	-
Norfloxacin	46 (31)	-	-	280 (1)	-	-
<i>Illicit drugs and their metabolites</i>						
6-acetylmorphine	100 (2)	-	-	72 (2)	-	-
MDMA	87 (3)	-	-	88 (2)	-	-
Methamphetamine	93 (4)	-	-	89 (0)	-	-
Cocaine	111 (3)	-	-	95 (2)	-	-
Benzoylcegonine	99 (1)	-	-	-	-	82 (0)
Codeine	102 (2)	-	-	101 (0)	-	-
Methadone	103 (2)	-	-	90 (1)	-	-
EDDP	102 (3)	-	-	92 (1)	-	-
THC-OH	-	-	97 (1)	10 (3)	10 (34)	33 (3)
THC	-	-	77 (6)	22 (1)	6 (3)	-
THC-COOH	-	87 (4)	-	18 (0)	33 (20)	26 (17)

Table 8. - continued.

Compound	Experiment 1 (without extraction)			Experiment 2 (incl. extraction)		
	Recovery % (X±RSD)			Recovery % (X±RSD)		
	Strata WCX	Strata WAX	Strata X	Strata WCX	Strata WAX	Strata X
<i>Plasticizers and flame retardants</i>						
Tributyl phosphate	-	-	79 (6)	-	-	45 (4)
Triphenyl phosphate	-	-	102 (4)	27 (1)	-	55 (1)
Bisphenol A	-	-	109 (5)	-	-	82 (0)
<i>Surfactants and their TPs</i>						
Benzyltrimethyl decylammonium chloride	108 (1)	-	-	75 (6)	-	-
Benzyltrimethyl dodecylammonium chloride	105 (2)	-	-	56 (5)	-	-
Benzyltrimethyl tetradecylammonium chloride	90 (1)	-	-	49 (1)	-	-
Benzyltrimethyl hexadecylammonium chloride	75 (2)	-	-	41 (13)	-	-
AEO, C ₁₂ , n=5	-	-	95 (3)	61 (3)	33 (24)	30 (0)
AEO, C ₁₂ , n=6	-	-	89 (6)	59 (5)	20 (36)	14 (11)
NPnEO, n=10	-	-	102 (3)	55 (0)	-	-
NPnEO, n=12	-	-	100 (8)	55 (0)	-	-
PEG, n=8	-	-	120 (4)	-	-	90 (8)
PEG, n=9	-	-	125 (4)	-	-	73 (8)
PEG, n=10	-	-	91 (2)	-	-	97 (8)
PEG, n=11	-	-	94 (1)	-	-	83 (15)
PEG, n=12	-	-	90 (1)	-	-	83 (6)
PEG, n=13	-	-	85 (4)	-	-	91 (18)
PEG, n=14	-	-	92 (5)	-	-	84 (10)
PEG, n=15	-	-	90 (9)	-	-	80 (8)
PEG, n=16	-	-	84 (9)	-	-	76 (13)
PPG, n=8	-	-	97 (3)	-	-	71 (0)
PPG, n=9	-	-	95 (2)	21 (39)	8 (10)	43 (0)
PPG, n=10	-	-	95 (2)	20 (1)	12 (3)	15 (0)
PPG, n=11	-	-	94 (1)	49 (17)	11 (2)	5 (0)
PPG, n=12	-	-	92 (4)	56 (8)	9 (9)	4 (0)
PPG, n=13	-	-	91 (8)	59 (4)	9 (9)	2 (0)
PPG, n=14	-	-	91 (7)	61 (0)	8 (2)	3 (0)
NP1EC	-	86 (3)	-	-	67 (1)	-
NP2EC	-	98 (1)	-	-	53 (1)	-

Table 8. - continued.

Compound	Experiment 1 (without extraction)			Experiment 2 (incl. extraction)		
	Recovery % (X±RSD)			Recovery % (X±RSD)		
	Strata WCX	Strata WAX	Strata X	Strata WCX	Strata WAX	Strata X
OP1EC	-	96 (1)	-	-	61 (0)	-
OP	-	92 (0)	-	-	60 (3)	-
NP	-	95 (1)	-	-	52 (3)	-
<i>Polycyclic aromatic hydrocarbons (PAHs)</i>						
Naphthalene	-	-	1 (58)	1	-	-
Acenaphthylene	-	-	14 (55)	1	-	-
Acenaphthene	-	-	12 (42)	2	-	1
Fluorene	-	-	34 (11)	6	-	2
Phenanthrene	-	-	79 (11)	17	-	10
Anthracene	-	-	69 (12)	13	-	3
Fluoranthene	-	-	102 (7)	28	-	2
Pyrene	-	-	107 (6)	33	-	2
Benzo[a]anthracene	-	-	122 (1)	38	1	5
Chrysene	-	-	125 (2)	28	1	4
Benzo[b]fluoranthene	-	-	112 (5)	30	2	8
Benzo[k]fluoranthene	-	-	106 (6)	36	3	15
Benzo[a]pyrene	-	-	113 (3)	34	2	5
Indeno[1,2,3-cd]pyrene	-	-	102 (9)	25	-	9
Dibenz[a,k]anthracene	-	-	104 (9)	24	-	6
Benzo[g,h,i]perylene	-	-	99 (11)	29	2	6
<i>Polychlorinated biphenyls (PCBs)</i>						
PCB 28	-	-	103 (6)	8	-	-
PCB 52	-	88 (3)	-	10	-	-
PCB 101	-	-	101 (6)	15	-	2
PCB 118	-	-	101 (3)	23	-	6
PCB 153	-	-	103 (6)	20	-	4
PCB 138	-	88 (3)	-	23	-	6
PCB 180	-	-	101 (6)	20	-	6

* Full compound names are listed in Table SI (Supplementary Material).

Cationic surfactants of benzyldimethyldecyl ammonium type are typical cationic molecules which are always present in the positively charged form. Consequently, they are expected to be strongly bound to the negative moieties of the cation-exchangers so that they should be easily separable from neutral and anionic compounds. This was fully achieved using silica-based ion-exchange sorbents. The same separation cannot be performed using sequential

elution from the polymeric mixed-bed sorbent where cationic surfactants were divided between the acidic and basic fractions.

The fractionation potential of silica-based sorbents was significantly altered when the same combination of sorbents was applied in the context of sequential extraction protocol. Behavior of small neutral but only moderately lipophilic molecules (pesticides, caffeine, PEG, TBP and BPA) was not strongly affected by the aqueous phase and these analytes were efficiently recovered in the neutral fraction like during sequential elution. Unfortunately, hydrophobic compounds (PAHs and PCBs) showed very low yields on the applied system, indicating significant losses during the enrichment and extract work-up. Moreover, the recovery of basic pharmaceuticals and illicit drugs was less consistent and showed some peculiar results. For example benzoylecgonine was found in the neutral fraction despite the fact that it contains both the amino and carboxylic moiety. Furthermore, we have no plausible explanation for the extremely high recoveries of fluoroquinolones (267 - 391%). Since the procedural blanks did not show any contamination, we can only speculate that this was an artefact of the matrix changes during the analysis. The strongest deviation from the envisaged fractionation behavior was observed the oxygen-rich compounds having a significant lipophilic moiety such as nonionic surfactants (AEO, NPnEO, and PPG), which were dispersed among all three fractions. Since these compounds do not possess any atoms carrying either positive or negative charge, it has to be assumed that their interaction with the stationary phases was mainly through the ion-dipole, dipole-dipole and lipophilic interactions. It is interesting to note that the recovery of PPGs in the first (WCX) fraction significantly increased for higher oligomers (21% for PPG9 vs 61% for PPG14), while the opposite trend was observed in the neutral fraction. Since a similar retention of nonionic polymers was not observed for closely related PEGs, the mechanism for the enhanced retention of PPGs is possibly linked to their higher lipophilicity. This is clearly reflected by their significantly higher retention times on the reverse-phase HPLC column as compared to PEGs.

The results described above indicate that the use of silica-based sorbents is a very promising approach for improving contaminant fractionation according to charge. However, it should be taken into account that the separation potential of sequential extraction is much lower than sequential elution. This means the optimum results can be achieved by combining comprehensive extraction protocols in combination with the fractionation of the total extract on a multi-sorbent system employing silica-based ion-exchange materials.

4.1.2. Fractionation protocol 3: Silica gel fractionation of the total SPE extract

As an alternative to fractionation protocols using solid-phase extraction based on polymeric sorbents, the potential of silica gel as a polar sorbent, which can separate the compounds according to their polarity, was also tested. This procedure was applied for the comprehensive GC-MS characterization of organic contaminants in wastewater and river water by Ahel and Giger¹⁴⁵ and further developed in the framework of an EDA protocol for the assessment of hazardous contamination in the Sava River.⁶⁵ However, detailed information on fractionation behavior of different compounds is still missing. The results of the fractionation experiment using the complex mixture of 90 model contaminants are presented in Table 9. Prior to the silica gel fractionation, mixed-bed SPE was performed as described in the experimental section above. Similarly to FP1 and FP2 protocols, recoveries of selected model compounds were determined in three different fractions representing a) non-polar (eluted with *n*-hexane), b) medium polar (eluted with dichloromethane) and c) polar (eluted with methanol) fraction.

Table 9. Fractionation and recoveries (%) of selected contaminants using partially deactivated (15 % water, w/w) silica gel column (n=3).

Compound	Recovery %	Recovery %	Recovery %	Instrument
	(X±RSD) Fraction a	(X±RSD) Fraction b	(X±RSD) Fraction c	
Atrazine	-	-	40 (9)	LC-QTOF
Atrazine-desethyl	-	-	25 (2)	LC-QTOF
Sebuthylazine	-	-	38 (4)	LC-QTOF
Simazine	-	-	33 (6)	LC-QTOF
Terbutylazine + propazine	-	-	42 (4)	LC-QTOF
Atrazine desisopropyl	-	-	23 (9)	LC-QTOF
Diuron	-	-	71 (6)	LC-QTOF
Isoproturon	-	-	92 (1)	LC-QTOF
Chlorphenvinphos	-	32 (6)	28 (11)	LC-QTOF
Pentachlorophenol	-	65 (7)	-	LC-QTOF
Tributyl phosphate	-	-	73 (3)	LC-QTOF
Triphenyl phosphate	-	89 (2)	8 (16)	LC-QTOF
Bisphenol A	-	-	43 (3)	LC-QTOF
Benzyl dimethyl decylammonium chloride	-	-	9 (9)	LC-QTOF
Benzyl dimethyl dodecylammonium chloride	-	-	16 (12)	LC-QTOF
Benzyl dimethyl tetradecylammonium chloride	-	-	13 (13)	LC-QTOF
Benzyl dimethyl hexadecylammonium chloride	-	-	11 (16)	LC-QTOF
AEO, C ₁₂ , n=5	-	-	18 (4)	LC-QTOF
AEO, C ₁₂ , n=6	-	-	14 (3)	LC-QTOF
NPnEO, n=10	-	-	27 (10)	LC-QTOF
NPnEO, n=12	-	-	21 (3)	LC-QTOF
Zolpidem	-	-	86 (5)	LC-QTOF
Warfarin	-	-	95 (1)	LC-QTOF
Torse mide	-	-	77 (6)	LC-QTOF
Terbinafine	-	-	67 (2)	LC-QTOF
Chlorthalidone	-	-	45 (9)	LC-QTOF
Caffeine	-	-	90 (1)	LC-QTOF
Sulfathiazole	-	-	-	LC-QTOF
Sulfapyridine	-	-	14 (15)	LC-QTOF
Sulfamerazine	-	-	21 (6)	LC-QTOF
Sulfamethazine	-	-	15 (3)	LC-QTOF
Sulfamethoxazole	-	-	25 (2)	LC-QTOF
Trimethoprim	-	-	70 (2)	LC-QTOF
N - acetyl sulfamethoxazole	-	-	94 (1)	LC-QTOF
Norfloracin	-	-	-	LC-QTOF
Ciprofloracin	-	-	-	LC-QTOF
Enrofloracin	-	-	-	LC-QTOF
Azithromycin	-	-	-	LC-QTOF
Erythromycin - H ₂ O	-	-	80 (3)	LC-QTOF
Roxithromycin	-	-	21 (9)	LC-QTOF

Table 9. – continued.

Compound	Recovery %	Recovery %	Recovery %	Instrument
	(X±RSD) Fraction a	(X±RSD) Fraction b	(X±RSD) Fraction c	
Clarithromycin	-	-	19 (6)	LC-QTOF
PPG, n=8	-	-	53 (1)	LC-QTOF
PPG, n=9	-	-	42 (1)	LC-QTOF
PPG, n=10	-	-	36 (12)	LC-QTOF
PPG, n=11	-	-	28 (5)	LC-QTOF
PPG, n=12	-	-	21 (12)	LC-QTOF
PPG, n=13	-	-	21 (13)	LC-QTOF
PPG, n=14	-	-	20 (9)	LC-QTOF
PEG, n=11	-	-	46 (28)	LC-QTOF
PEG, n=12	-	-	42 (24)	LC-QTOF
PEG, n=13	-	-	46 (5)	LC-QTOF
PEG, n=14	-	-	38 (9)	LC-QTOF
PEG, n=15	-	-	41 (3)	LC-QTOF
PEG, n=16	-	-	34 (8)	LC-QTOF
6-acetylmorphine	-	-	72 (8)	LC-QTOF
MDMA	-	-	58 (1)	LC-QTOF
Methamphetamine	-	-	37 (2)	LC-QTOF
Cocaine	-	-	53 (12)	LC-QTOF
Benzoylcegonine	-	-	58 (3)	LC-QTOF
Codeine	-	-	14 (10)	LC-QTOF
Methadone	-	-	26 (7)	LC-QTOF
EDDP	-	-	3 (8)	LC-QTOF
THC-OH	-	-	9 (4)	LC-QTOF
THC	-	10 (10)	-	LC-QTOF
THC-COOH	-	-	17 (4)	LC-QTOF
OP1EC	-	-	34 (12)	LC-QTOF
NP1EC	-	-	27 (5)	LC-QTOF
Naphthalene	-	-	-	GC-MS
Acenaphthylene	-	-	-	GC-MS
Acenaphthene	-	-	-	GC-MS
Fluorene	-	-	-	GC-MS
Phenanthrene	5	-	-	GC-MS
Anthracene	6	-	-	GC-MS
Fluoranthene	46	-	-	GC-MS
Pyrene	49	-	-	GC-MS
Benzo[a]anthracene	104	-	-	GC-MS
Chrysene	105	-	-	GC-MS
Benzo[b]fluoranthene	113	-	-	GC-MS
Benzo[k]fluoranthene	114	-	-	GC-MS
Benzo[a]pyrene	108	-	-	GC-MS
Indeno[1,2,3-cd]pyrene	116	-	-	GC-MS
Dibenz[a,k]anthracene	116	-	-	GC-MS
Benzo[g,h,i]perylene	114	-	-	GC-MS
PCB 28	7	-	-	GC-MS

Table 9. – continued.

Compound	Recovery %	Recovery %	Recovery %	Instrument
	(X±RSD) Fraction a	(X±RSD) Fraction b	(X±RSD) Fraction c	
PCB 52	14	-	-	GC-MS
PCB 101	34	-	-	GC-MS
PCB 118	52	-	-	GC-MS
PCB 153	54	-	-	GC-MS
PCB 138	61	-	-	GC-MS
PCB 180	71	-	-	GC-MS

*For PAHs and PCBs recovery results for silica gel separation are presented (without previous SPE); Full compound names are listed in Table SI (Supplementary Material).

The silica gel fractionation proved to be efficient for isolation of non-polar compounds amenable for GC-MS analyses. Non polar compounds such as PAHs and PCBs were recovered exclusively in the *n*-hexane fraction (non-polar, fraction a), and were fully separated from the medium polar and polar compounds. Recoveries for PAHs with four and more aromatic rings were in the range of 46 - 116%. Low recoveries for low molecular weight PAHs and for some PCBs were obtained due to the evaporation losses during the extract work up, including evaporation to dryness and solvent exchange (Table 9).

Also, significant losses of some ionized polar compounds were observed. The incomplete recoveries of polar compounds suggested that some polar compounds such as norfloxacin, ciprofloxacin, enrofloxacin and azithromycin cannot be eluted from the applied silica gel sorbent using the applied neutral solvents. Recoveries of analysed pesticides were in the range of 23 - 92%, which is lower than the recoveries based on SPE protocols (70 - 110%). This suggests that a significant loss happened either during the solvent exchange prior to silica gel fractionation or by irreversible sorption. Other authors also reported that silica gel stationary phases are generally problematic in respect of recovery of polar compounds.^{146,147} Therefore, further optimization of this protocol is necessary to achieve fully quantitative results for these compounds.

4.2. Application of simplified effect-directed analysis for the field study on the Sava River

4.2.1. Chemical analyses with focus on polar contaminants in the dissolved fraction

As a part of the European demonstration programme, carried out during the project EDA-EMERGE, we performed a preliminary screening of the Sava River for potential hazardous chemical contaminants. One of the priorities of the European demonstration programme was to put a strong emphasis on hydrophilic water contaminants, in particular those predominantly associated with the dissolved fraction. Dissolved fraction is supposed to be a compartment carrying primarily polar contaminants highly soluble in water, including those forming ionized species in aqueous solutions at pH values usually encountered in the environment. Such polar compounds are recognised to be of a special interest as the target group, because recent literature reports increasingly suggest that most of the possible emerging contaminants belong to this class, while their possible adverse ecotoxicological effects remain largely unknown. Furthermore, it was assumed that, due to their high water solubility, polar compounds in the real water samples remain in the dissolved fraction. This assumption is very important in light of the fact that water quality standards of organic contaminants, as defined by current WFD regulation, refer to their total concentration in water (thus including both dissolved and particulate fraction). Obviously, this might be rather critical when assessing some more hydrophobic priority contaminants such as PAHs and chlorinated hydrocarbons, which are often mainly associated with the suspended particles, while, for most of the polar compounds, the percentage associated with the suspended particles is expected to be low. Such prediction of the partitioning behavior for polar compounds allows us to consider the dissolved fraction fairly representative of the total concentration, which leads to a significant simplification of the overall procedure. Focusing solely on dissolved fraction is also important from the practical point of view because it allows the samples to be filtered before solid-phase extraction to prevent clogging. This is essential when processing larger samples (50 L or more) with the aim to perform a comprehensive EDA analysis, which comprises the need to carefully plan the division of the sample extract into numerous aliquots for different chemical and biological methods. Along the lines of possible physico-chemical discrimination during the sample filtration, the major focus of all downstream steps, including fractionation, analysis and biotesting, was put on putative contaminants having a medium-to-low

hydrophobicity. One of the important goals of the study was to indicate which chemical contaminants can be linked with the observed toxic effects. Taking into account that a comprehensive identification of true unknowns is a very tedious and time-consuming process, the elucidation of unknowns was complemented by a wide-spectrum target analysis. In order to cover different classes of environmentally and toxicologically relevant environmental contaminants and their transformation products, 151 chemical compounds were selected for the target analyses (Table 10). Moreover, the toxic effects and the amount of analytes recovered in acidic and basic fractions were relatively low in comparison with neutral fractions due to ability of neutral sorbent (polystyrene-divinylbenzene copolymer) to capture most of the polar analytes, including different organic cations and anions (Figures 8-18). Therefore, the neutral fraction was assumed to be representative of the total extract. The list of the target compounds consisted of 55 pharmaceuticals, 43 pesticides, 11 surfactants and surfactant-derived compounds, and several compounds from other classes like industrial compounds, polycyclic aromatic hydrocarbons, corrosion inhibitors, sunscreen agents, sweeteners and flame retardants. The target compounds list included thirty one WFD priority substances and three compounds from the WFD Watch list. Apart from the expected loss of some classic contaminants, this methodological restriction reduced the requirements/problems during the sample transfer and solvent exchange in biotests and chemical analyses, which might be caused by adsorption losses of highly lipophilic compounds. Moreover, pronounced polar character of the expected compounds made LC-MS - based techniques the key tool for the analytical screening.

4.2.1.1. Target analyses

Table 10 lists the target compounds investigated in the Sava River samples, collected at four sampling locations in the wider area of the city of Zagreb. Neutral extracts were selected for the target analyses. The target analyses provided profound chemical characterization of the LVSPE Sava River extracts. A large number of contaminants were identified, covering a wide range of chemical structures and physico-chemical properties. Out of total 151 compounds, 63 compounds were detected at least at one sampling site, while 88 compounds were not detected in any of the investigated samples.

Table 10. Summary results of target analyses of contaminants in the Sava River samples, collected during the European demonstration study, August-September 2013.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB				
					SO	PO	OB	SC	
		(ng/L)							
17-alpha-ethinylestradiol	pharmaceutical, estrogen, WFD watch list	0.40	ND	ND	ND	ND	ND	ND	ND
17-β-estradiol	hormone, estrogen, WFD watch list	0.30	ND	ND	ND	ND	ND	ND	ND
1H-Benzotriazole	corrosion inhibitor, industrial compound	1.0	ND	NA	<LOQ	<LOQ	90.1	135.7	
2,4-Dichlorophenoxyacetic acid	herbicide	1.0	ND	ND	ND	1.3	1.6	<LOQ	
2,4-Dinitrophenol	herbicide, transformation product	1.0	ND	ND	3.7	7.1	1.8	4.8	
2,6-Dichlorobenzamide	herbicide, transformation product	1.0	ND	ND	ND	ND	ND	ND	ND
4-Androstene-3,17-dione	hormone, androgen	1.50	ND	ND	ND	ND	ND	ND	ND
4-Hydroxytamoxifen	pharmaceutical, anti-estrogen	0.60	ND	ND	ND	ND	ND	ND	ND
4-Nitrophenol	industrial compound	5.0	ND	ND	<LOQ	ND	ND	ND	ND
4-Toluenesulfonamide	industrial compound	10	ND	ND	<LOQ	71	238	245	
5-Methyl-1H-benzotriazole	corrosion inhibitor	1.0	<LOQ	ND	19.5	39.5	83.5	48.5	
6-alpha-Methylprednisolone	pharmaceutical, glucocorticoid	0.63	ND	ND	ND	ND	ND	ND	ND
Acesulfame	artificial sweetener, marker compound	1.0	ND	ND	1.1	1.4	2.8	1.7	
Acetyl-Sulfamethoxazole	pharmaceutical, transformation product	1.0	ND	ND	8.1	7.9	11.9	10.3	
Aclonifen	herbicide, WFD priority	0.1	ND	NA	ND	ND	ND	ND	ND
Alachlor	herbicide, WFD priority	1.0	ND	NA	ND	ND	ND	ND	ND
Aldosterone	hormone, glucocorticoid	14	ND	ND	ND	ND	ND	ND	ND
Aminonide	pharmaceutical, glucocorticoid	0.88	ND	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
(ng/L)								
Anastrozole	pharmaceutical, anti-estrogen	0.15	ND	ND	ND	ND	ND	ND
Androsterone	hormone, androgen	2.0	ND	ND	ND	ND	ND	ND
Anthracene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Atrazine	herbicide, WFD priority	1.0	ND	ND	3.4	4.0	6.5	5.4
Atrazine-desethyl	herbicide, transformation product	1.0	ND	ND	3.2	6.2	11.0	11.0
Azithromycin	pharmaceutical, antibiotic	1.0	ND	NA	1.9	370	ND	ND
Azoxystrobin	fungicide	1.0	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
Bentazone	herbicide	1.0	ND	ND	2.9	2.4	27.2	6.3
Benzo(a)pyrene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Benzo(b)fluoranthene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Benzo(g,h,i)perylene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Benzophenone-3	sunscreen agent	1.0	ND	ND	ND	1.7	<LOQ	3.6
Benzophenone-4	UV filter	1.0	ND	ND	22.1	21	111.6	51.6
Betamethasone	pharmaceutical, glucocorticoid	0.52	ND	ND	ND	ND	ND	ND
Bezafibrate	pharmaceutical, lipid regulator	1.0	ND	ND	ND	ND	ND	ND
Bifenox	herbicide, WFD priority	100	ND	ND	ND	ND	ND	ND
Bisphenol A	plasticizer, xenoestrogen	4.0	<LOQ	ND	5.8	17.0	12.0	<LOQ
Caffeine	stimulant in beverages, marker compound	1.0	ND	ND	79	103	378	211
Canrenone	pharmaceutical, diuretic, anti-androgen	0.70	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB				
					SO	PO	OB	SC	
(ng/L)									
Carbamazepine	pharmaceutical, antiepileptic, marker compound	1.0	<LOQ	ND	14.5	5.5	23.5	21.5	
Carbendazim	fungicide	1.0	ND	ND	2.6	3.0	2.6	2.8	
Chlorfenvinphos	insecticide, WFD priority	1.0	<LOQ	ND	ND	ND	ND	ND	ND
Chlorotoluron	herbicide	1.0	ND	ND	ND	ND	ND	ND	ND
Chlorpyrifos	insecticide, WFD priority	1.0	ND	ND	ND	ND	5.3	ND	ND
Ciprofloxacin	pharmaceutical, antibiotic	10	ND	NA	ND	ND	ND	ND	ND
Clarithromycin	pharmaceutical, antibiotic	1.0	<LOQ	ND	5.6	11.5	48.5	8.8	
Clobetasol propionate	pharmaceutical, glucocorticoid	0.93	ND	ND	ND	ND	ND	ND	ND
Clobetasone butyrate	pharmaceutical, glucocorticoid	0.39	ND	ND	ND	ND	ND	ND	ND
Clopidogrel	pharmaceutical, anti-coagulant	0.20	ND	ND	ND	ND	ND	ND	ND
Clozapine	pharmaceutical, anti-psychotic	0.25	ND	ND	0.35	ND	0.64	0.36	
Cortisone	hormone, glucocorticoid	1.0	ND	ND	1.8	ND	<LOQ	ND	ND
Cyproterone	pharmaceutical, anti-androgen	0.70	ND	ND	ND	ND	ND	ND	ND
Desoximethasone	pharmaceutical, glucocorticoid	0.42	ND	ND	ND	ND	ND	ND	ND
Dexamethasone	pharmaceutical, glucocorticoid	0.54	ND	ND	ND	ND	ND	ND	ND
Diazinon	insecticide	1.0	ND	ND	9.9	ND	10.0	ND	ND
Diclofenac	pharmaceutical, analgesic, WFD watch list	1.0	ND	1.5	<LOQ	<LOQ	4.3	ND	ND
Dicofol	insecticide, WFD priority	10	ND	ND	ND	ND	ND	ND	ND
Didecyldimethylammonium	surfactant	1.0	ND	ND	ND	ND	ND	ND	ND
Diethyltoluamid	repellent	1.0	<LOQ	ND	10.5	11.5	13.5	12.5	
Difluprednate	pharmaceutical, glucocorticoid	0.73	ND	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
(ng/L)								
Diglyme	industrial solvent	1.0	ND	NA	ND	ND	ND	ND
Dihydrotestosterone	hormone, androgen	1.5	ND	ND	ND	ND	ND	ND
Dichlorvos	insecticide, WFD priority	1.0	ND	ND	ND	ND	ND	10.6
Dimethenamid-p	herbicide	1.0	ND	NA	ND	ND	ND	ND
Dimethoate	insecticide	1.0	ND	ND	ND	ND	ND	1.2
Diuron	herbicide, WFD priority	1.0	ND	ND	<LOQ	2.0	2.0	1.8
Drospirenone	pharmaceutical, progesterone	1.0	ND	ND	ND	ND	ND	ND
Endosulfan alpha and beta	insecticide, WFD priority	1.0	ND	ND	ND	ND	<LOQ	ND
Epi-Androsterone	hormone, androgen	2.0	ND	ND	ND	ND	ND	2.1
Erythromycin	pharmaceutical, antibiotic	10	ND	ND	ND	86	27	39
Estriol	hormone, estrogen	1.0	ND	ND	ND	ND	ND	ND
Estrone	hormone, estrogen	0.10	ND	ND	ND	0.43	0.45	1.28
Fipronil	insecticide	0.10	ND	ND	ND	0.16	0.16	0.41
Flunisolide	pharmaceutical, glucocorticoid	0.65	ND	ND	ND	ND	ND	ND
Fluoranthene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Fluorometholone	pharmaceutical, glucocorticoid	0.41	ND	ND	ND	ND	ND	ND
Gestoden	pharmaceutical, progesterone	1.0	ND	ND	ND	ND	ND	ND
Heptachlor	insecticide, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Heptachlor epoxide	insecticide, transformation product	1.0	ND	ND	ND	ND	ND	ND
Hexachlorobenzene	fungicide, WFD priority	1.0	ND	ND	ND	ND	ND	<LOQ
Hexachlorocyclohexane	insecticide, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Hexamethoxymethylmelamine	industrial compound, marker compound	5.0	ND	ND	ND	ND	ND	147.6

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
(ng/L)								
Hydrocortisone	hormone, glucocorticoid	0.86	ND	ND	ND	ND	2.56	ND
Ibuprofen	pharmaceutical, analgesic	10	ND	ND	ND	ND	13	11
Indeno(1,2,3-cd)pyrene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Irgarol (cybutryn)	biocide, anti-fouling agent	1.0	ND	ND	ND	ND	<LOQ	ND
Isoprotruron	herbicide, WFD priority	1.0	ND	ND	<LOQ	1.2	140.0	5.5
Ketoprofen	pharmaceutical, analgesic	1.0	ND	ND	ND	<LOQ	1.0	ND
Lauryl diethanolamide	surfactant	9.4	ND	ND	ND	ND	ND	<LOQ
Levo-norgestrel	pharmaceutical, progesterone	1.0	ND	ND	ND	ND	ND	ND
Mecoprop	herbicide	1.0	ND	ND	1.4	1.0	1.3	<LOQ
Medroxyprogesterone	pharmaceutical, progesterone	0.40	ND	ND	ND	ND	ND	ND
Metoprolol	pharmaceutical, beta blocker	1.0	ND	ND	1.2	1.1	1.3	ND
N,N-Dimethyldodecylamine-N-oxide	surfactant	1.0	ND	ND	ND	ND	<LOQ	ND
Naphthalene	PAH, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Naproxen	pharmaceutical, analgesic	1.0	ND	ND	8.8	7.5	8.9	3.8
Nonylphenol	surfactant TP, WFD priority, xenoestrogen	10	ND	14	22	22	17	25
Nonylphenoxyacetic acid	surfactant TP, xenoestrogen	1.0	ND	ND	12.0	6.2	12.0	2.6
Nonylphenoxydiethoxyacetic acid	surfactant TP, xenoestrogen	1.0	ND	ND	2.5	2.0	2.7	ND
Norethindrone	pharmaceutical, progesterone	1.0	ND	ND	ND	ND	ND	ND
Norfloxacin	pharmaceutical, antibiotic	10	ND	NA	ND	ND	ND	ND
Norgestimate	pharmaceutical, progesterone	0.30	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
(ng/L)								
Octylphenol	surfactant TP, xenoestrogen	3.0	ND	4.5	ND	ND	ND	28.0
Octylphenoxyacetic acid	surfactant TP, xenoestrogen	1.0	ND	ND	ND	ND	ND	ND
Pentabromodiphenylether (congener numbers 28, 47, 99, 100, 153 and 154)	flame retardant, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Pentachlorobenzene	industrial compound, WFD priority	1.0	ND	ND	ND	ND	<LOQ	ND
Pentachlorophenol	fungicide, WFD priority	100	ND	ND	ND	ND	ND	ND
Perfluorooctanesulfonic acid	surfactant, WFD priority	10	ND	ND	21	<LOQ	20	25
Perfluorooctanoic acid	surfactant	1.0	<LOQ	ND	9.2	10.5	18.5	12.5
Phenazone	pharmaceutical, analgesic	1.0	ND	ND	ND	ND	ND	<LOQ
Phenylbenzimidazolesulfonic acid	sunscreen agent	1.0	<LOQ	ND	26.6	23.1	52.0	42.0
Pirimicarb	insecticide	1.0	ND	ND	<LOQ	ND	ND	ND
Prednicarbate	pharmaceutical, glucocorticoid	0.41	ND	ND	ND	ND	ND	ND
Prednisolone	pharmaceutical, glucocorticoid	0.88	ND	ND	ND	ND	ND	ND
Prednisone	pharmaceutical, glucocorticoid	0.76	ND	ND	ND	ND	ND	ND
Progesterone	hormone, progesterone	1.0	ND	ND	ND	ND	ND	ND
Propiconazole	fungicide	1.0	ND	ND	<LOQ	<LOQ	2.1	<LOQ
Prothioconazole-desthio	fungicide, transformation product	1.0	ND	ND	ND	ND	ND	<LOQ
Quinoxifen	fungicide, WFD priority	10	ND	ND	ND	ND	ND	ND
Raloxifene	pharmaceutical, anti-/estrogen	0.40	ND	ND	ND	ND	ND	ND
Rimexolone	pharmaceutical, glucocorticoid	0.39	ND	ND	ND	ND	ND	ND
Roxithromycin	pharmaceutical, antibiotic	1.0	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
(ng/L)								
Simazine	herbicide, WFD priority	1.0	ND	ND	1.6	<LOQ	<LOQ	1.4
Sotalol	pharmaceutical, beta blocker	1.0	ND	ND	1.4	4.1	4.1	2.7
Spiroxamine	fungicide	1.0	ND	ND	ND	ND	ND	ND
Sucralose	artificial sweetener, marker compound	10	ND	ND	61	68	110	82
Sulfamethazine	pharmaceutical, sulfonamide antimicrobial	1.0	ND	ND	ND	ND	<LOQ	<LOQ
Sulfamethoxazole	pharmaceutical, sulfonamide antimicrobial	1.0	ND	ND	4.3	8.0	12.3	15.9
Sulfapyridine	pharmaceutical, sulfonamide antimicrobial	1.0	ND	ND	ND	ND	4.7	2.4
Sulfathiazole	pharmaceutical, sulfonamide antimicrobial	1.0	ND	NA	ND	ND	ND	ND
Tamoxifen	pharmaceutical, anti-estrogen	0.70	ND	ND	ND	ND	ND	ND
Tebuconazole	fungicide	1.0	<LOQ	ND	ND	ND	<LOQ	ND
Terbuthylazine	herbicide	1.0	ND	ND	ND	2.0	6.2	2.7
Terbuthylazine-desethyl	herbicide, transformation product	1.0	ND	ND	<LOQ	2.2	1.3	1.6
Terbutryn	herbicide, WFD priority	1.0	ND	ND	1.7	2.2	2.8	1.9
Testosterone	hormone, androgen	0.40	ND	ND	ND	ND	ND	ND
Tetraglyme	industrial solvent	0.10	ND	NA	3.90	2.70	3.50	2.60
Thiacloprid	insecticide	1.0	ND	ND	ND	ND	ND	ND
Trenbolone	pharmaceutical, growth promoter	2.0	ND	ND	2.7	ND	ND	ND
Triamcethacetonide	pharmaceutical, glucocorticoid	18	ND	ND	ND	ND	ND	ND
Triamcinolone	pharmaceutical, corticosteroid	5.0	ND	ND	ND	ND	ND	ND

Table 10. – continued.

Compound name	Compound group / Usage pattern	LOQ	Fabrication blank	Laboratory blank	Sava RB			
					SO	PO	OB	SC
					(ng/L)			
Triamcinolone acetonide	pharmaceutical, glucocorticoid	0.59	ND	ND	ND	ND	0.82	<LOQ
Triclosan	biocide	1.0	ND	ND	ND	ND	73.5	29.4
Triethylcitrate	plasticizer	1.0	ND	ND	ND	ND	ND	10.6
Trifluralin	herbicide, WFD priority	1.0	ND	ND	ND	ND	ND	ND
Triglyme	industrial solvent	10	ND	ND	ND	1	1	0.2
Trimethoprim	pharmaceutical, antibiotic	1.0	ND	ND	<LOQ	<LOQ	1.9	1.2
Trimethyloctylammonium	surfactant	1.0	ND	ND	ND	ND	ND	ND
Triphenylphosphate	plasticizer, flame retardant	5.0	ND	ND	ND	<LOQ	ND	ND
Triphenylphosphine oxide	industrial compound, marker compound	5.0	<LOQ	ND	ND	<LOQ	<LOQ	<LOQ
Tris(2-butoxyethyl)phosphate	flame retardant	5.0	ND	ND	ND	19.0	7.8	22.1
Verapamil	pharmaceutical, antihypertensive	1.0	<LOQ	ND	ND	<LOQ	<LOQ	ND

LOQ - limit of quantification; NA - not analysed; ND - not detected; OB - Oborovo, PO - Podused, SC - Sisak Crmac; SO - Samoborski Otok.

The concentration ranges of different classes of analysed compounds (pharmaceuticals, pesticides, surfactants, PAHs and several compounds from other classes like industrial compounds, corrosion inhibitors, sunscreen agents, plasticizers, flame retardants and sweeteners) showed differences between studied locations (Figures 19a-e). As expected, the least contaminated spot corresponded to Samoborski Otok (SO), the reference site, where the lowest concentration and the lowest number of compounds (36) were detected.

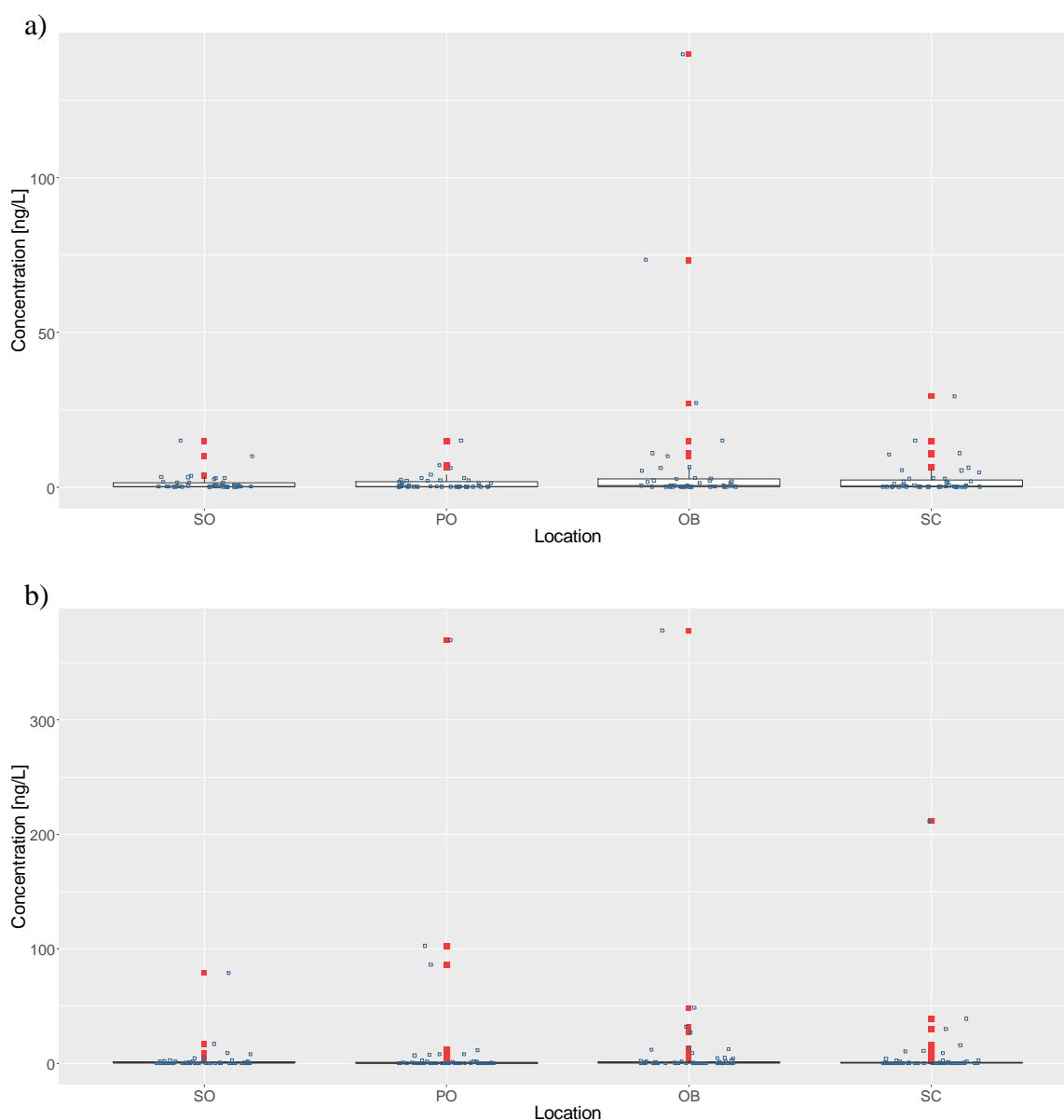


Figure 19. Box-plot diagrams of different contaminant classes: a) pesticides, b) pharmaceuticals found in the Sava River at four sampling locations (SO -Samoborski Otok, PO - Podsused, OB - Oborovo, SC - Sisak Crnac).

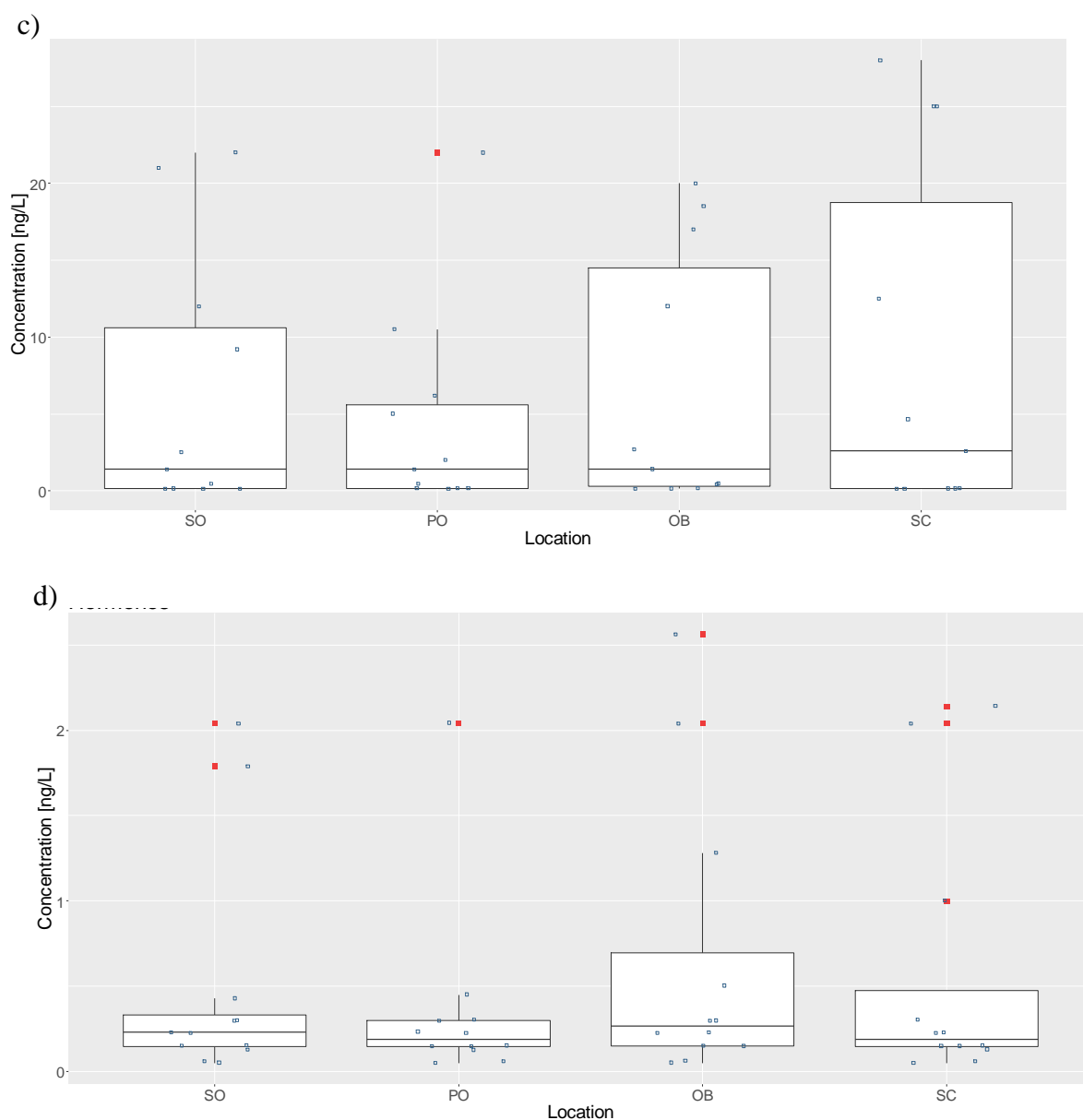


Figure 19. Box-plot diagrams of different contaminant classes: c) surfactants and surfactant-derived compounds, d) natural hormones found in the Sava River at four sampling locations (SO -Samoborski Otok, PO - Podsused, OB - Oborovo, SC - Sisak Crnac).

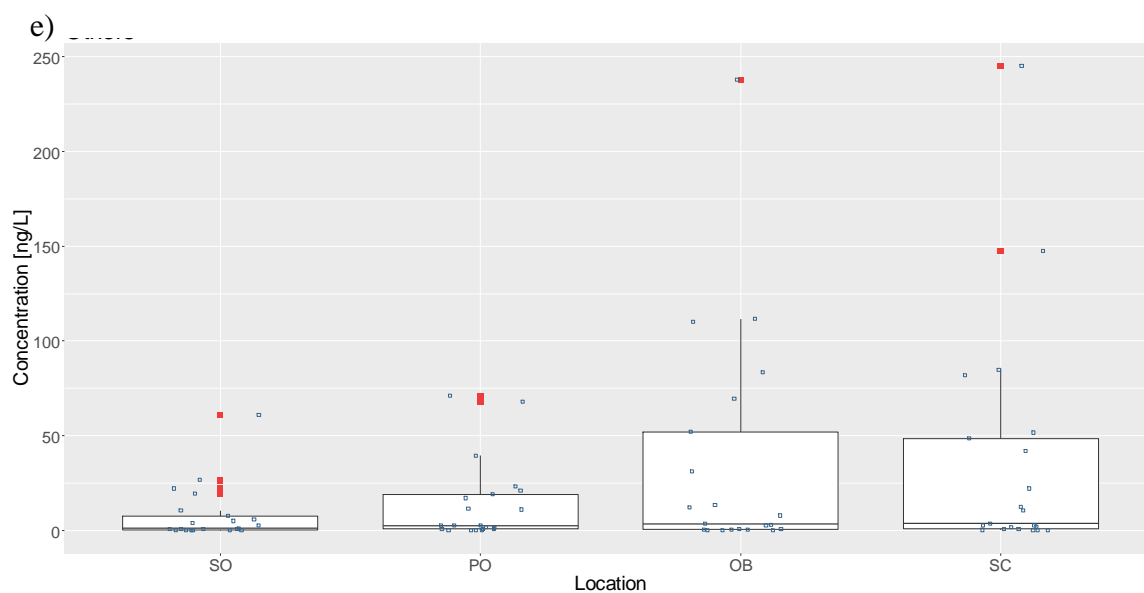


Figure 19. Box-plot diagrams of different contaminant classes e) other organic contaminants found in the Sava River at four sampling locations (SO -Samoborski Otok, PO - Podsused, OB - Oborovo, SC - Sisak Crnac).

In general, levels of pesticides measured at four sampling locations along the Sava River basin were relatively low (Figure 20). This could be explained by the fact that sampling was conducted during August and September, which is not the main pesticide application season. Among different groups of pesticides analysed in this study, maximum concentrations were measured for WFD priority herbicide isoproturon (140 ng/L), following by biocide triclosan (73.5 ng/L) at Oborovo sampling site. Triclosan has been also found at Sisak Crnac site at concentration of 29.4 ng/L, which is the highest measured concentration for pesticides at this location. Organophosphate insecticides dimethoate and dichlorvos were detected at only one sampling site (Sisak Crnac) at 1.2 ng/L and 10.6 ng/L, respectively, while fungicide propiconazole was detected exclusively at Oborovo site at 2.1 ng/L. Six analysed pesticides (2,4-dinitrophenol, atrazine, carbendazim, fipronil, terbutryn, bentazone) were detected at all sampling sites. WFD priority herbicide atrazine was found at all four sampling sites (3.4 - 6.5 ng/L), while simazine was detected at Podsused (1.6 ng/L) and Sisak Crnac site (1.4 ng/L). Despite the ban on their use in many countries, they continue to be observed in water samples in Europe.^{148,149} One of the main metabolites of herbicide atrazine, atrazine-desethyl was also found in all analysed samples, indicating the importance of investigation of transformation

products when investigating the environmental impact of contaminants. The lowest concentration of atrazine-desethyl (3.2 ng/L) was measured at Samoborski Otok site, while maximum concentration of 11 ng/L was measured both at Oborovo and Sisak Crnac sites. Compounds which are used instead of simazine and atrazine, such a terbuthylazine and terbutryn, were also detected in the Sava River with maximum concentrations of 6.2 ng/L and 2.8 ng/L, respectively at Oborovo site. Terbuthylazine-desethyl, transformation product of herbicide terbuthylazine was also detected in Sava River, with concentrations lower than 2.2 ng/L. In general, low pesticide levels found in Sava River samples are in agreement with the levels found by Loos et al.¹⁵⁰ during the basin-wide monitoring campaign on the Danube River and its tributaries.

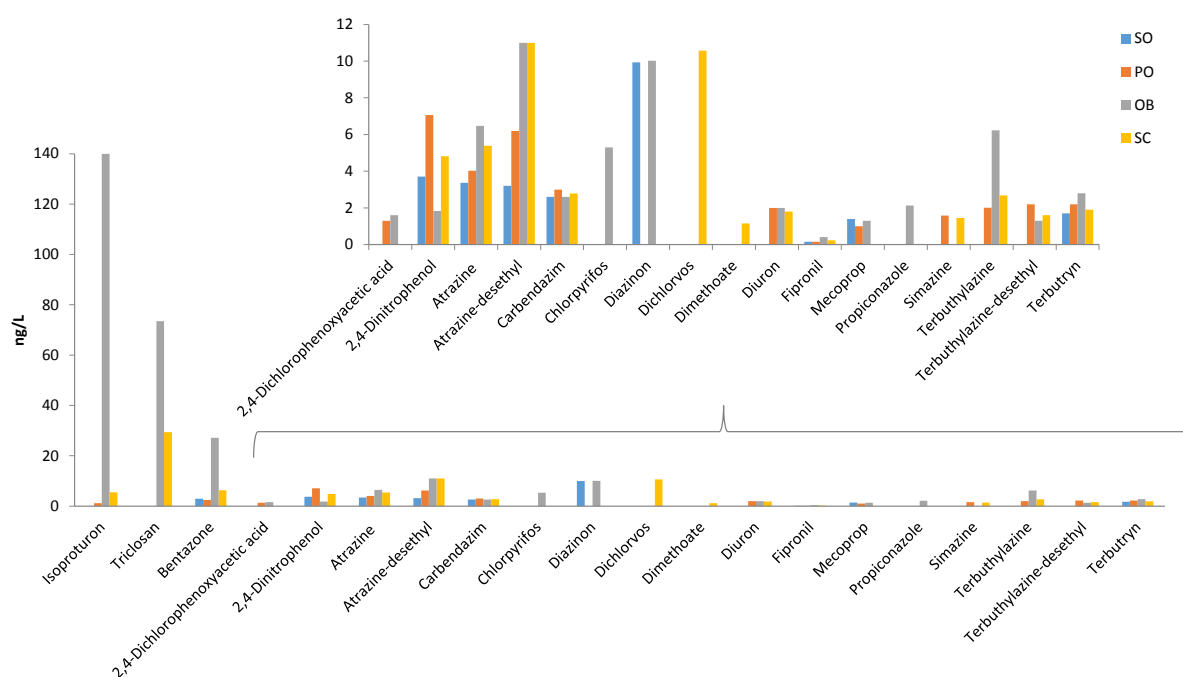


Figure 20. Concentrations of pesticides (ng/L) in the Sava River.

Another prominent group of contaminants determined in this study were pharmaceuticals, which encompass a great variety of compounds belonging to different therapeutic classes. Eighteen (18) out of fifty six (56) analysed pharmaceutical compounds were detected at minimum one sampling site with concentration above the quantification limit (Figure 21). The highest concentrations were measured for macrolide antibiotics azithromycin (370 ng/L) and erythromycin (86 ng/L) at the Podsused site. Azithromycin is a semi-synthetic antibiotic made

from erythromycin as a precursor. It was discovered by the Croatian pharmaceutical company and it has been continuously produced in large quantities in the facilities situated near the city of Zagreb for almost 30 years.¹⁵¹ Therefore the measured concentrations of macrolide antibiotics were expected since the investigated site is impacted by the effluents from the pharmaceutical industry. However, the maximum concentration of macrolide antibiotic clarithromycin (48.5 ng/L) was detected at Oborovo site, impacted by the wastewater effluents of the cities of Zagreb and Velika Gorica. The typical levels of macrolide antibiotics reported for other European countries¹⁵² and USA¹⁵³ are much lower, indicating significant impact of the local production on the macrolide levels in the Sava River. Sulfonamide antibiotic sulfamethoxazole, and its transformation product acetyl-sulfamethoxazole, were detected along the whole river section in the range of 4.3 - 15.9 ng/L and 7.9 - 11.9 ng/L, respectively. The slightly increasing concentrations of sulfamethoxazole were detected in the more downstream part of the Sava River. The antiepileptic marker compound carbamazepine was detected at all sampling sites (5.5 - 23.5 ng/L). The elevated concentrations of carbamazepine measured downstream of the city of Zagreb are caused by untreated or insufficiently treated effluents from the cities of Zagreb and Velika Gorica. Carbamazepine was found at similar concentrations in Sava and Tamiš River in Serbia¹⁵⁴ and Llobregat River in Spain.¹⁵⁵ However, lower concentrations of carbamazepine were observed by Loos et al.¹⁵⁰ and Nödler et al.¹⁵⁶ Naproxen, a pharmaceutical belonging to analgesic group, was detected at all samples with maximum concentration at Oborovo site (8.9 ng/L). Ibuprofen levels were slightly higher, 13 ng/L and 11 ng/L were measured at Oborovo and Sisak Crnac site, respectively. Comparable results for ibuprofen were reported by Pedrouzo et al.¹⁵⁵ Levels of other pharmaceuticals detected in this study (sulfapyridine, trenbolone, triamcinolone acetonide, trimethoprim, metoprolol and sotalol) were lower than 5 ng/L.

Caffeine has been the most abundant individual compound detected in the Sava River samples showing its abundant use, primarily due to the consumption of coffee, tea and some soft drinks. Caffeine is ubiquitous in surface waters, and it has been proposed as a marker of anthropogenic pressure on the environment.^{157,158} Maximum concentration of caffeine (378 ng/L) was detected at Oborovo site as a consequence of wastewater inputs from the cities of Zagreb and Velika Gorica.

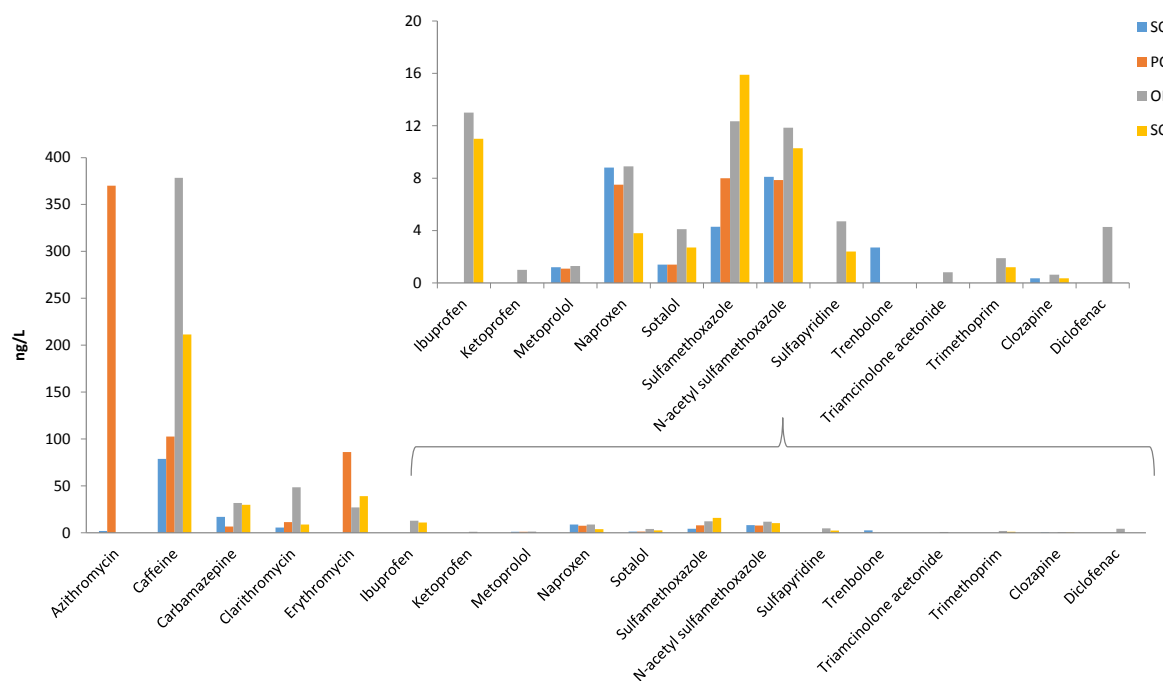


Figure 21. Concentrations of pharmaceuticals and caffeine (ng/L) in the Sava River.

Out of eleven analysed surfactants and surfactant-derived compounds, 6 have been detected with concentrations higher than limit of detection (Figure 22). The most toxic endocrine disrupting compound derived from NPEO surfactants, nonylphenol (NP), was detected at all four sampling sites with concentrations ranging from 17 - 25 ng/L, while octylphenolic analogue of NPEOs, octylphenol, was detected at only one sampling site (Sisak Crnac) with concentration of 28 ng/L. Comparing these results with those obtained in other countries, levels of alkyphenolic compounds in the Sava River were much lower.^{159,160,161} However, Micić and Hofmann¹⁶² also reported low concentration levels, close to the LOQ for NP and OP in Danube River samples. Similar concentrations of alkyphenolic compounds were measured in rivers of Portugal.¹⁶³

The maximum concentration of perfluorinated compounds (25 ng/L) was measured for perfluorooctanesulfonic acid at the Sisak Crnac site, affected by both urban and industrial WW.

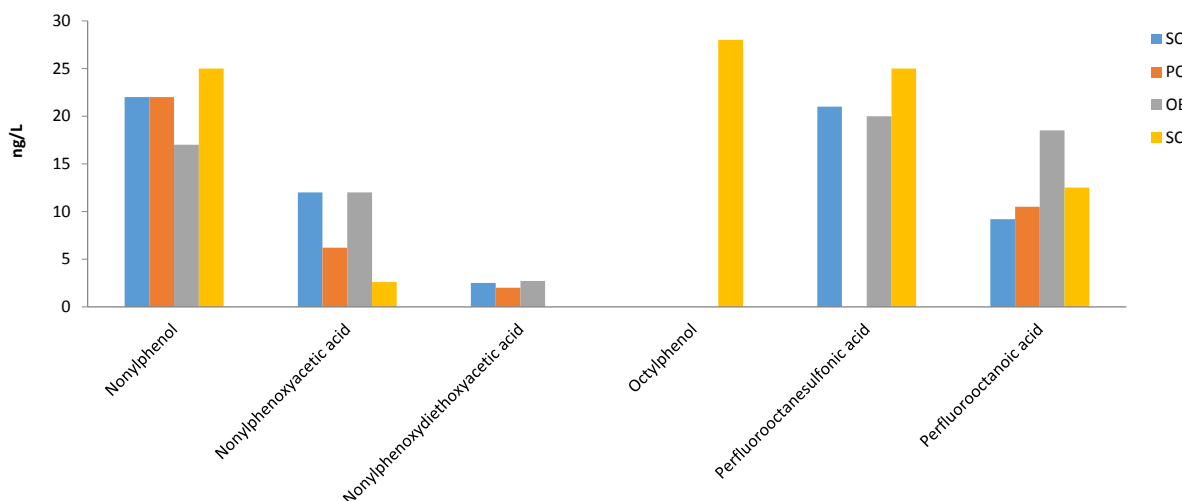


Figure 22. Concentrations of surfactants and surfactant-derived compounds (ng/L) in the Sava River.

Highly hydrophobic compounds such as PAHs were not detected in any of the sample (LOQ = 1 ng/L). This could be explained by the fact that the results represent mainly the dissolved fraction, since the water samples were filtered through a 0.63 μm glass fiber filters prior to the SPE.

The presence of estrogens in surface waters is of particularly relevance because of their high estrogenic potency. Out of 12 investigated natural hormones, 4 were detected at concentrations higher than the LOQ (Figure 23). The most abundant naturally occurring estrogenic hormone detected at all sampling sites was estrone (E1), an oxidation product of estradiol that may be also formed in wastewater treatment plants.¹⁶⁴ The lowest concentration of E1 was measured at Samoborski Otok at concentration of 0.43 ng/L, while the maximum concentration of 1.28 ng/L was measured at Oborovo site. This reflects the increasing contamination of the Sava River due to significant percentage of sewage effluents from human sources at Oborovo sampling site. Measured concentrations of E1 were in accordance with concentrations generally found in surface waters varying between 0.1 and a few ng/L.^{165,166} Epiandrosterone, a dehydroepiandrosterone metabolite and a precursor of testosterone and estradiol, was detected at only one sampling site (Sisak Crnac) at 2.1 ng/L. It is interesting to note that naturally occurring glucocorticoid compound, cortisone, was detected only at Samoborski Otok site at 1.8 ng/L, while hydrocortisone was exclusively measured at Oborovo site at 2.56 ng/L.

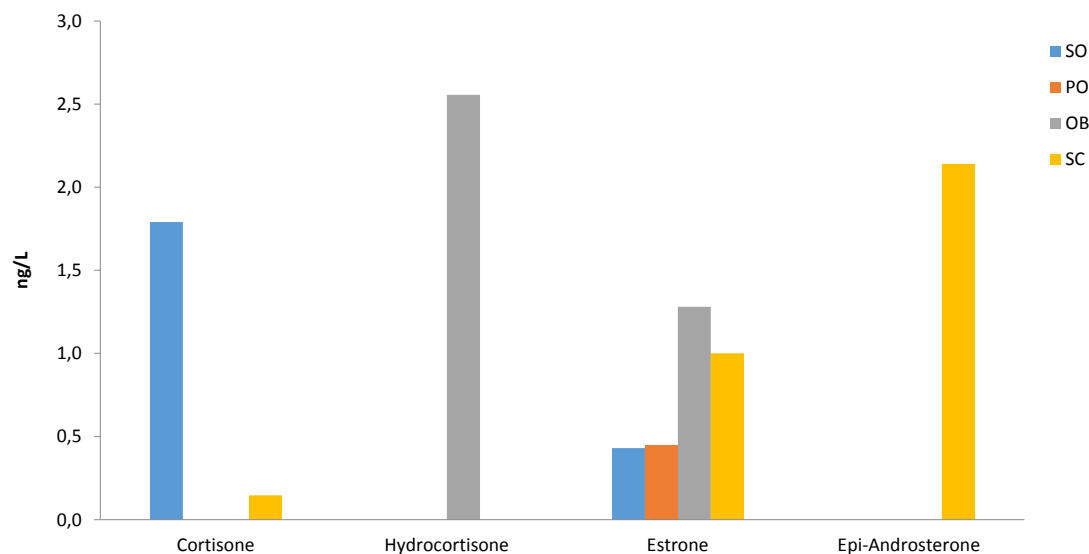


Figure 23. Concentrations of natural hormones (ng/L) in the Sava River.

The two sulfonates sunscreen compounds, benzophenone-4 and phenylbenzimidazolesulfonic acid, were found in all Sava River samples ranging from 21 - 111.6 ng/L and 23.1 - 52 ng/L, respectively. Maximum concentrations of these compounds were measured at Oborovo site (Figure 24). Measured concentrations could be linked with the indirect inputs through wastewater resulting from the use of personal care products (wash-off from skin during showering), washing clothes and industrial discharges. The data on benzophenone-4 on Sava River samples seems to match the findings in the UK, where benzophenone-4 is the main UV filter among the benzophenones.¹⁶⁷ The medium polarity compound, benzophenone-3, was detected at two sampling sites (Podsused and Sisak Crnac), with concentrations of 1.7 ng/L and 3.6 ng/L, respectively. Similar levels of concentrations for benzophenone-3 were obtained by Cabeza et al.¹⁶⁸ and Kasprzyk-Hordern et al.¹⁶⁷ Diethyltoluamide (DEET), the active component of many insect repellents and among the most frequently detected compounds in aqueous environments, was detected at all sampling sites along the Sava River basin with concentration range of 10.5 - 13.5 ng/L. After product uses by dermal application, DEET is typically washed from the skin when bathing, so its occurrence in municipal wastewater is ensured.¹⁶⁹ In fact, DEET has been previously reported in 67% of the wastewater samples in Croatia¹⁷⁰, with average concentration of 840 ng/L showing that DEET is still the main insect repellent in WB region. Concentrations of DEET found in Sava River samples were in agreement to other studies.^{29,171}

Among flame retardants, tris(2-butoxyethyl)phosphate was detected at three investigated sampling sites with maximum concentration of 22.1 ng/L at Sisak Crnac site. Bisphenol A (BPA), a chemical under discussion to be added to the priority substance list of the WFD was detected at three investigated sampling sites along the Sava River basin. As a polar monomer of polycarbonate plastic, used in canned foods as well as an intermediate in the synthesis of epoxy resins, flame retardants and many other products, BPA has prolific use and it is ubiquitous in the environment.¹⁷² Maximum concentration of BPA was measured at Podsused site at 17 ng/L. Compared to the data from other studies, detected concentrations are similar to the ones reported in the Netherlands,¹⁶⁰ central area of Italy,¹⁷³ and Portugal,¹⁶³ with BPA levels of 68 ng/L, 43 ng/L, 29 ng/L and 5.4 ng/L, respectively. Loos et al.,¹⁵⁰ reported higher concentrations of BPA in Arges (up to 490 ng/L), and in the Sava River (up to 246 ng/L), while lower concentrations were found after the Morava influent in Bratislava 116 ng/L, downstream Budapest 12 ng/L, downstream the Drava influent 27 ng/L, and the Sava influent 15 ng/L. Another analysed plasticizer, triethylcitrate, was detected at only one sampling site (Sisak Crnac) with concentration of 10.6 ng/L. There was a general trend of higher concentrations of industrial compounds at the fourth location (Sisak Crnac) in comparison with other three sampling locations of the river. Higher concentrations of industrial compounds encompassed by this study found at Sisak Crnac site indicated their intensive use in this area, and could be linked with the fact that this site is affected by both urban and industrial WW.

The highest concentrations of industrial compounds were found for 4-toluenesulfonamide, main hydrolysis product of the antimicrobial agent Chloramine T (Figure 24). This compound is used as a plasticizer. The highest concentration of 4-toluenesulfonamide was measured at Sisak Crnac (245 ng/L) following by Oborovo (238 ng/L) and Podsused site (71 ng/L). At the reference site (Samoborski otok), level of 4-toluenesulfonamide was below the quantification limit. Similar concentration levels of 4-toluenesulfonamide were measured in Germany by Richter et al.,¹⁷⁴ while slightly lower concentrations were detected in several river waters in Spain.¹⁷⁵ Benzotriazole and 5-Methyl-1H-benzotriazole (tolytriazole), compounds that are mainly used as anticorrosives in industrial and household applications (e.g. in engine coolants, aircraft deicing fluids, or antifreezing liquids; silver protection in dishwasher detergents) were also detected in Sava River samples. Due to their low biodegradability and limited sorption tendency, they are only

partly removed in wastewater treatment.¹⁷⁶ Two common compound forms used as corrosion inhibitors, 1-H benzotriazole and 5-Methyl-1H-benzotriazole, were also detected in Sava River samples. 1-H benzotriazole was detected at two sampling sites, Oborovo and Sisak Crnac with concentrations of 90 ng/L and 136 ng/L, respectively. Benzotriazole derivate 5-Methyl-1H-benzotriazole was detected at all sampling sites with concentration range of 19.5 - 83.5 ng/L. In comparison to other river water monitoring results from Europe, concentration levels found in the Sava River appear to be rather low. Nödler et al.¹⁵⁶ reported mean concentration of 670 ng/L of 1H-benzotriazole and 741 ng/L of tolyltriazole for river Leine in Germany. Giger et al.,¹⁷⁷ determined concentrations of benzotriazole and tolytriazole in ambient surface waters in Switzerland, including seven rivers which have distinct water flows and receive treated wastewater effluents at various dilution ratios. A maximum benzotriazole concentration of 6.3 µg/L was found in the Glatt River, while concentration of tolytriazole, in most cases was about 5 - 10 times less abundant. Hexamethoxymethylmelamine was detected at two sampling sites downstream of the city of Zagreb (Oborovo and Sisak Crnac), with concentrations of 31.2 ng/L and 147.6 ng/L, respectively. In Europe, industrial applications for hexamethoxymethylmelamine include coatings for cans, coils, and automobiles.^{178,179} The higher concentration of this pollutant was found in well-known contaminated site, downstream from the city of Sisak and near industrial area. This compound was not present in samples from the Samoborski Otok and Podsused site. The results obtained in this study were in the line with the study of Dsikowitzky and Schwarzbauer.¹⁸⁰ Levels of industrial solvents detected in this study (triglyme and tetraglyme) were relatively low, being in the range of 0.2 - 3.9 ng/L.

Analyses of artificial sweeteners confirmed the presence of two marker compounds, acesulfame and sucralose in all analysed samples, with concentration range of 1.1 - 2.8 ng/L and 61 - 110 ng/L, respectively (Figure 24). The maximum concentrations of these compounds were measured at Oborovo site. The concentration of the persistent sweeteners acesulfame and sucralose lie in the nanogram per liter to microgram per liter range in rivers and lakes which confirmed that artificial sweeteners are water contaminants that are highly specific markers of municipal wastewater.¹⁸¹ Detected levels of sucralose in Sava River were in the line with the levels detected in the river Rhine being in the range from < LOD to 100 ng/L and from < LOQ to 180 ng/L.^{182,183} Surprisingly, measured concentrations of acesulfame in the Sava River were much lower compared to other studies.^{184,185,186}

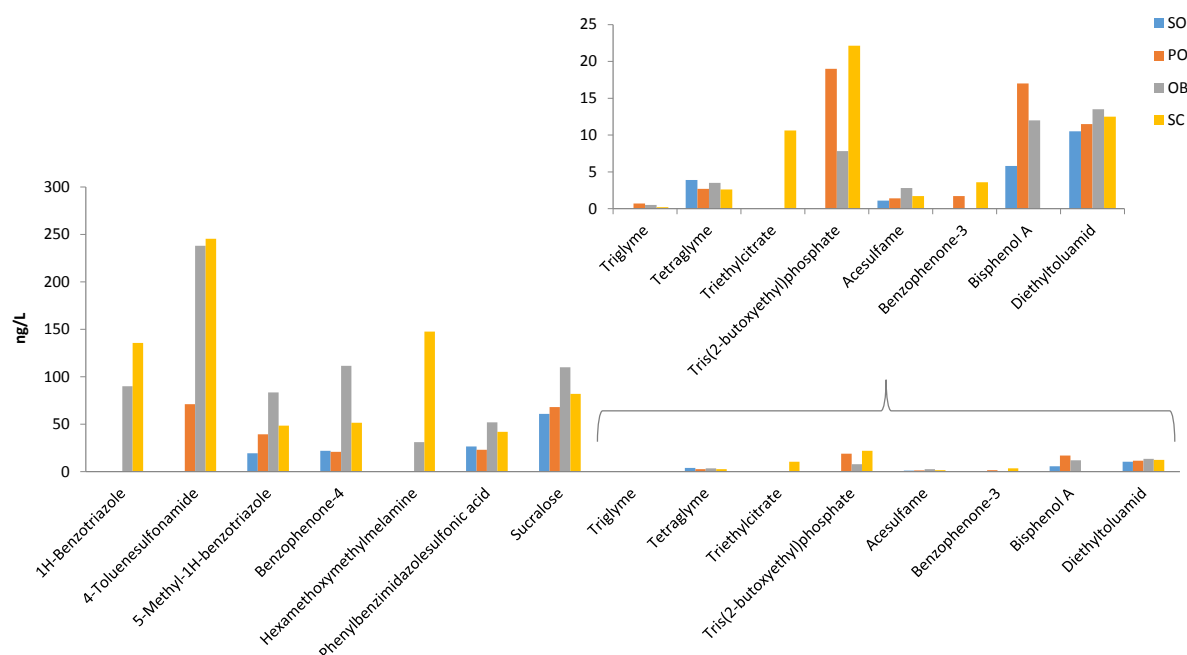


Figure 24. Concentrations of other organic contaminants (ng/L) in the Sava River.

4.2.2. Results of bioassays

A battery of ecotoxicological *in vitro* and *in vivo* bioassays that encompassed various toxicity end-points was applied for the assessment of the Sava River samples collected during the EDP study. These measurements provided important complementary information about the contaminant levels in the Sava River. The most frequently observed effects were estrogenicity, zebrafish acute embryo toxicity and algal growth inhibition, while glucocorticoid activity was measured only at one sampling site. Most of the observed biological activity was associated with neutral extracts, whereas acidic and basic extracts exhibited only occasionally very weak or no detectable effects. Therefore, in the following section, results of bioassays obtained for neutral extracts will be discussed. The results of bioassays for neutral, acidic and basic extracts are summarized in the Supplementary material (Table SII).

4.2.2.1. Receptor-mediated *in vitro* bioassays

The presence of estrogenic compounds in Sava River extracts were tested in the MELN reporter cell line. Estrogenic activity was observed for three sampling sites, Samoborski Otok (SO), Posdused (PO) and Oborovo (OB) (Table 11). The biological estradiol equivalents (E2-EQs) ranged from 0.16 ng/L to 0.42 ng/L, with the highest activity at Oborovo sampling site (Figure 25). The measurements of estrogenicity in the Sava River samples were in good agreement with a relatively low estrogenic potential (EEQ < 1 ng/L) of surface water reported previously for Sava River samples (Smital et al.⁶⁵). The detected levels were also in the line with the results reported by several other authors^{187,188,189} for the surface water samples.

Mass balance calculations for estrogenic and glucocorticoid activities in Sava River samples are given in the Table 12. Chemical equivalents (Chem-EQ), calculated by multiplying measured concentrations of known agonists with their relative potencies in the particular bioassays, were compared to biological equivalents (Bio-EQ) and the resulting ratio (expressed as a percentage) showed the extent to which the observed biological activity can be explained by target compounds (Table 12). The mass balance calculations resulted in ratios of observed estrogenic activity that could be partly explained by the detected ER agonists. Estrone (E1) was a dominant driver of the observed estrogenicity for all three sites where its contribution ranged from 21% to 34%. Nonylphenoxyacetic acid and bisphenol A also partly contributed to the observed estrogenicity at investigated sites. Interestingly, none of the other target estrogens (17- α -ethinylestradiol (EE2), 17- β -estradiol (E2) or estriol (E3) were detected at these sites. For the OB sampling site with the highest estrogenic activity (0.42 ng/L E2-EQ), the effect could be partially explained by these three chemicals, estrone, nonylphenoxyacetic acid and bisphenol A, which contributed with 34%, 1.2%, and 0.1%, respectively. Typical xenobiotic candidates for the estrogenic activity reported in different studies are alkylphenols,¹⁹⁰ benzophenone¹⁹¹ and phthalates.¹⁹²

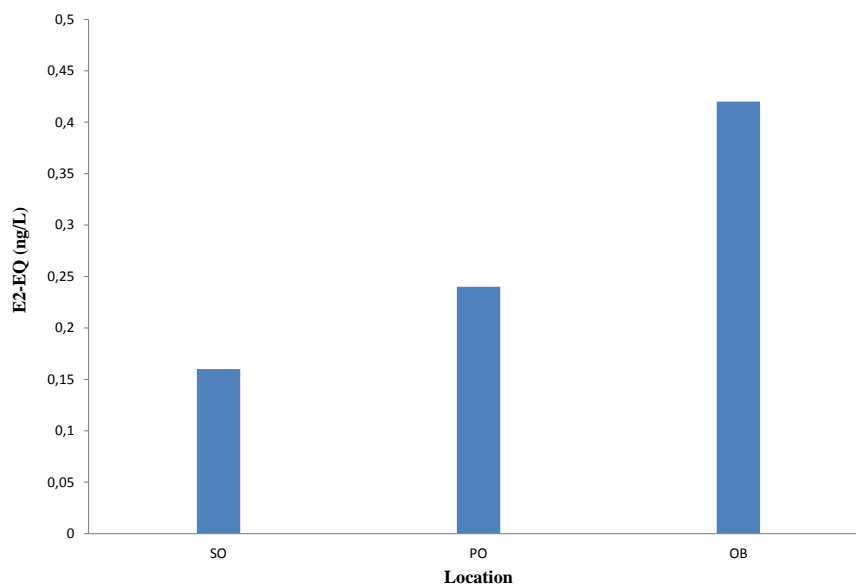


Figure 25. Estrogenic potential of the Sava River water samples (SO - Samoborski Otok, PO - Podsused, OB - Oborovo) tested in the MELN reporter cell line. Results are measured in monoplicate and expressed in biological estradiol equivalents (E2-EQs, ng/L).

It is important to note that despite decreasing LODs of chemical analytical methods, a mixture of estrogens, where each component occurs at a concentration below LOD may still exhibit measurable biological effects.¹⁹³ The analysis of estrogens in environmental samples still remains a challenge due to high sample complexity, need of appropriate clean-up, special analytical procedures, e.g. derivatization, and their very low physiologically active concentrations.¹⁹⁴ Having in mind that some portions of the observed effects still cannot be explained by target chemical analysis, the use of bioanalytical tools for detection of estrogenicity, as a highly relevant endpoint for monitoring of European surface waters is necessary.¹⁹⁵

Table 11. Results of receptor-mediated *in vitro* bioassays expressed by biological effect equivalents (BEQs). The results are shown for the neutral sorbent (HR-X) extracts. For more details see Supplementary material (Table SII).

Bioassay (maximal tested REF)	Endpoint, BEQ, unit	LOD	LOQ	Sava River basin			
				SO	PO	OB	SC
GR-CALUX® (100)	Glucocorticoid activity Dex-EQ [ng/L]	0.4	1.2	<LOD	<LOD	0.32	<LOD
ER-mediated activity – MELN cells (100)	Estrogenicity E2-EQ [ng/L]	0.009	0.024	0.16	0.24	0.42	<LOD
	Anti- estrogenicity OH-Tam-EQ [ng/L]	2.4	6.6	<LOD	<LOD	<LOD	<LOD
AR-mediated activity -MDA-kb2 cells (100)	Androgenicity DHT-EQ [ng/L]	0.021	0.085	<LOD	<LOD	<LOD	<LOD
	Anti- androgenicity Flu-EQ [ng/L]	120	618	<LOD	<LOD	<LOD	<LOD

AR - androgen receptor; Dex-EQ - dexamethasone equivalent; DHT-EQ - dihydrotestosterone equivalent; E2-EQ - 17- β -estradiol equivalent; ER - estrogen receptor; Flu-EQ - flutamide equivalent; GR - glucocorticoid receptor; LOD - limit of detection; LOQ - limit of quantification; OB - Oborovo; PO - Podsused; REF - relative effect potencies; SC - Sisak Crnac; SO - Samoborski Otok.

Neither anti-estrogens nor anti-estrogenic activity was detected at any of the investigated sampling sites. Similarly, androgenic activity was not detected in any of the Sava River samples. Glucocorticoid activity was measured by both GR-CALUX® and MDA-kb2 cells, and the GR-CALUX® assay showed to be more sensitive. Such a difference in sensitivity could be explained by differences in the specificity of the assays towards glucocorticoids.¹⁹⁶ Metabolic capacity of the cells used for GR-CALUX® assay could also explain observed differences.¹⁹⁷

Table 12. Mass balance calculations for estrogenic, androgenic and glucocorticoid activities in the Sava River samples.

	Compound name	LOQ (ng/L)	REP	Reference	Sava River basin		
					SO	PO	OB
Estrogenicity (MELN)	Benzophenone-3	1	7.04E-07	Molina et al. ¹⁹⁸	ND	1.7	<LOQ
	Bisphenol A	4	4.49E-05	Neale et al. ¹⁹⁹	5.8 (0.2%)	17 (0.3%)	12 (0.1%)
	Estrone	0.1	0.11	Neale et al. ²⁰⁰	0.43 (30%)	0.45 (21%)	1.28 (34%)
	Nonylphenol	10	3.30E-06	Creusot et al. ⁷⁰	22	22	17
	Nonylphenoxyacetic acid	1	4.10E-04	Creusot et al. ⁷⁰	12 (3%)	6.2 (1%)	12 (1.2%)
	Bio-E2-EQ				0.16	0.24	0.42
	Chem-E2-EQ				0.05	0.05	0.15
	Chem/Bio ratio (%)				33	22	35
Glucocorticoid activity (GR-CALUX)	Cortisone	1	8.00E-04	Schriks et al. ¹³⁹	1.8	ND	<LOQ
	Hydrocortisone	0.86	3.60E-02	Macikova et al. ²⁰¹	ND	ND	2.56 (29%)
	Bio-Dex-EQ				-	-	0.32
	Chem-E2-EQ				0	0	0.09
	Chem/Bio ratio (%)				-	-	28.9

Bio-Dex-EQ - biological dexamethasone equivalent; Bio-E2-EQs - biological estradiol equivalents; Chem-E2-EQ - chemical estradiol equivalents, LOQ - limit of quantification; ND - not detected; OB - Oborovo; PO - Podsused; REP - relative effect potencies; SC - Sisak Crnac; SO - Samoborski Otok.

Glucocorticoid activity using GR CALUX assay was detected exclusively at Oborovo sampling site, where biological dexamethasone equivalent (Bio-Dex-EQ) reached 0.32 ng/L. This level is similar to a study reported by Van der Linden et al.,⁷¹ where glucocorticoid activity was detected in the levels ranging from 0.39 - 1.3 ng/L (Bio-Dex-EQ) in surface waters. Out of 20 targeted glucocorticoids measured in the Sava River samples,

hydrocortisone was only detected at OB site at concentration of 2.56 ng/L, which could explain only 29% of the biological activity measured at this site. The chemical results also showed a small amount of steroid hormone cortisone at Samoborski Otok site (1.8 ng/L), however no GR activity was detected at this site. At present, there are insufficient data to predict the environmental concentrations of glucocorticoid compounds in surface waters. However, Chang et al.,²⁰² showed that glucocorticoids seem to be relatively well removed in sewage treatment plants. This is consistent with the fact that environmental concentrations of glucocorticoids in surface waters measured so far were in the (low) ng/L range.^{71,202}

4.2.2.2. *In vivo* bioassays

In order to investigate embryotoxic effect potentials of Sava River samples, the *in vivo* zebrafish embryo acute toxicity test assay (FET) was used. Acute toxicity was observed at three locations, Samoborski Otok, Podsused and Oborovo (Table 13). Multitude of chemicals from different classes could be related to embryotoxicity of surface waters, however, micropollutants originating from wastewater, especially pharmaceuticals are considered to play an important role for this endpoint.^{203,204} Surprisingly, large embryotoxic effect was measured at the upstream reference location Samoborski Otok, the sampling site with no immediate impact of wastewater effluents. At SO, lethality reached approximately 50% for the relative enrichment factor (REF) of 19.1. Out of 151 target compounds, only 36 were detected at this site, all in concentrations below 80 ng/L. Neale and Escher²⁰⁵ showed that co-extracted dissolved organic carbon may affect bioassays by having an effect by itself. However, having in mind that the extracts collected on other downstream locations are supposed to have an even larger percentage of co-extracted dissolved organic material, due to significant inputs from the WWTPs Zaprešić, Zagreb and Velika Gorica, this assumption doesn't seem plausible. More probably, it can be assumed that the observed effect should be attributed to some unknown contaminants that were not covered by our target analyses. Medium embryotoxic effect was measured (EC50 = REF 72) in sample collected at the location Podsused. This probably reflected an input of embryotoxic compounds such as macrolide antibiotics from pharmaceutical industry effluents. Embryotoxic effect was also measured at Oborovo site, downstream of the main wastewater outlets of WWTPs of the cities of Zagreb and Velika Gorica, where lethality reached 50% for the REF of 23.3. Several

main contributors to embryotoxicity appeared at multiple sampling sites, e.g. surfactants-derived compounds like nonylphenol, nonylphenoxyacetic acid, nonylphenoxydiethoxyacetic acid and octylphenol (detected only at Oborovo location), macrolide pharmaceutical azithromycin (detected at locations Samoborski Otok and Podsused) or pesticides such as diazinon, fipronil and chlorpyrifos (detected only at location Oborovo). The values for FET were approximately one order of magnitude lower than those reported by Neale et al.¹⁹⁹ for samples collected in highly polluted Danube River.

Table 13. Results of bioassays expressed as LC50, EC50 or LOEC values, where relative enrichment factor (REF) is used as the unit. Shown are the results for the neutral sorbent (HR-X) extracts (algal growth inhibition, AChE inhibition and zebrafish embryo acute toxicity) and whole water samples (*in vivo* thyroid activity). Active samples with resulting REF < 20 are marked in red, REF between 20 and 100 in orange, equaling 100 in yellow and > 100 in green.

Bioassay (maximal tested REF)	Endpoint, unit	Sava River basin			
		SO	PO	OB	SC
Algal growth inhibition (100)	Growth rate inhibition EC50 [REF]:	259	94.6	132	NA
	Growth rate inhibition LOEC [REF]:	100	33	33	100
AChE inhibition (500)	AChE inhibition EC50 [REF]:	NA	NA	NA	NA
	AChE inhibition LOEC [REF]:	>250	>250	>250	>250
Zebrafish embryo acute toxicity (100)	Survival LC50 [REF]	19.1	72	23.3	NA
	Survival LOEC [REF]	12.5	25	25	>100
	Sublethal endpoints EC50 [REF]	NA	NA	NA	NA
	Sublethal endpoints LOEC [REF]	NA	NA	NA	>100
<i>In vivo</i> thyroid activity (1)	Thyroid activity LOEC [REF]	>1	>1	>1	>1

NA - not assessed; EC50 - concentration at which the effect reaches 50% of the effect in untreated control; LC50 - lethal concentration that causes 50% of the maximal lethal effect; LOEC - lowest observed effect concentration; REF - relative enrichment factor.

In vivo thyroid activity in transgenic *Xenopus*, using whole water samples, was not detected in any of the Sava River samples.

Also, no mutagenicity in *Salmonella typhimurium* strain TA100 was observed for any of the Sava River samples (data not shown). This is in agreement with previous report by Smital et al.⁶⁵ who showed that the mutagenic/genotoxic potential of the Sava River samples was generally very low.

Algal growth inhibition was detected at three sampling sites, Samoborski Otok, Podsused and Oborovo and the effect concentrations were relatively low (Table 13). Extracts from those sites elicited toxicity (EC50 = REF 94.6 - 259) to algae, with the highest measured toxicity in the sample collected at PO site. Tang and Escher²⁰⁶ indicated that herbicides dominated the algal toxicity in treated wastewaters and surface waters, and that the contribution by the non-herbicides was negligible. Several pesticides were also detected at these sites that could cause algal growth inhibition such as herbicides isoproturon, atrazine or atrazine-desethyl, terbuthylazine, diuron or biocide triclosan. Algal growth inhibition detected at SO, PO and OB sites could be caused by the combined effect of several compounds. Azithromycin, detected at Podsused site at concentration of 140 ng/L could also contribute to the observed toxicity. Erythromycin, detected at PO and OB sites at concentration of 86 ng/L and 27 ng/L, respectively could also cause effects on algae. González-Pleiter et al.,²⁰⁷ examined individual and combined toxicities of several pharmaceuticals in two organisms representative of the aquatic environment, the cyanobacterium *Anabaena* and the green alga *Pseudokirchneriella* and showed that erythromycin was highly toxic for both organisms.

In the AChE inhibition assay, two basic extracts were active (PO and OB: LOEC = 500 REF), while in neutral extracts showed no activity.

In spite of extensive target analysis of a wide range of environmental contaminants, the observed level of biological activity could only partially be explained by the contaminants determined by the target chemical analyses. Therefore, as a further step in characterization of hazardous contamination in the Sava River, a more detailed EDA (higher tier EDA) study was conducted at the selected locations in order to further investigate causative agents responsible for the detected activities.

4.3. Detailed EDA study of organic contaminants in the Sava River

Based on the experiences gained during the preliminary EDA study (chapter 4.2) the methodology of the detailed EDA study on the Sava River was slightly modified. Since the sequential extraction using serially coupled sorbents did not provide the expected fractionation during the in-situ extraction, the extracts obtained from the neutral and both ionic sorbents were combined before further processing of the extracts. Moreover, a more detailed fractionation of the total extract was performed using preparative HPLC separation on an octadecylsilica column. Finally, in order to facilitate linking the observed effects with pollutant signatures of the analysed samples, an extended range of target contaminants was included in the chemical analyses as compared to the preliminary study discussed above.

4.3.1. Results of chemical screening

The detailed EDA study on the Sava River focused on the most polluted section of the Sava River in the wider area of the city of Zagreb situated between Podsused and Oborovo. Moreover, in addition to river water samples the sampling included composite samples of the most important wastewater inputs (WWTP Zaprešić, wastewater effluents of the city of Zagreb collected at the discharge point at Hruščica, WWTP Velika Gorica) into the river. All wastewater samples were also collected using LVSPE device (Figure 7).

Out of the 226 compounds analyzed in the total SPE fractions, 131 compounds were detected at least once in the samples, including 30 compounds at Zaprešić site (ZA) site, 69 compounds at Podsused (PO), 110 at Hruščica canal (HC), 72 at Hruščica (H), 106 in secondary effluent from the WWTP of the city of Velika Gorica (WWTP VG) and 62 at Oborovo (OB). Table 14 summarizes the analytical results for the compounds detected at least ones in the samples (above the lowest detectable level, LDL). Concentrations of detected compounds at each sampling site are also presented in Figures 26a-f (for the correspondence between numbers and chemicals see Table 14). The list of all target compounds is given in the Supplementary material (Table SIII).

At Zaprešić site, the discharge point of the pharmaceutical industry into the Sava River, only 30 compounds were detected. Interestingly, the maximum concentration of 324 ng/L was measured for 4-bromophenol, a phenolic organohalogen, often used as flame

retardant. Perfluorooctanoic acid was detected at concentration of 220 ng/L. As expected, pharmaceuticals were the most frequent detected group of contaminants at ZA site, with 18 detected compounds. However, concentrations of detected pharmaceuticals were rather low, ranging from 0.5 - 156 ng/L. The highest concentration was measured for analgesic ibuprofen. Pharmaceuticals enalapril, warfarin, metformin, erythromycin, clarithromycin, carbamazepine, 10,11-Dihydro-10,11-dihydroxycarbamazepine and penicillin G were detected in the range between 10 and 100 ng/L.

At PO site, situated 5 km downstream from the discharge point of the WW from the WWTP of the city of Zaprešić, only one compound was detected in the concentration higher than 1 µg/L. The anti-diabetic drug metformin, thought to be the pharmaceutical most deposited into the aquatic environment by mass,²⁰⁸ was found in concentration of 1.3 µg/L. Metformin is one of the most abundant pharmaceuticals found in recent studies of WWTP effluents and surface-waters.²⁰⁹ Three compounds were detected in the concentration range between 100 ng/L and 1 µg/L including pharmaceutical carbamazepine (137 ng/L), perfluorinated compound perfluorooctanoic acid (270 ng/L) and the stimulant caffeine (271 ng/L). Caffeine, carbamazepine and its metabolites have been typically used as indicators of municipal wastewater pollution.^{157,210} Perfluorooctanoic acid has been identified as a major industrial contaminant present in European rivers.^{211,212} The Po River in northern Italy was identified as a major perfluorooctanoic acid source from the European continent; around 200 ng/L of this compound was found at a median river flow of ~1500 m³/s.²¹² Several other large rivers were found to be contaminated with this industrial chemical such as River Danube in Austria (25 ng/L), River Scheldt in Belgium and The Netherlands (88 and 73 ng/L), River Rhone in France (116 ng/L), and the River Wyre in the UK (100 ng/L).²¹³ Twenty three compounds were detected in the concentration range between 10 and 100 ng/L, including different pharmaceuticals (enalapril, naproxen, tramadol, sulfamethoxazole), two transformation products of the analgesic metamizole (N-acetyl-4-aminoantipyrine and N-formyl-4-aminoantipyrine), transformation products of pharmaceutical carbamazepine (10,11-dihydro-10,11-dihydroxycarbamazepine and 2-hydroxycarbamazepine), together with plasticizers (bisphenol A and tri(butoxyethyl)phosphate)), some industrial compounds, herbicides and their transformation products etc.

The highest number of compounds (110) was detected at HC site, which is the main outlet of WWTP effluent of the city of Zagreb, additionally affected with some portion of untreated WW from the eastern suburbs of the city of Zagreb. Six compounds exceeded 1 µg/L, including caffeine (4.3 µg/L), triethyl citrate (2.8 µg/L), metformin (1.7 µg/L) carbamazepine (1.5 µg/L), UV filter benzophenone-4 (1.3 µg/L) and 10,11-dihydro-10,11-dihydroxycarbamazepine (1.1 µg/L). Twenty-six compounds reached concentrations in the range between 100 ng/L and 1 µg/L, while 47 compounds were detected in the range between 10 ng/L and 100 ng/L. The measured concentrations of chemicals presented in the sample collected at HC showed that not only the total concentrations were higher, and a large number of chemicals were present that had been below the detection limit in the Sava river at the Hruščica site (situated downstream of the HC), but also sample collected at HC had a pattern with larger number of pharmaceuticals, pesticides, natural hormones and surfactants than in all other investigated sites.

The single compounds with the highest maximum concentrations detected in river water at Hruščica site (H), located downstream of the Hruščica canal were: metformin (666 ng/L), perfluorooctanoic acid (217 ng/L), carbamazepine (172 ng/L), caffeine (109 ng/L) and triethyl citrate (109 ng/L). Thirty-one compounds were detected in concentrations between 10 ng/L and 100 ng/L. Concentrations of several chemicals detected in the Sava River at the location Hruščica were one or more orders of magnitude lower than detected in Hruščica canal. These chemicals include benzophenone-4, carbamazepine transformation products (10,11-dihydro-10,11-dihydroxycarbamazepine and 2-hydroxycarbamazepine), caffeine, industrial chemical (2-naphthalenesulfonic acid), pharmaceuticals (trimethoprim, diclofenac, cetirizine, propyphenazone), plasticizers (triethyl citrate, tri(butoxyethyl)phosphate)), insecticide dimethoate, surfactant N,N-dimethyldodecylamine-N-oxide etc. The lower concentrations of these compounds at location Hruščica are an indication for possible dilution occurring along the river. In general, predominant classes of compounds were pharmaceuticals and their transformation products, together with herbicides and their transformation products.

High concentration of the target contaminants were found also in the secondary effluent from the WWTP of the city of Velika Gorica. It should be kept in mind that municipal WW, which contain mainly domestic sewage, in the Western Balkan countries often include a significant contribution of wastewaters having industrial and institutional

origin.²¹⁴ Thirteen compounds reached concentrations higher than 1 µg/L. The highest concentration was measured for pharmaceutical metformin (18.9 µg/L). Measured concentration of metformin in the secondary effluent from the WWTP was in accordance with concentrations generally found in WWTP effluents ranging from 1 to 47 µg/L.^{208,215,216} The detected concentrations of pharmaceutical carbamazepine (3.6 µg/L) and its transformation products, 10,11-dihydro-10,11-dihydroxycarbamazepine (2.8 µg/L) and 2-hydroxycarbamazepine (3.3 µg/L) demonstrated that these compounds are convenient indicators of municipal wastewater pollution. The widespread occurrence of carbamazepine in municipal WW was also confirmed by many other studies in Europe²¹⁷ and North America.²¹⁸ Caffeine, another indicator of municipal wastewater pollution was detected at concentration of 1.3 µg/L. The measured concentration of caffeine was in the line with the previous study of Smital et al.²³ Benzophenone-4 was detected at significant concentration of 3.4 µg/L. In 2011, benzophenone was identified in the untreated WW and secondary effluent from the WWTP of the city of Zagreb, Croatia at concentrations between 1 and 10 µg/L.²³ Another widely distributed environmental pollutant, perfluorooctanoic acid, best known for its use in the production of Teflon and other stain-resistant materials was detected at the concentration of 1.8 µg/L, which demonstrated the contribution of industrial wastewater to this effluent. Four pharmaceuticals, including enapril, sulfamethoxazole, gemfibrozil and ibuprofen were detected at levels higher than 1 µg/L. Among plasticizers, the most abundant ones were tri(butoxyethyl)phosphate (2.4 µg/L) and triethyl citrate (2.1 µg/L). On the other hand, another widespread alkylphenolic xenoestrogen bisphenol A was found in the concentration of 180 ng/L. A very similar concentration levels were recently reported for WW samples from the Western Balkan Region.¹⁷⁰ Twenty-nine compounds were detected in the range between 100 ng/L and 1 µg/L, including various classes of pharmaceuticals, pesticides, plasticizers, industrial chemicals and hormones.

At OB site, located several km downstream of the main WW outlets of WWTPs of the cities of Zagreb and Velika Gorica, maximum concentration was also measured for pharmaceutical metformin (1.1 µg/L). Similarly to H location, four compounds were found at concentrations higher than 100 ng/L, including perfluorooctanoic acid (475 ng/L), caffeine (338 ng/L), carbamazepine (189 ng/L) and plasticizer triethyl citrate (121 ng/L). Triethyl citrate was also detected in Danube River at maximum concentration of 243 ng/L.¹³⁷ Nineteen compounds were found in the concentration range between 10 and 100 ng/L including

pesticides and their transformation products, pharmaceuticals and their transformation products, UV filters, hormones, plasticizers, industrial chemicals and nicotine transformation product. Concentrations of those chemicals were similar to those measured at PO site.

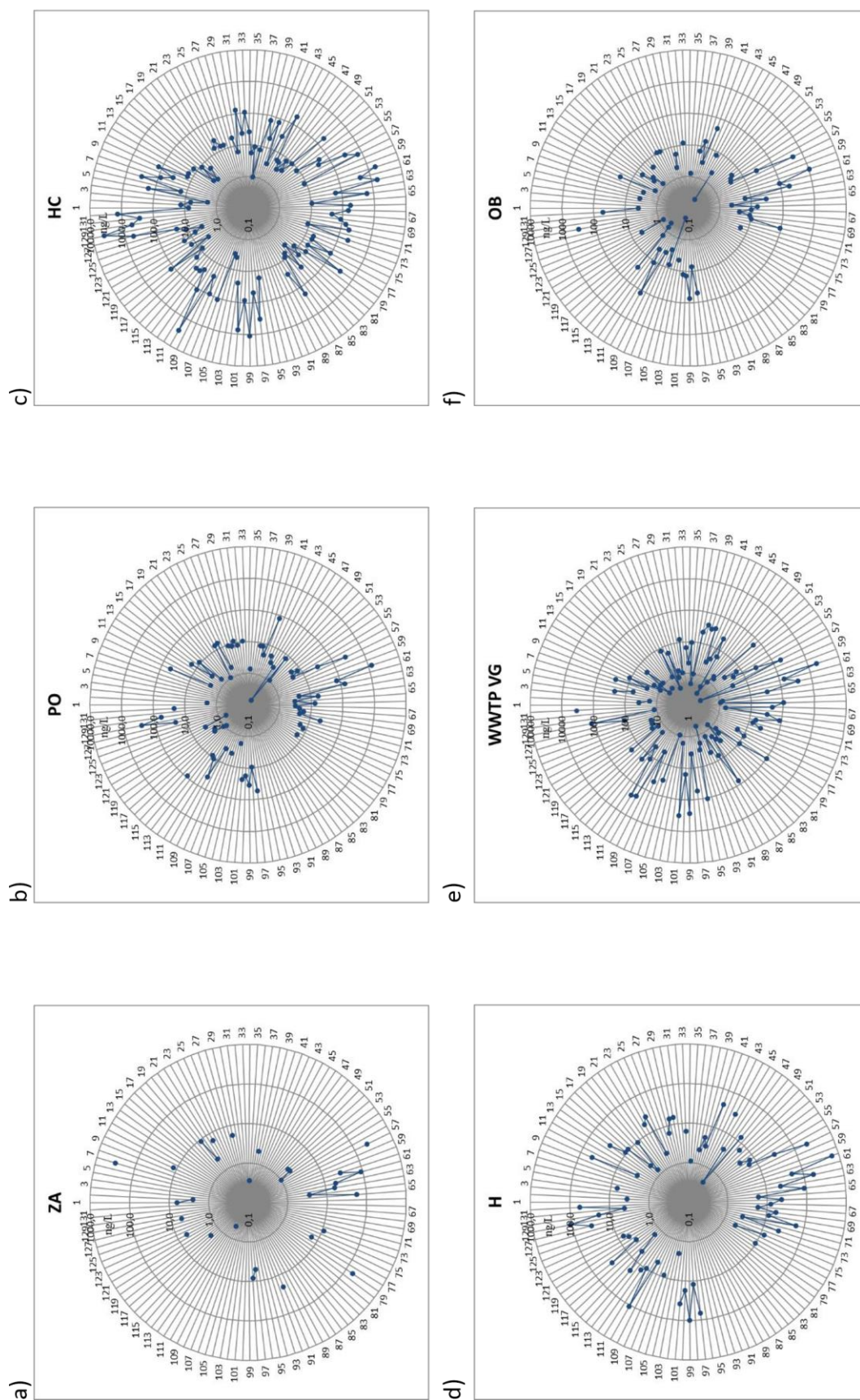


Figure 26. Concentrations of individual detected chemicals at each sampling site: a) ZA - Zaprešić; b) PO - Podsused; c) HC - Hrušćica canal, d) H – Hrušćica, e) WWTP Velika Gorica effluent, f) OB - Oborovo expressed in ng/L. Each number refers to one chemical and is listed in Table 14.

Table 14. Concentrations (ng/L) of detected compounds in WW and Sava River water.

Nr.	Compound	LDL	Procedural blank	ZA	PO	HC	H	WWTP VG	OB	Compound group/Usage pattern
1	2-Methylbenzothiazole	nr	nd	6.4	nd	7.5	nd	nd	3.8	aroma constituent of tea leaves
2	Thiabendazole	2	nd	2.5	2	13	3.6	37	<LDL	biocide/preservative
3	Imazalil	5	nd	nd	nd	6.7	nd	16.7	nd	biocide/preservative
4	Triclosan	0.5*	nd	nd	nd	1.9	nd	nd	nd	biocide/preservative
5	Denatonium	nr	nd	nd	nd	164	6.9	227	3.7	bittering compound
6	Acid orange 7	10	nd	nd	<LDL	15	<LDL	62	<LDL	dye
7	4-Bromophenol	nr	nd	324	nd	326	nd	nd	nd	flame retardant
8	Tris(2-chloroethyl)phosphate	nr	nd	nd	nd	83	nd	274	3.2	flame retardant, plasticizer
9	Tris(1,3-dichloro-2-propyl)phosphate	nr	nd	nd	nd	36.4	nd	25.1	nd	flame retardant/plasticizer
10	Carbendazim	1	nd	12	51	129	49	109	24	fungicide
11	2-Phenylphenol	1*	nd	nd	2.1	15	2.9	10	1.6	fungicide
12	Boscalid	12.5	nd	nd	nd	15	<LDL	18	nd	fungicide
13	Azoxystrobin	5	<LDL	nd	<LDL	<LDL	<LDL	5.1	<LDL	fungicide
14	Metolachlor	2	nd	<LDL	16	13	28	12	6.3	herbicide
15	Bentazone	1	nd	<LDL	2.8	2.4	11	46	2.4	herbicide
16	Terbutylazine	2	nd	<LDL	11	8.9	11	11	3.1	herbicide
17	Mecoprop	1	<LDL	<LDL	1.5	2.1	2.1	nd	1.3	herbicide
18	Isoproturon	1	<LDL	<LDL	<LDL	5.2	1.3	5.4	<LDL	herbicide
19	MCPA	5	5.1	nd	nd	nd	9.4	nd	5.2	herbicide
20	Chlorpropham	nr	nd	nd	nd	nd	nd	nd	nd	herbicide
21	Bromoxynil	1	nd	2.2	nd	nd	nd	nd	nd	herbicide
22	Pethoxamid	5	nd	nd	1.1	nd	nd	nd	nd	herbicide
23	Terbutylazine-2-hydroxy	nr	nd	6.3	14	15	18	11	10	herbicide TP
24	Metolachlor ESA	5	<LDL	<LDL	15	25	26	80	10	herbicide TP
25	Atrazine desisopropyl	1	<LDL	<LDL	2	14	2.3	3.9	<LDL	herbicide TP
26	Atrazine-desethyl	10	<LDL	<LDL	<LDL	13.6	<LDL	56.1	<LDL	herbicide TP
27	Terbutylazine-desethyl	5	nd	<LDL	9.2	<LDL	<LDL	8.5	<LDL	herbicide TP

Table 14. – continued.

Nr.	Compound	LDL	Procedural blank	ZA	PO	HC	H	WWTP VG	OB	Compound group/Usage pattern
28	2,4-Dinitrophenol	1	<LDL	<LDL	8.4	<LDL	6.5	13.5	2.2	herbicide, TP
29	Diuron	5	nd	5.5	12	18	15	nd	5.6	herbicide, WFD priority
30	Simazine	5	nd	<LDL	8.2	6.2	15	11	<LDL	herbicide, WFD priority
31	2,4-Dichlorophenoxyacetic acid	25	nd	nd	<LDL	130	<LDL	122	<LDL	herbicide/biocide
32	Androsterone	2*	nd	nd	10	23	nd	nd	12	hormone, androgen
33	Epi-Androsterone	2*	nd	nd	nd	105	6.1	65	nd	hormone, androgen
34	4-Androstenedione	1.5*	nd	nd	nd	26	nd	21	nd	hormone, androgen
35	Estrone	0.1*	nd	0.4	1.4	5.6	1.1	96	1.3	hormone, estrogen
36	Estradiol	1*	nd	nd	nd	8.9	nd	4.8	nd	hormone, estrogen
37	17- β -estradiol	0.3*	nd	nd	nd	1	nd	10	nd	hormone, estrogen, WFD watch list
38	Bisphenol S	0.2*	0.4	2.1	8.2	7.7	2.3	222	6.9	industrial chemical
39	2-Naphthalenesulfonic acid	1	<LDL	<LDL	8.3	71	4.9	409	15	industrial chemical
40	Diphenyl phosphate	2	<LDL	<LDL	4.5	20	3.0	326	3.3	industrial chemical
41	Benzothiazole	1	nd	nd	81	75	43	374	48	industrial chemical
42	4-Methylcoumarin	1	<LDL	<LDL	<LDL	3.2	<LDL	29	<LDL	industrial chemical
43	p-Nitrophenol	1	<LDL	<LDL	5.2	32	<LDL	40	<LDL	industrial chemical/TP
44	DEET	1	<LDL	<LDL	3.3	188	33	241	7.7	insect repellent
45	Icaridin	1	<LDL	<LDL	<LDL	5.9	<LDL	59	<LDL	insect repellent
46	Diazinon	2	2	nd	2.7	5.4	4	5	2.2	insecticide
47	Fipronil	0.1*	nd	nd	0.2	3.3	0.4	2.9	0.2	insecticide
48	Carbaryl	nr	nd	nd	7.5	8.3	13	8.8	nd	insecticide
49	Acetamiprid	nr	nd	nd	1.1	3.9	nd	16	nd	insecticide
50	Bendiocarb	5	nd	nd	<LDL	9.5	7.1	14	nd	insecticide
51	Dimethoate	5	<LDL	nd	nd	174	<LDL	645	<LDL	insecticide
52	3,5,6-Trichloro-2-pyridinol	2	nd	2	nd	nd	nd	nd	nd	insecticide TP
53	Atrazine	2	<LDL	2.1	5.3	8.5	4.3	28	5.2	legacy herbicide
54	Daidzein	0.5*	nd	1	7.7	52	7.7	71	4.3	natural food isoflavone
55	Genistein	1*	nd	nd	4.6	42	6.5	49	3.7	natural food isoflavone

Table 14. – continued.

Nr.	Compound	LDL	Procedural blank	ZA	PO	HC	H	WWTP VG	OB	Compound group/Usage pattern
56	Cotinine	1	<LDL	<LDL	32	306	31	276	29	nicotine TP
57	Perfluorooctanoic acid	1	nd	220	270	723	217	1800	475	perfluorinated compound
58	Perfluorohexanoic acid	1	<LDL	<LDL	<LDL	8.6	<LDL	35	<LDL	perfluorinated compound
59	Perfluoroheptanoic acid	nr	nd	nd	nd	nd	nd	2.6	nd	perfluorinated compound
60	Metformin	5	nd	28	1256	1666	666	18872	1056	pharmaceutical
61	Enalapril	nr	nd	89	79	476	52	1383	98	pharmaceutical
62	Carbamazepine	5	6.3	19	137	1494	172	3584	189	pharmaceutical
63	Clarithromycin	2	<LDL	16	4.3	65	23	338	6.8	pharmaceutical
64	Tramadol	2	<LDL	4	17	615	91	13	30	pharmaceutical
65	Warfarin	1	<LDL	57	3.1	10	6.0	11	2.4	pharmaceutical
66	Sulfamethoxazole	10	nd	<LDL	13	173	23	1057	15	pharmaceutical
67	Trimethoprim	2	nd	<LDL	3.2	152	12	464	9.9	pharmaceutical
68	Lidocaine	2	nd	<LDL	4.2	45	5.8	16	4.2	pharmaceutical
69	Sulfapyridine	5	nd	<LDL	5.8	89	17	325	9.0	pharmaceutical
70	Propyphenazone	2	<LDL	<LDL	4.5	138	12	852	11	pharmaceutical
71	Naproxen	nr	nd	nd	21	185	62	159	94	pharmaceutical
72	Ketoprofen	1	<LDL	<LDL	<LDL	9.0	2.6	132	<LDL	pharmaceutical
73	Cetirizine	25	nd	<LDL	<LDL	207	25	257	<LDL	pharmaceutical
74	Penicillin G	2	nd	11	5.8	nd	nd	nd	5.8	pharmaceutical
75	Clotrimazole	5	nd	nd	8.0	18	12	nd	nd	pharmaceutical
76	Citalopram	2	<LDL	<LDL	<LDL	18	2.1	54	<LDL	pharmaceutical
77	Ketoconazole	5	nd	6.7	nd	94	nd	104	nd	pharmaceutical
78	Pantoprazole	nr	nd	nd	6.5	16.9	9.8	nd	nd	pharmaceutical
79	Ibuprofen	nr	nd	156	nd	345	nd	1024	nd	pharmaceutical
80	Metoprolol	5	<LDL	<LDL	<LDL	19	<LDL	107	<LDL	pharmaceutical
81	Phenazone	1	nd	<LDL	<LDL	7.6	<LDL	14	<LDL	pharmaceutical
82	Lorazepam	nr	nd	nd	nd	15	nd	20	nd	pharmaceutical
83	Crotamiton	2	nd	<LDL	<LDL	4.3	<LDL	7.4	<LDL	pharmaceutical

Table 14. – continued.

Nr.	Compound	LDL	Procedural blank	ZA	PO	HC	H	WWTP VG	OB	Compound group/Usage pattern
84	Verapamil	2	<LDL	<LDL	<LDL	32	<LDL	33	<LDL	pharmaceutical
85	Diazepam	2	nd	nd	nd	6.8	<LDL	25	<LDL	pharmaceutical
86	Propranolol	2	nd	nd	<LDL	8.7	<LDL	18	<LDL	pharmaceutical
87	Furosemide	25	nd	nd	nd	184	<LDL	748	nd	pharmaceutical
88	Primidone	12.5	<LDL	<LDL	<LDL	28	<LDL	73	nd	pharmaceutical
89	Mefenamic acid	5	nd	nd	<LDL	<LDL	<LDL	13	<LDL	pharmaceutical
90	Bezafibrate	5	nd	nd	<LDL	nd	nd	47	<LDL	pharmaceutical
91	Erythromycin	5	nd	20	<LDL	nd	<LDL	<LDL	<LDL	pharmaceutical
92	Sulfamethazine	5	nd	<LDL	<LDL	<LDL	<LDL	5.1	<LDL	pharmaceutical
93	Diphenhydramine	5	nd	nd	nd	<LDL	<LDL	18	nd	pharmaceutical
94	Paroxetine	12.5	nd	nd	nd	nd	nd	30	nd	pharmaceutical
95	Gemfibrozil	1	nd	nd	nd	nd	nd	1049	nd	pharmaceutical
96	Finasteride	0.25*	nd	nd	nd	16.9	nd	nd	nd	pharmaceutical
97	N-Acetyl-4-aminoantipyrine	5	nd	5.1	54	336	67	542	51	pharmaceutical TP
98	Acetyl-Sulfamethoxazole	2	nd	8.3	9.4	49	12	36	7.3	pharmaceutical TP
99	10,11-Dihydro-10,11-dihydroxycarbamazepine	12.5	<LDL	<LDL	35	1123	97	2769	74	pharmaceutical TP
100	N-Formyl-4-aminoantipyrine	5	nd	<LDL	18	84	17	162	14	pharmaceutical TP
101	2-Hydroxycarbamazepine	10	<LDL	<LDL	24	743	39	3265	13	pharmaceutical TP
102	4-Formyl-antipyrine	5	nd	<LDL	<LDL	24	<LDL	16	<LDL	pharmaceutical TP
103	Exemestane	0.7*	nd	nd	1.8	nd	2.1	nd	4.7	pharmaceutical, androgen
104	Raloxifene	0.4*	0.6	nd	nd	4.0	nd	nd	nd	pharmaceutical, anti-/estrogen
105	Anastrozole	0.15*	nd	nd	nd	3.1	nd	9.7	0.2	pharmaceutical, anti-/estrogen
106	Clozapine	0.25*	nd	nd	4.2	115	9.1	328	7.8	pharmaceutical, anti-psychotic
107	Levo-norgestrel	1*	nd	nd	nd	nd	nd	nd	2.6	pharmaceutical, progesterone
108	Diclofenac	10	nd	nd	<LDL	95	<LDL	457	<LDL	pharmaceutical, WFD watch list
109	Bicalutamide	0.05*	nd	0.5	3.5	26	5.1	45	3.7	pharmaceutical/hormone

Table 14. – continued.

Nr.	Compound	LDL	Procedural blank	ZA	PO	HC	H	WWTP VG	OB	Compound group/Usage pattern
110	Triethyl citrate	1	<LDL	<LDL	41	2771	109	2083	121	plasticizer
111	Tri(butoxyethyl)phosphate	1	<LDL	<LDL	11	112	10	2392	11	plasticizer
112	Bisphenol A-(2,3-dihydroxypropylether)	nr	nd	nd	8.7	25	13	124	4.0	plasticizer
113	Monoethyl-phthalate	1.0	<LDL	<LDL	<LDL	32	<LDL	48	<LDL	plasticizer TP
114	Bisphenol A	1*	nd	nd	89	32	19	180	29	plasticizer, xenoestrogen
115	Bisphenol F	1*	nd	nd	nd	nd	5.2	15.3	1.5	plasticizer, xenoestrogen
116	Ethylparaben	0.5*	nd	nd	0.9	18.0	1.5	nd	0.7	preservative
117	Propylparaben	0.5*	nd	1.8	1.1	8.3	nd	nd	0.5	preservative
118	Benzyl(dimethyl)dodecylammonium	1	<LDL	<LDL	2.2	127	30	196	<LDL	quat. ammonium surfactant/biocide
119	Didecyl(dimethyl)ammonium	1	<LDL	<LDL	2.0	3.5	4.3	14	<LDL	quat. ammonium surfactant/biocide
120	Hexadecyl(trimethyl)ammonium	1	<LDL	<LDL	<LDL	13	<LDL	23	<LDL	quat. ammonium surfactant/biocide
121	Benzyl(dimethyl)hexadecylammonium	1	<LDL	<LDL	<LDL	<LDL	5.4	<LDL	<LDL	quat. ammonium surfactant/biocide
122	Progesterone	1*	2.0	5.6	3.3	19	7.2	19	4.5	steroid
123	Testosterone	0.4*	nd	nd	0.6	6.9	nd	18	0.7	steroid
124	Canrenone	0.7*	nd	nd	nd	3.8	nd	18	nd	steroid
125	4-Androstene-3,17-dione	12.5*	nd	nd	nd	12.2	nd	nd	nd	steroid
126	Trenbolone	2*	nd	nd	nd	22.7	nd	nd	nd	steroid
127	Cholic acid	2	nd	5.4	23	539	34	121	<LDL	steroid TP
128	Caffeine	25	<LDL	<LDL	271	4323	109	1311	338	stimulant in beverages, marker compound
129	Lauryl diethanolamide	1	<LDL	<LDL	60	516	20	12	<LDL	surfactant
130	N,N-Dimethyldodecylamine-N-oxide	nr	nd	nd	nd	278	4.2	nd	nd	surfactant
131	Benzophenone-4	5	<LDL	<LDL	22	1346	57	3416	51	UV filter

*method detection limit is presented; LDL – lowest detectable level; nd – not detected; nr – not reported.

4.3.2. Results of bioassays

In order to get a deeper insight into ecotoxicological potential of environmental samples analysed in this study, a battery of selected ecotoxicological *in vitro* and *in vivo* bioassays that encompassed various toxicity end-points was applied for the assessment of the collected Sava River and WW samples. Furthermore, the most significant samples were selected for a fine HPLC fractionation in order to facilitate identification of the most critical compounds contributed to a given endpoint.

4.3.2.1. Unfractionated SPE extracts

Estrogenicity in the Sava River and WW extracts was tested in the MELN reporter cell line. Estrogenic activity was observed at all investigated sampling sites, Zaprešić, Podsused, Hruščica canal, Hruščica, WWTP Velika Gorica and Oborovo. The biological estradiol equivalents (E2-EQs) ranged from 0.06 ng/L to 36.02 ng/L (Figure 27). Measured estrogenicity in samples collected at locations Podsused (0.32 ng/L E2-EQ) and Oborovo (0.38 ng/L E2-EQ) were in the line with the results obtained during the European demonstration programme (Table 11). As expected, higher estrogenicity (3.95 ng/L E2-EQ) was measured in sample collected at Hruščica canal, the main outlet of WWTP effluent of the city of Zagreb. Estrogenicity measured in Sava River downstream of the Hruščica canal at the location Hruščica was ten folds lower (0.38 ng/L E2-EQ), indicating fast dilution of the discharged wastewater in the Sava River. A very high estrogenic activity was found in the WWTP effluent of the city of Velika Gorica (36.02 ng/L E2-EQ). This estrogenic activity was much higher than in effluents reported in previous studies.^{23,219} The observation of the high E2-EQ of effluent from WWTP in Velika Gorica could be attributed to relatively poor WWTP efficacy compared to other plants and/or to a special composition of chemical constituents in the plant influent. The measured total estrogenic activity in sample collected at the location Oborovo, downstream of the WWTP Velika Gorica, was relatively low (0.38 ng/L E2-EQ).

The mass balance calculations resulted in ratios of observed estrogenic activity that could be explained by the detected ER- agonists ranging from 33% to 80% (Table 15). As observed during the EDP study, estrone was a major candidate for the measured estrogenicity in investigated samples where its contribution ranged from 16 to 66%.

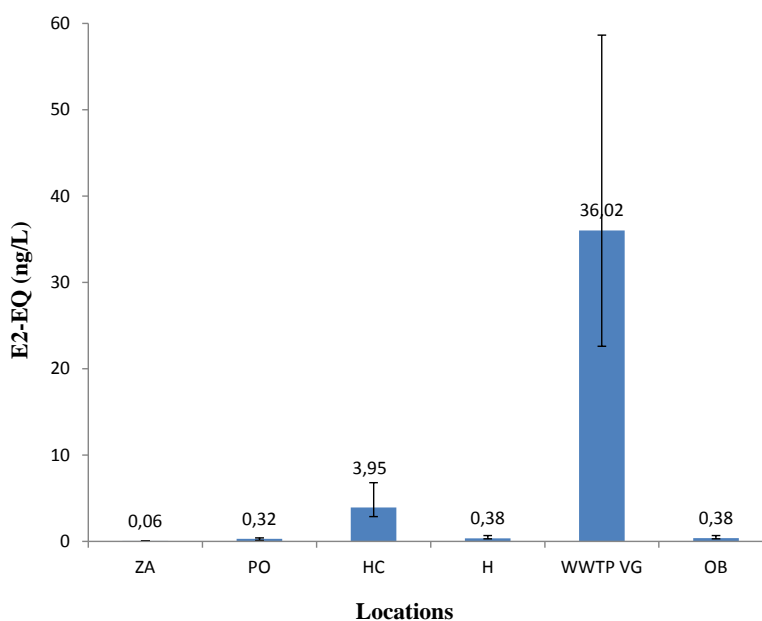


Figure 27. Estrogenic potential of the selected water samples (ZA - Zaprešić, PO - Podsused, HC - Hruščica canal, H - Hruščica, WWTP VG - WWTP Velika Gorica effluent, OB - Oborovo) tested in the MELN reporter cell line. Data are expressed in biological estradiol equivalents (E2-EQs, ng/L). Values represent mean \pm SD of samples tested in quintuplicate.

For the sample collected at HC with the estrogenic activity of 3.95 ng/L E2-EQ, 80% of the effect could be explained by the four chemicals 17- β -estradiol, estriol, estrone and genistein which contributed with 25%, 38%, 16% and 0.6%, respectively. A small part of estrogenic activity in the samples could be explained by the presence of bisphenol A (0.2 - 1.3%) and isoflavone genistein (0.1 - 0.9%). Measurable levels of steroids, parabens, plasticizers, phthalates and pharmaceuticals at investigated locations (Table 14) could also partly contribute to the observed activities. Niemuth and Klaper²⁰⁹ demonstrated that pharmaceutical metformin, which was detected in all investigated samples, has a significant impact on the reproductive system of freshwater fish fathead minnow and can cause intersex in male fish at concentrations emerging from WWTPs.

Jarošová et al.²²⁰ proposed safe concentrations of estrogenic equivalents (EEQs-SSE) regarding steroid estrogens that could be compared with the measured E2-EQs in order to assess the risk of the observed estrogenic activities. Based on the MELN cells, authors derived EEQ-SSEs, threshold values for detected bioactivity expressed as bio-E2-EQ, below which no adverse effects are expected in aquatic biota due to estrogens. The EEQ-SSE for short term exposure (0.8 ng/L bio-E2-EQ) was exceeded at two sites (Hruščica canal and WWTP Velika Gorica) and the EEQ-SSE for chronic exposure (0.2 ng/L bio-E2-EQ) was exceeded even at three sampling sites on the Sava River (Podsused, Hruščica and Oborovo). It could be concluded that all sampling locations, except Zaprešić, were characterized by estrogenic activity above the threshold for concern.

Table 15. Mass balance calculations for estrogenic activities in the selected water samples. Chemical estradiol equivalents (Chem-E2-EQ), calculated by multiplying measured concentrations (ng/L) of known agonists with their relative potencies in the particular bioassays, were compared to biological estradiol equivalents (Bio-E2-EQ) and the resulting ratio (expressed as a percentage) shows the extent to which the observed biological activity can be explained by target compounds.

Compound name	REP	Reference	Sampling locations					
			ZA	PO	HC	H	WWTP VG	OB
4-Androstenedione	9.70E-07	Creusot et al. ³⁰	nd	nd	26	nd	21	nd
Bisphenol A	4.49E-05	Neale et al. ¹⁹⁹	nd	89 (1.3%)	32	19 (0.2%)	180	29 (0.3%)
Bisphenol S	1.18E-06	Neale et al. ¹⁹⁹	2.1	8.2	7.7	2.3	222	6.9
17- β -estradiol	1	Kinani et al. ¹⁴¹	nd	nd	1 (25%)	nd	10 (28%)	nd
Estrinol	0.17	Kinani et al. ¹⁴¹	nd	nd	8.9 (38%)	nd	4.8 (2%)	nd
Estrone	0.11	Neale et al. ²⁰⁰	0.4 (66%)	1.4 (48%)	5.6 (16%)	1.1 (32%)	96 (29%)	1.3 (36%)
Gemfibrozil	4.70E-08	Creusot et al. ³⁰	nd	nd	nd	nd	1049	nd
Genistein	5.35E-04	Neale et al. ¹⁹⁹	nd	4.6 (0.8%)	42 (0.6)	6.5 (0.9%)	49 (0.1%)	3.7 (0.5)
Progesterone	7.50E-07	Creusot et al. ³⁰	5.6	3.3	18.6	7.2	19	4.5
		Bio-E2-EQ	0.06	0.32	3.95	0.38	36.02	0.38
		Chem-E2-EQ	0.04	0.2	3.2	0.13	21	0.14
		Chem/Bio ratio (%)	66	50	80	33	59	37

nd - not detected; REP - relative effect potencies.

Androgenic activity was not detected in any of the investigated unfractionated SPE extracts, since none of the samples was found to significantly induce luciferase in the MDA-kb2 cells.

The results of *in vivo* ChgH-gfp medaka test used for monitoring of estrogenic activity in WW and Sava River samples showed that the only estrogenic sample was the secondary effluent collected at WWTP of the city of Velika Gorica, with the measured estradiol equivalent of 21.6 ng/L.

Initial cytotoxicity evaluation of the Sava River and WW samples was measured using MTT reduction assay, a widely accepted rapid and sensitive *in vitro* method. Since the intention was to test a wide dilution series for all samples (1:100–1:100000), using *in vitro* bioassays, the information about the overall cytotoxicity was important for further sample analyses and the interpretation of results. This information is important also for the interpretation of the results of other bioassays since some specific end-point responses can be partly masked due to the cytotoxicity at higher extract doses.⁶⁵ Results of MTT test showed in Figure 28 revealed low cytotoxic potential of investigated river water samples.

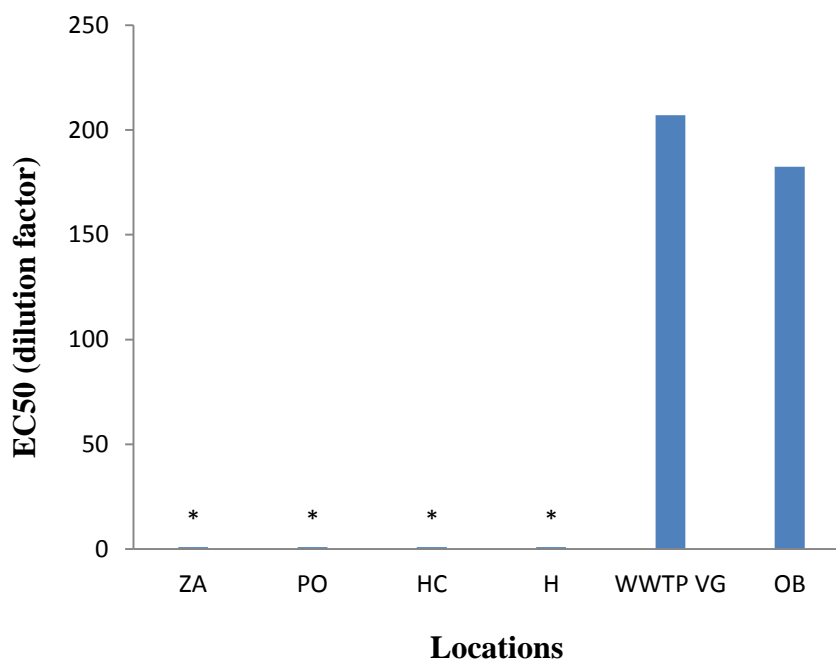


Figure 28. Acute cytotoxicity of the selected water samples (ZA - Zaprešić, PO - Podsused, HC - Hruščica canal, H - Hruščica, WWTP VG - WWTP Velika Gorica effluent, OB - Oborovo) as determined by the MTT test in PLHC-1 cells. Asterisks denote that toxicity of ZA, PO, HC and H samples was below the detection limit of the method. Results are measured in monoplicate.

In general, cytotoxicity of the WW samples was much lower than reported for *in vitro* bioassays elsewhere.^{23,103} Toxicity measured downstream of the WWTP VG, at the location Oborovo, was similar to acute toxicity measured in WW extract, indicating the impact of contaminants from the WWTP effluent on the toxicity of the Sava River. Furthermore, the toxicity of the samples collected at the locations Zaprešić, Podsused, Sava Hruščica and Hruščica canal was negligible, below the detection limit of the method.

The chronic toxicity using AlgaeTox assay showed that all investigated Sava River samples inhibited the growth of freshwater alga, while the sample collected at location Oborovo showed the most potent toxic potential (Figure 29).

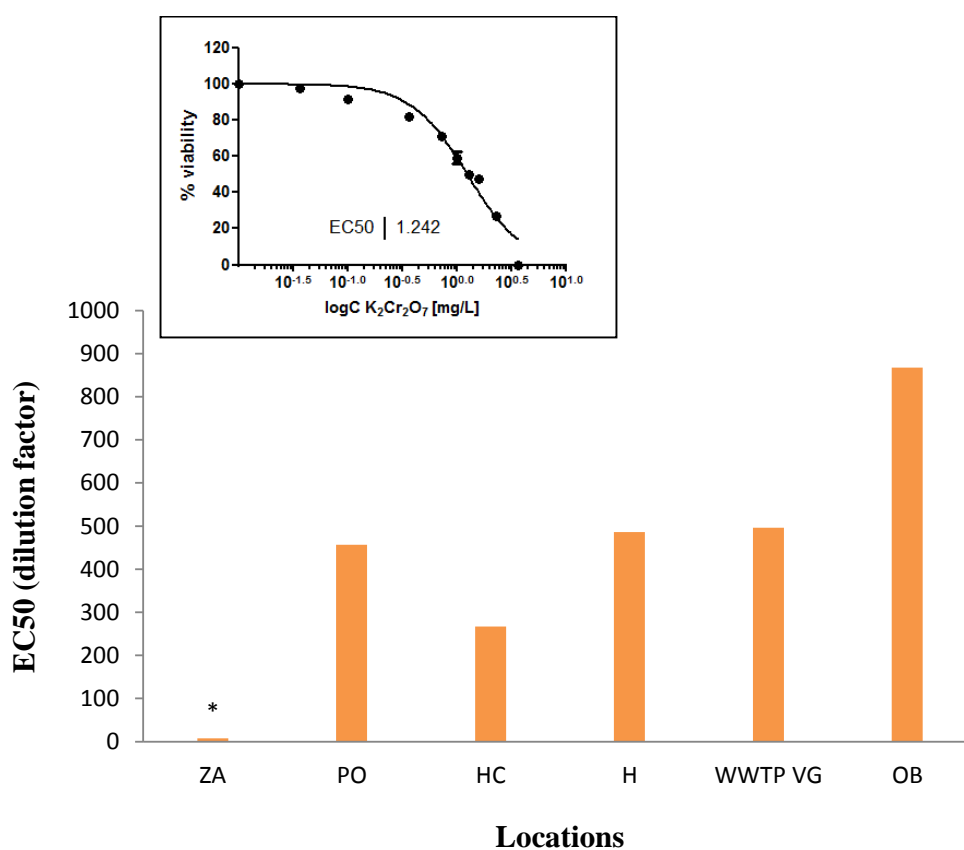


Figure 29. Chronic cytotoxicity of the selected water samples (ZA - Zaprešić, PO - Podsused, HC - Hruščica canal, H - Hruščica, WWTP VG - WWTP Velika Gorica effluent, OB - Oborovo) as determined by the AlgaeTox assay with unicellular green alga *Scenedesmus subspicatus*. Asterisk denotes that toxicity of ZA was below the detection limit of the method. Dose response curve for the model toxicant $K_2Cr_2O_7$ is given in the inserted chart. Results are measured in monoplicate.

The measured chronic toxicity in the Oborovo sample (EC50 was obtained for 1:867 dilution) was even higher than the toxicity of the WWTP VG sample (EC50 was obtained for 1:496 dilution) indicating that the complex interactions between numerous constituents present in the WW extract can mask some specific effects. Similarly, in the Sava Hruščica sample EC50 value was obtained for higher dilution factor (1:486) compared to WW effluent collected upstream, in Hruščica canal, whose EC50 value was measured for 1:267 dilution. It was also interesting to note that industrial WW sample collected at location Zaprešić was below the detection limit of the method. On the contrary, nutrients presented in ZA extract caused the growth of the *Scenedesmus subspicatus* alga. Downstream, at location Podsused chronic toxicity was measured and EC50 value was obtained for 1:457 dilution. It can be assumed that among various categories of compounds detected in the WW and Sava River samples, the most probable candidates for the observed cytotoxicity are pharmaceuticals. They might have contributed to the overall toxicity, due to their comparatively high concentrations detected in samples (Table 14).

Results of the determination of an EROD induction potential are presented in Figure 30. All Sava River samples were characterized with low EROD induction potential, with the highest activity determined for the Oborovo location downstream of the main Zagreb and Velika Gorica WW outlets. These data are generally in agreement with a data from bioassay-assisted monitoring of the Sava River basin.¹¹⁶ Podsused and Hruščica SPE extracts showed lower EROD/CYP1A induction potential, while the induction potential of the ZA sample was below the method detection limit. As expected, a significantly enhanced EROD induction potential was measured in samples collected at WWTP Velika Gorica and Hruščica canal, the main outlet of WWTP effluent of the city of Zagreb. EC50 values of WWTP VG and HC were obtained for 1:920 and 1:412 dilutions, respectively.

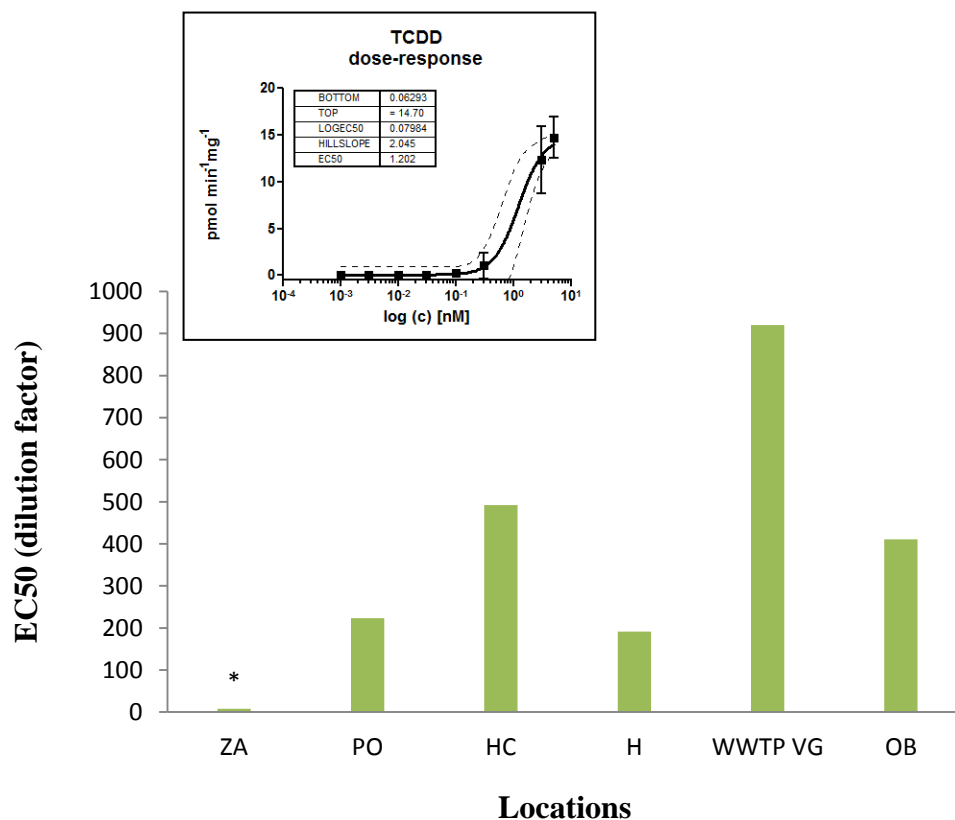


Figure 30. CYP1A induction potential of the selected water samples (ZA - Zaprešić, PO - Podsused, HC - Hruščica canal, H - Hruščica, WWTP VG - WWTP Velika Gorica effluent, OB - Oborovo) as determined by the measurement of the EROD activity in PLHC-1 cells. Asterisk denotes that EROD induction potential of the ZA sample was below the method detection limit. Dose-response curve for model inducer TCDD is inserted. Results are measured in monoplicate.

4.3.2.2. HPLC fractionated extracts

Based on the results obtained for the unfractionated SPE extracts, samples collected at locations Oborovo and WWTP Velika Gorica were chosen for further fractionation using semi-preparative RP HPLC. SPE extracts were fractionated into 37 fractions and subsequently subjected to both biological and chemical analyses. EROD activity, chronic and acute cytotoxicity were not analysed in fractions 1, 2, 4, 5, 6, 8 and 9. The resulting toxicological profiles of the estrogenic, androgenic activity, chronic and acute cytotoxicity and EROD activity are shown in Figures 31-36.

In order to obtain an initial characterization of the physico-chemical properties of active ligands, estrogenic activity was measured in OB and WWTP VG fractions (Figure 31). The fractionation step allowed good isolation of ER activities in individual fractions. Both OB and WWTP VG proved to be highly contaminated samples, with several active fractions. In some fractions super induction was observed for WWTP VG (e.g. 13-15, 19, 21) and OB (e.g. 13-16, 18), probably due to mixture effects of pure ligands and some unspecific effects. For WWTP VG sample, the highest activity was measured in fractions 14 and 19 (6.4 E2-EQ), while for OB sample the strongest estrogenic activity was detected in fraction 13 (3.7 E2-EQ). It should be noted that HPLC fractionation of OB sample may have highlighted an inhibitory mixture effect in unfractionated extract since the sum of E2-EQ in individual fractions was higher than that measured in unfractionated extract. Fractionation of WWTP VG confirmed that the sample was highly contaminated, in line with effect observed for the unfractionated extract.

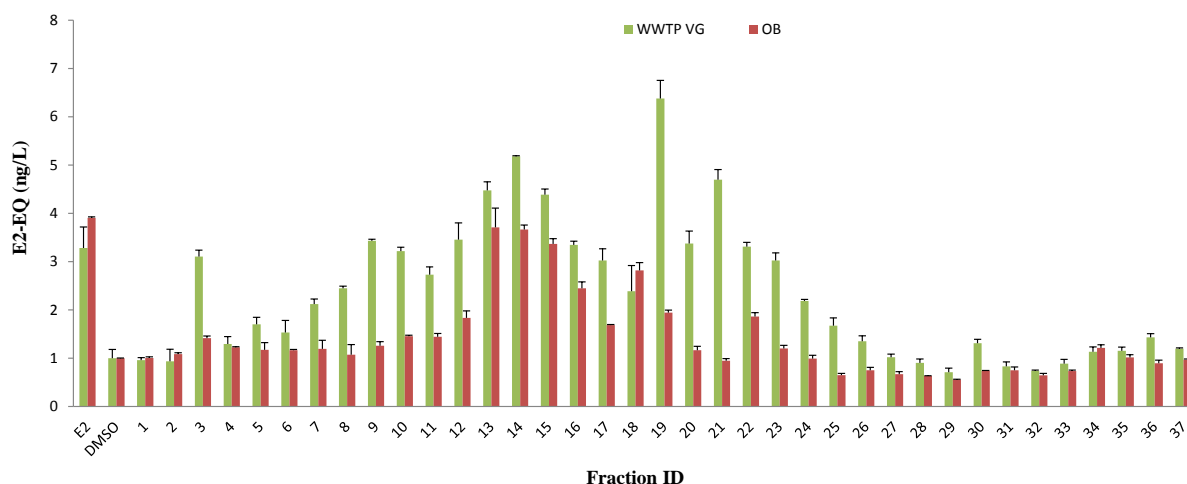


Figure 31. Comparison of estrogenic potential of WWTP VG - WWTP Velika Gorica effluent and OB - Oborovo fractions tested in the MELN reporter cell line. Data are expressed in biological estradiol equivalents (bio-E2-EQs, ng/L). E2: 17 β -estradiol was used as positive control, while DMSO - dimethyl sulfoxide was used as negative control. Values represent mean \pm SD of samples tested in triplicate.

Results for androgenic activity of WWTP VG and OB fractions are presented in the Figure 32. Interestingly, HPLC fractionation revealed some high potent fractions (15, 16, 20, 21, 23, 24) in WWTP VG sample that were not evidenced when testing the unfractionated extract. This data indicated again the importance of simple HPLC fractionation procedures for the prevention of masking effects, which often occur in complex environmental matrices such as WW effluents.

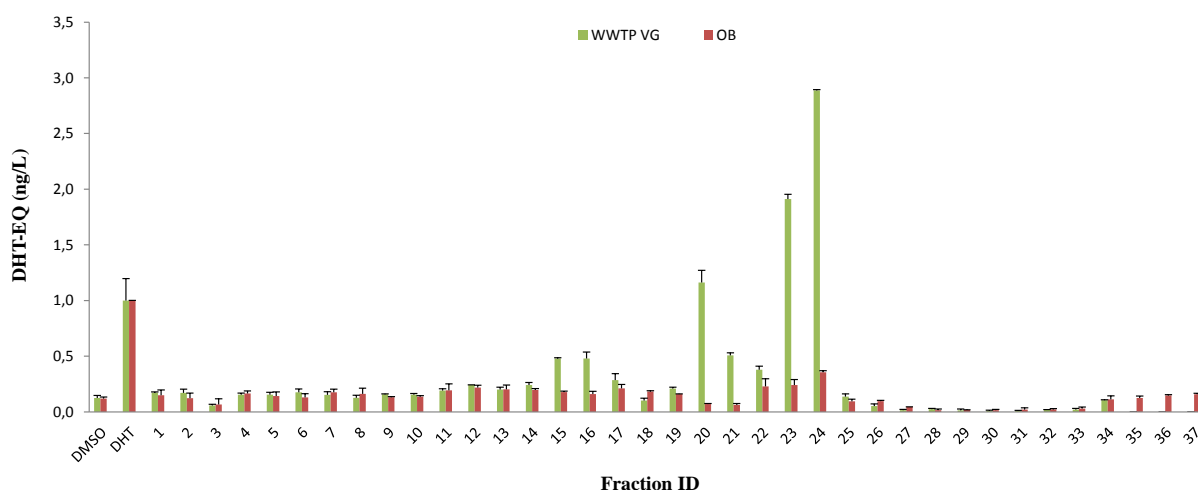


Figure 32. Comparison of androgenic potential of WWTP VG - WWTP Velika Gorica effluent and OB - Oborovo fractions tested in the MDA-kb2 cell line. Data are expressed in dihydrotestosterone equivalents (DHT-EQs, ng/L). DHT- dihydrotestosterone was used as positive control, while DMSO - dimethyl sulfoxide was used as negative control. Values represent mean \pm SD of samples tested in triplicate.

This assay co-detects both AR and GR ligands, and the luciferase response induced by GR ligands is higher than that induced by androgens. The positive control used in this assay was dihydrotestosterone (DHT). GR agonist such as dexamethasone (DEX) is about four times more potent than DHT. Therefore, it was assumed that in WWTP VG fractions 20, 21, 23 and 24 we can expect GR ligands. In order to detect is agonistic activity detected in some HPLC fractions mediated by AR or GR ligands, WWTP VG fractions 15, 16, 20, 21, 23 and 24 were tested either alone or in combination with specific AR antagonist flutamide (Fluta), which blocks AR but not GR- mediated response, or with specific GR antagonist RU486 (RU) which blocks GR but not AR-mediated response. Finally, the co-exposure experiment confirmed the hypothesis in fractions 20, 21 and 24 that GR agonists are present, while in

fraction 23 androgens are likely to be present. Inconclusive results were obtained for fractions 15 and 16 indicating that probably a mix of AR and GR ligands or some unspecific effects are present.

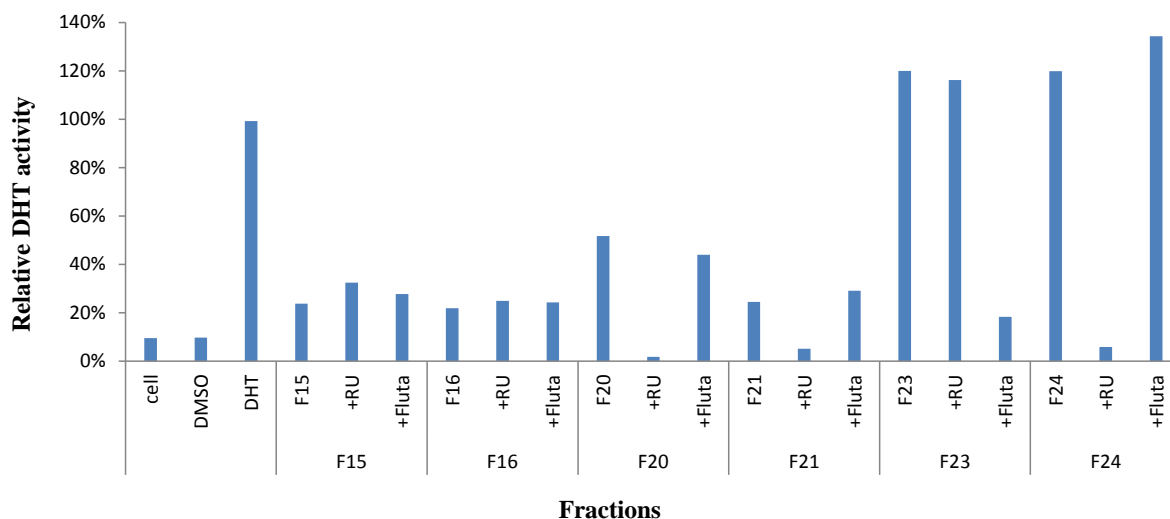


Figure 33. Luciferase inhibition by WWTP VG fractions tested either alone or in combination with specific AR antagonist flutamide (Fluta) or with specific GR antagonist RU486 (RU) in MDA-kb2 cells. DHT - dihydrotestosterone was used as positive control, while DMSO- dimethyl sulfoxide was used as negative control.

Six concentrations ranging from 1:100 to 1:5000 were tested for analysis of acute toxic potential of WWTP VG and OB fractions. Sixteen out of 30 analysed WWTP VG fractions had an effect on survival of PLHC-1/wt cells (Figure 34). The cytotoxicity of other fourteen fractions was negligible, below the detection limit of the method. The highest acute toxicity was observed in fraction 29 (EC₅₀ was obtained for 1:375 dilution). HPLC fractionation revealed an inhibitory mixture effect in unfractionated WWTP VG extracts since the EC₅₀ values in several individual fractions (3, 14, 17, 20, 23, 26, 29, 30-33) were higher than the measured in unfractionated extract. Regarding OB fractions, cytotoxicity was detected in only six fractions, while the fractions 20 and 33 showed even higher toxicity in comparison with toxicity measured in unfractionated extract.

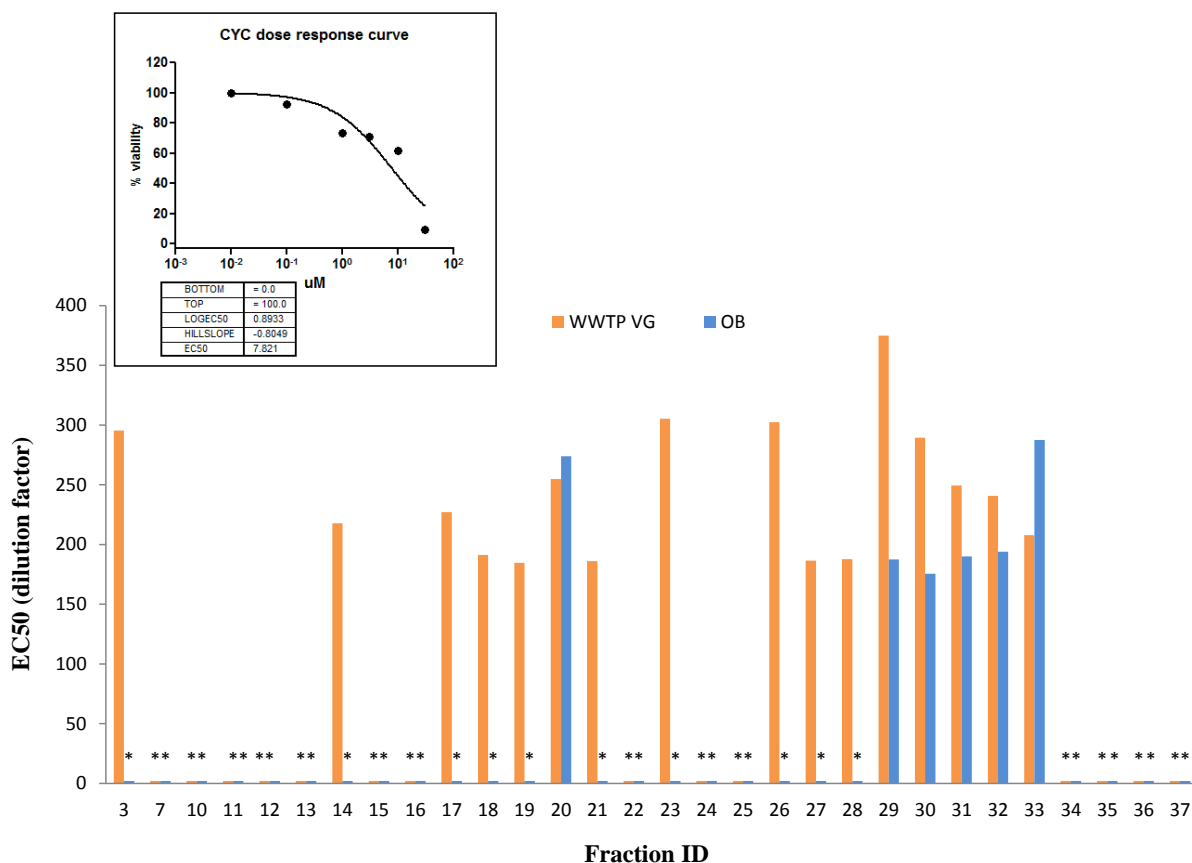


Figure 34. Comparison of acute toxicity of WWTP VG - WWTP Velika Gorica effluent and OB - Oborovo fractions, as determined by the the MTT test in PLHC-1 cells. Asterisks denote that toxicity of fraction was below the detection limit of the method. Dose response curve for the model toxicant CYC (cyclosporin A) is given in the inserted chart.

Algal chronic toxicity potential of the WWTP VG and Oborovo fractions was very widely distributed in the preparative HPLC fractions (Figure 35). The most active WWTP VG fractions were fractions 15, 29, 31, and 32 with similar EC50 values to those obtained for unfractionated SPE extract. For Oborovo sample, the most potent were fractions 3 and 29, whose EC50 values were obtained for 1:473 dilution and 1:451 dilution, respectively. Several WWTP VG fractions (4, 11, 34-37) and Oborovo fractions (4, 10, 11-18, 20-25, 35, 37) were below the detection limit of the method. Comparison of toxicological profiles of WWTP VG effluent and the Sava River (Figure 35) shows a very nice resemblance of the two curves for fractions 3, 19, 26-33, which additionally demonstrates common origin of the present contaminants.

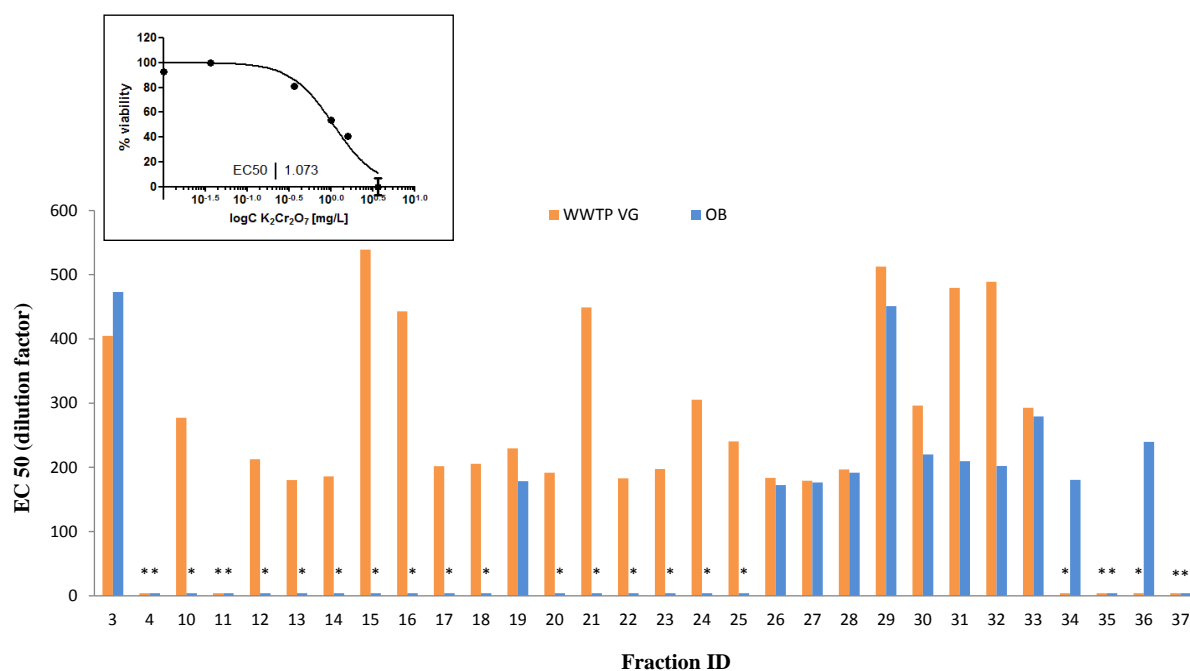


Figure 35. Comparison of chronic toxicity of WWTP VG - WWTP Velika Gorica effluent and OB - Oborovo fractions, as determined by the AlgaeTox assay with unicellular green alga *Scenedesmus subspicatus*. Asterisks denote that toxicity of fraction was below the detection limit of the method. Dose response curve for the model toxicant $K_2Cr_2O_7$ is given in the inserted chart.

Results of the determination of an EROD induction potential including WWTP VG and Oborovo fractions are presented in Figure 36. A range of 6 concentrations was evaluated for EROD induction with dilution range from 1:100 to 1:5000. Several WWTP VG fractions significantly induced CYP1A1 enzyme activity in PLHC1/wt cells, while all of them resulted in stronger induction than Oborovo fractions. The highest CYP1A1 induction was observed for WWTP VG fractions 16 and 21. Eight Oborovo fractions were found to induce CYP1A1 above the method detection limit, while the highest EROD induction potential was identified in the fraction 20.

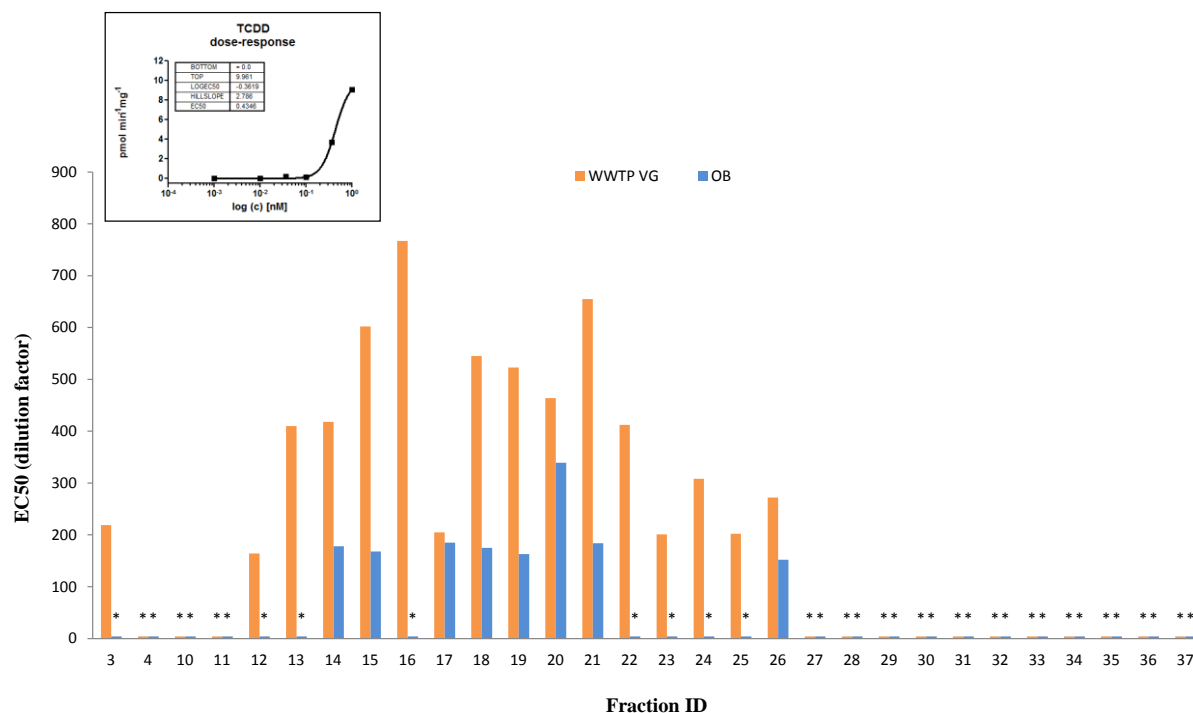


Figure 36. Comparison of the CYP1A induction potential of the WWTP VG - WWTP Velika Gorica effluent and OB - Oborovo fractions, as determined by the measurement of an EROD activity in PLHC-1 cells. Asterisks denote that EROD induction potential of the fraction was below the method detection limit. The inside graph represents the standard curve and calculated EC50 value for TCDD obtained by the EROD test.

4.3.3. Chemical characterization of HPLC fractions

The chromatograms of the WWTP VG and OB fractions recorded in both positive and negative ionization modes using UHPLC-QTOFMS were rather complex, exhibiting presence of a large number of different organic compounds. A detailed Q-TOFMS analysis of the most active fractions for possible unknowns indicated various groups of common surfactants and their metabolites as the most abundant contaminant classes. Accurate mass and retention time for those contaminants were sufficient for a reliable identification. The list of compounds, identified in positive and negative electrospray ionization mode, are presented in Supplementary material (Table SIV). The major problem confronting analytical determination of surfactants in WW as well in river waters arises from the complexity of surfactant mixtures, often composed of several tens to hundreds of homologues, oligomers and isomers. Some surfactants were detected in multiple fractions. This could be explained by the fact that

they can be present in different forms (dissociated, non-dissociated, or bonded to cations). Another reason could be overloading of the HPLC column used for fractionation or lower column efficiency.

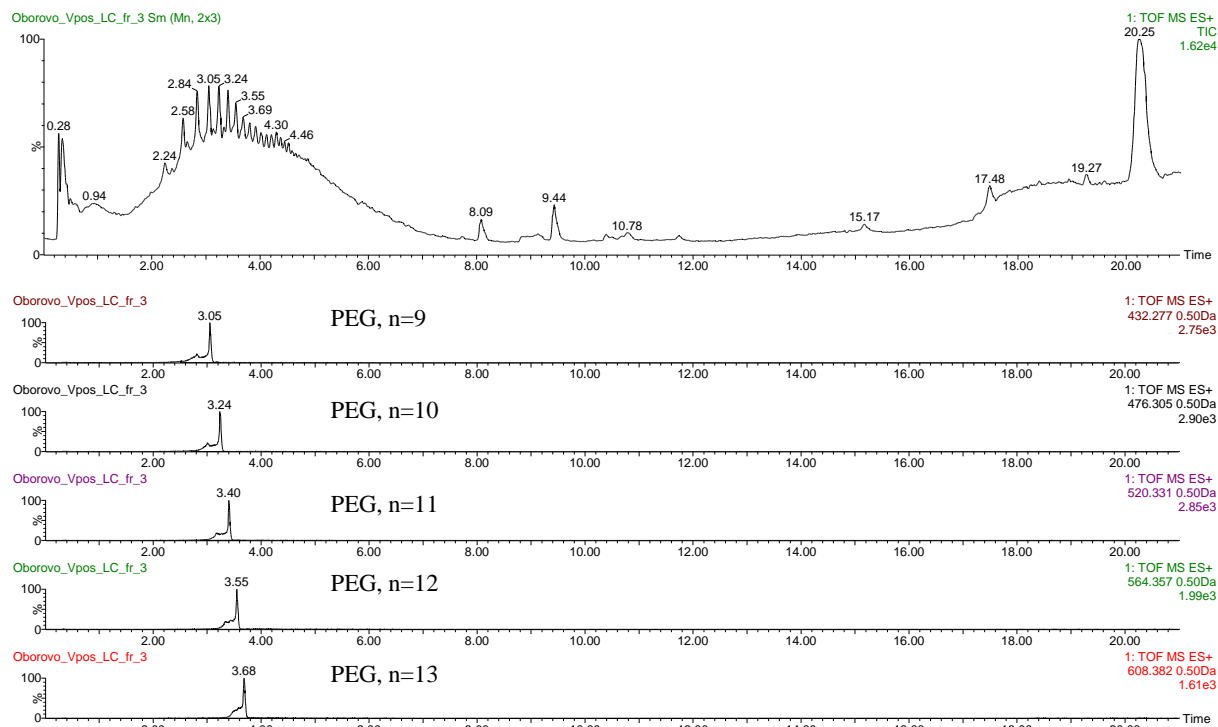
Generally, surfactants are only moderately toxic to aquatic life,²²¹ however they should not be neglected when assessing the overall toxic potential since their concentrations in the river water are often 1,000 times higher than the concentrations of the classical hydrophobic contaminants. Moreover, recent *in vitro* studies have shown that surfactants can enhance the toxicity of other contaminants,¹⁹² most probably because surfactants may enhance the transfer of contaminants through the cell membrane²²² or enhance their bioavailability.²²³ Nevertheless, some information is still lacking in relation to the aquatic toxicity of surfactants, especially knowledge regarding the toxicity of the degradation products, the effect of surfactants on marine species, the ecotoxicity of mixtures of chemical compounds with surfactants, the relationship between toxicity and chemical residue and the effect of surfactant presence in specific environmental compartments (water, particulate matter, pore-water, sediment).²²⁴

A detailed inspection of the Oborovo fraction 3 (showed to be the most toxic for algae) in PI mode, revealed a significant presence of polyethylene glycols (PEGs) as the most abundant contaminant class (Figure 37). PEGs are the important group of non-ionic synthetic water-soluble polymers of ethylene oxide and main degradation metabolites of alcohol polyethoxylates (AEO). Their usage is widespread and includes various applications such as the production of emulsifiers, surfactants, detergents, cosmetics etc. Therefore, they are common constituents of municipal wastewaters.²²⁵ The most prominent PEGs found in the fraction 3 were those having 9-15 ethoxy units, which can be recognized in the chromatogram reconstructed using their corresponding $[M + NH_4]^+$ ions and in QTOF mass spectrum (Figure 37). The chromatographic pattern of PEGs is characterized by regularly increasing retention times of individual PEG oligomers, which were accompanied by m/z values increasing at a regular interval of the ethoxy unit (C_2H_4O).

The main peak in the total ion current chromatogram of the fraction 3 at the retention time (RT) of 6.21 min in NI mode was identified as anionic surfactant C_{12} alkylsulfate (AS) with m/z 265.1458 (Figure 38). Alkylsulfates are also commonly used in consumer products (shampoos, hand dish washing liquids, laundry detergents, etc.). Moreover, these compounds

are the basis of synthesis in the production of anionic surfactants alkyether sulfates (AES) type.

a)



b)

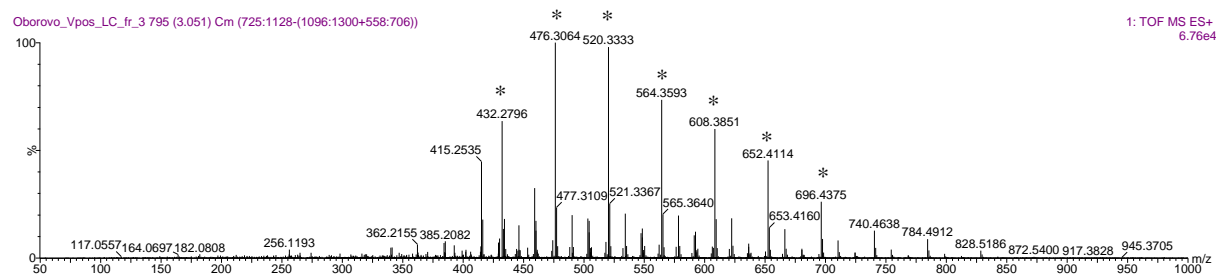
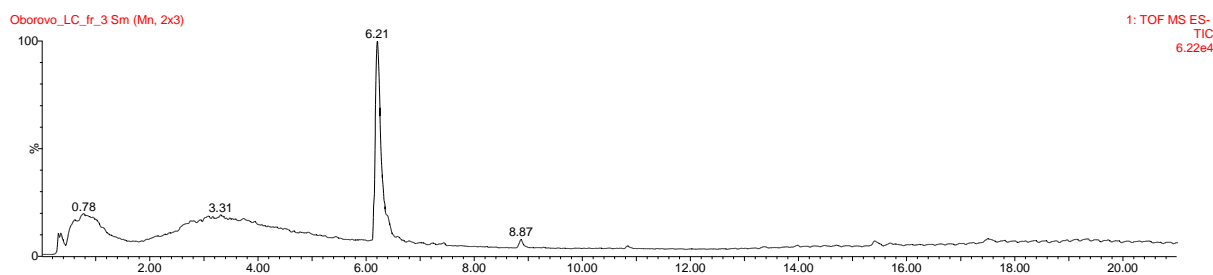


Figure 37. a) UHPLC-QTOFMS chromatogram of the Oborovo fraction 3 in PI mode, showing total ion current chromatogram and extracted ion chromatograms reconstructed using accurate masses of the PEGs adducts with ammonia; b) mass spectrum showing strong predominance of PEGs oligomers (* n=9-15).

This fraction also revealed a significant presence of sulfophenyl carboxylic acid (SPC) homologues, the major biotransformation metabolites of the most abundantly used anionic surfactant linear alkyl benzenesulfonates (LAS). SPCs originating from commercial LAS constitute even more complex mixtures than the parent compound, since the initial attack on

the alkyl chain can take place on either side of the phenyl substituent such that a wider range of alkyl homologues ranging from C₄ to C₁₃ SPC can be produced. The most prominent homologues of SPC identified in the Oborovo fraction 3 were C₈, and C₉ with corresponding masses at m/z 299.0916 and m/z 313.1065, respectively (Figure 38).

a)



b)

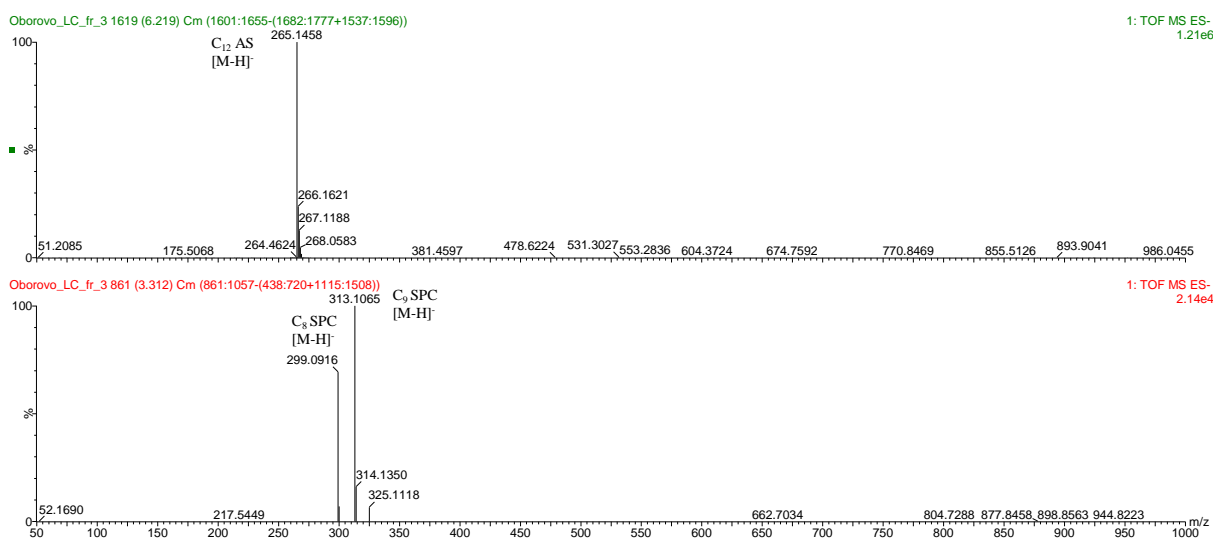


Figure 38. a) UHPLC-QTOFMS chromatogram of the Oborovo fraction 3 in NI mode showing total ion current chromatogram b) mass spectrum of the C₁₂ alkyl sulfate (C₁₂ AS) and sulfophenyl carboxylic acid homologues C₈ SPC and C₉ SPC.

The strongest estrogenic activity in Oborovo fractions was detected in fraction 13, and the detailed UPLC-QTOFMS analysis of this fraction was performed (Figure 39).

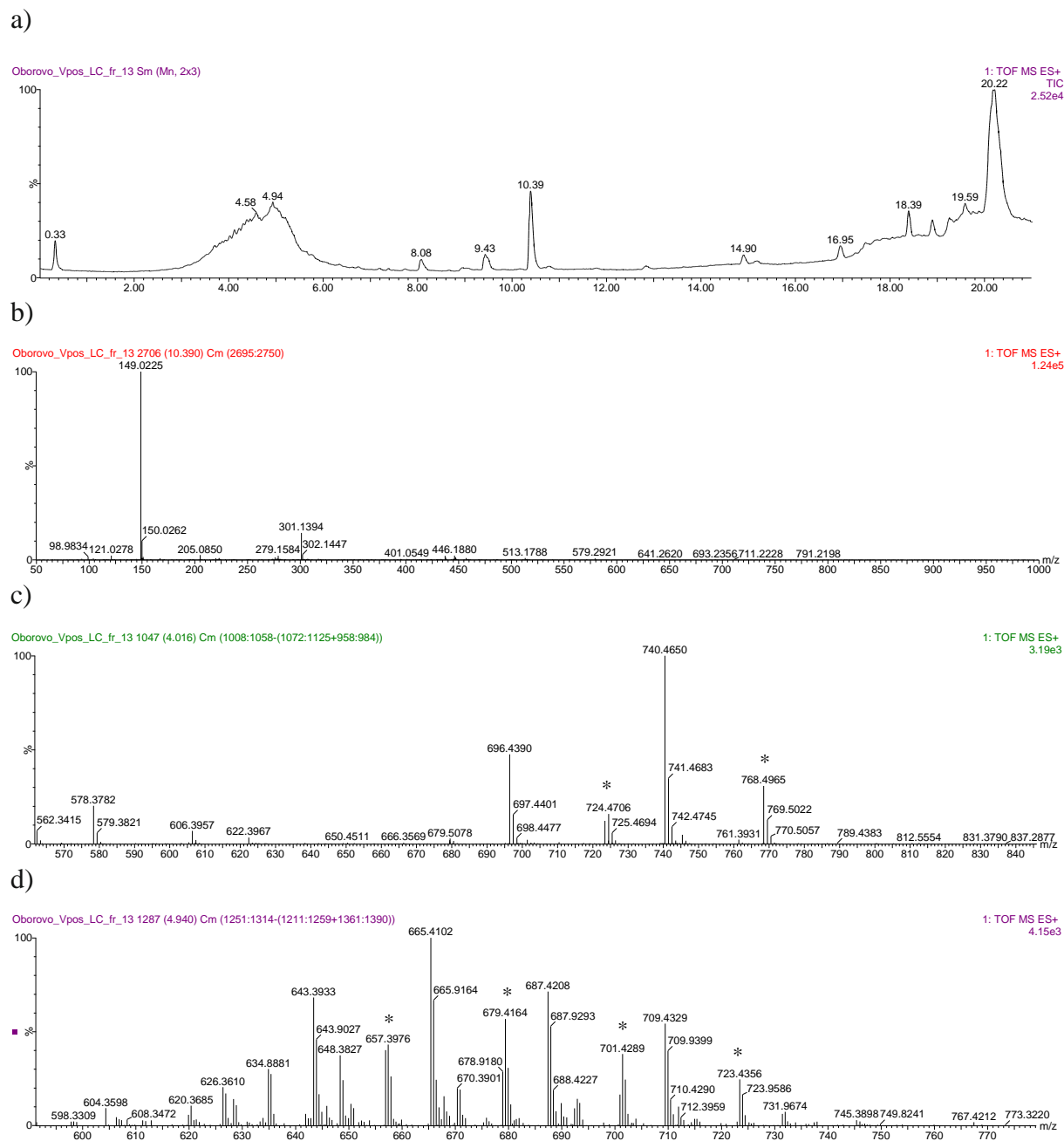


Figure 39. a) UHPLC-QTOFMS chromatogram of the Oborovo fraction 13 in PI mode showing total ion current chromatogram; b) mass spectrum of the dibutyl phthalate; c) mass spectrum of the single charged polyethylene glycol dimethyl ethers (* $n=15-16$); d) mass spectrum of the double charged species of the polyethylene glycol dimethyl ethers (* $n=28-31$).

One of the major peaks found in the TIC chromatogram, at the RT of 10.39 min in PI mode revealed mass spectrum with prominent ions including m/z 279.1584, m/z 301.1394 and m/z 149.0225, which corresponded to protonated dibutyl phthalate (DBP), DBP adduct with sodium $[M+Na]^+$ and the characteristic fragment ion of DBP, respectively. Phthalates are used for many decades in a wide range of plastics and products, from furnishings to packaging and toys. Since they are used as additives (i.e. not chemically bound to containing materials) they easily migrate into the environment. They are known as endocrine disruptors, therefore they have been included in the priority lists of dangerous substances in most of the industrialized countries. The peak at RT 4.58-4.94 min revealed complex mass spectrum composed of a large number of oligomers. The isotopic pattern of the ions shown in Figure 39c. revealed the presence of a single charged polyethylene glycol dimethyl ether oligomers (PEGDME). The even number of the ions (e.g. m/z 724.4706 and m/z 768.4965) suggested the PEGDME ammonia adducts $[M+NH_4]^+$ with 15 and 16 ethoxy units. A mass difference of exactly 44.0259 Da corresponds with regular interval of the ethoxy unit (C_2H_4O). The presence of the ions at m/z 696.4390 and m/z 740.4650 corresponded to fragment ions of PEGDME with 15 and 16 ethoxy units, respectively, showing a mass difference of 28 Da which correlated with loss of a C_2H_4 group. The mass spectrum shown in Figure 39d. illustrate the predominant pattern of the doubly charged ions (with a mass difference of 0.5 Da) which corresponded well to PEGDME having higher number of ethoxy groups ($n=28-31$). Polyethylene glycol dimethyl ethers, similar as PEGs are widely used in many fields, such as technical, pharmaceutical and biochemical applications.

Similar to fraction 3, a detailed analysis of the Oborovo fraction 13 in NI mode, revealed the presence of anionic surfactant C_{12} AS as well as C_8 and C_9 SPC homologues.

The highest CYP1A induction potential of the Oborovo fractions as determined by the measurement of an EROD activity in PLHC-1 cells was measured in the fraction 20. This fraction also showed acute cytotoxic potential to PLHC-1 cells in MTT assay. Most of the distinct peaks apparent in the UHPLC trace, acquired in the NI mode belong to anionic surfactants including C_{12} and C_{14} alkylsulfates (AS) and C_{12} alkylether sulfates (AES) containing 1-4 ethoxy units, and linear alkyl benzenesulfonates (LAS) having 12 carbon atoms in the alkyl chain, and a commercially available surfactant commonly used in agrochemicals 1,4-Bis(2-ethylhexyl) sulfosuccinate (DOSS). The peak at RT of 6.19 min revealed m/z 265.1470 (C_{12} AS), while the peak at RT of 6.78-7.17 min exhibited rather

complex TOF mass spectrum with several prominent ions including m/z 293.1784 (C_{14} AS), m/z 309.1729 (C_{12} AES, $n=1$), m/z 325.1812 (C_{12} LAS), m/z 353.1989 (C_{12} AES, $n=2$), m/z 397.2280 (C_{12} AES, $n=3$), m/z 421.2218 (DOSS) and m/z 441.2526 (C_{12} AES, $n=4$). Total ion chromatogram (TIC), as well as QTOFMS spectrum of the peak at RT of 6.78-7.17 min showing deprotonated molecules identified in Oborovo fraction 20 are presented in Figure 40.

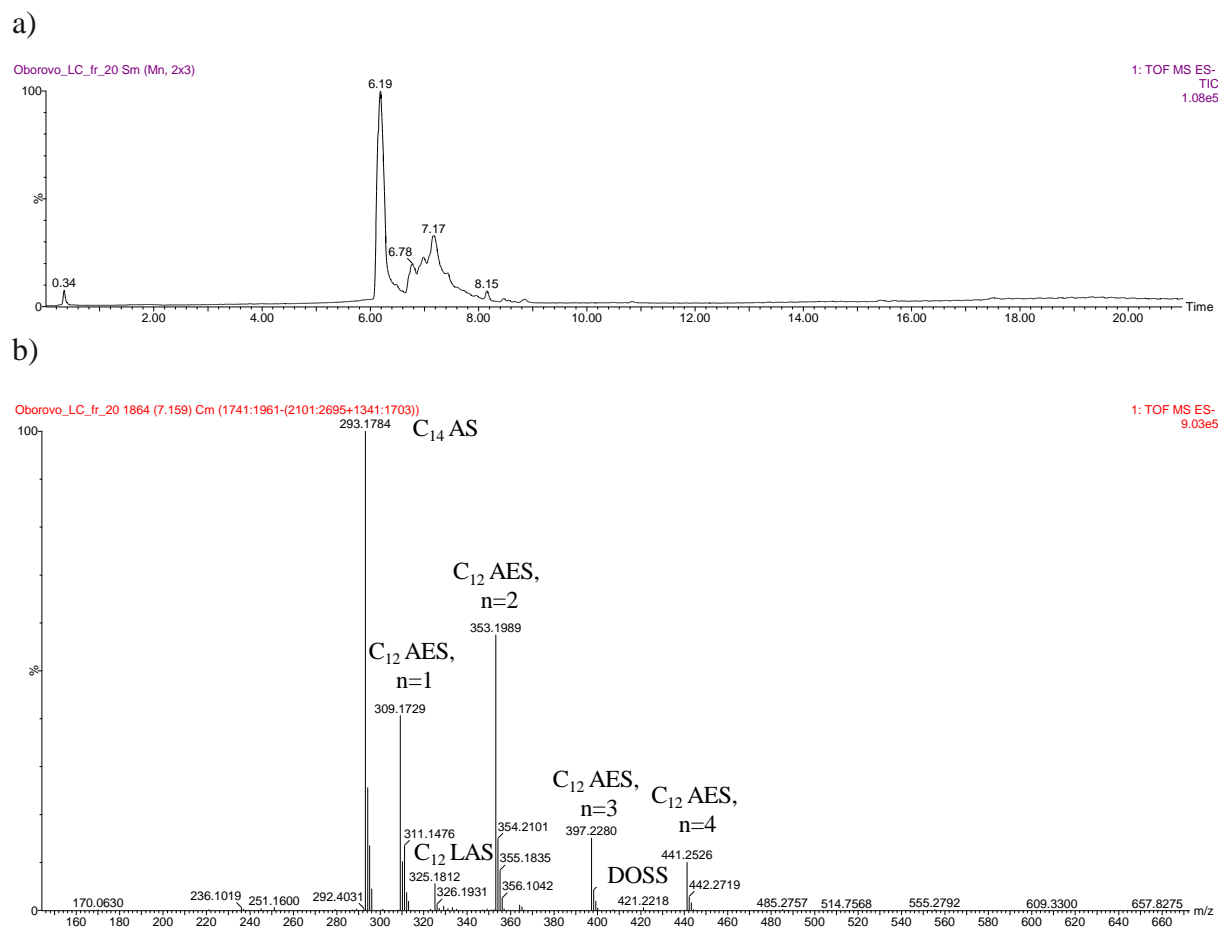


Figure 40. a) UHPLC-QTOFMS chromatogram of the Oborovo fraction 20 in NI mode, showing total ion current chromatogram b) mass spectrum of the peak at RT of 6.78-7.17 min.

The highest CYP1A induction potential of the WWTP VG fractions was measured in the fraction 16.

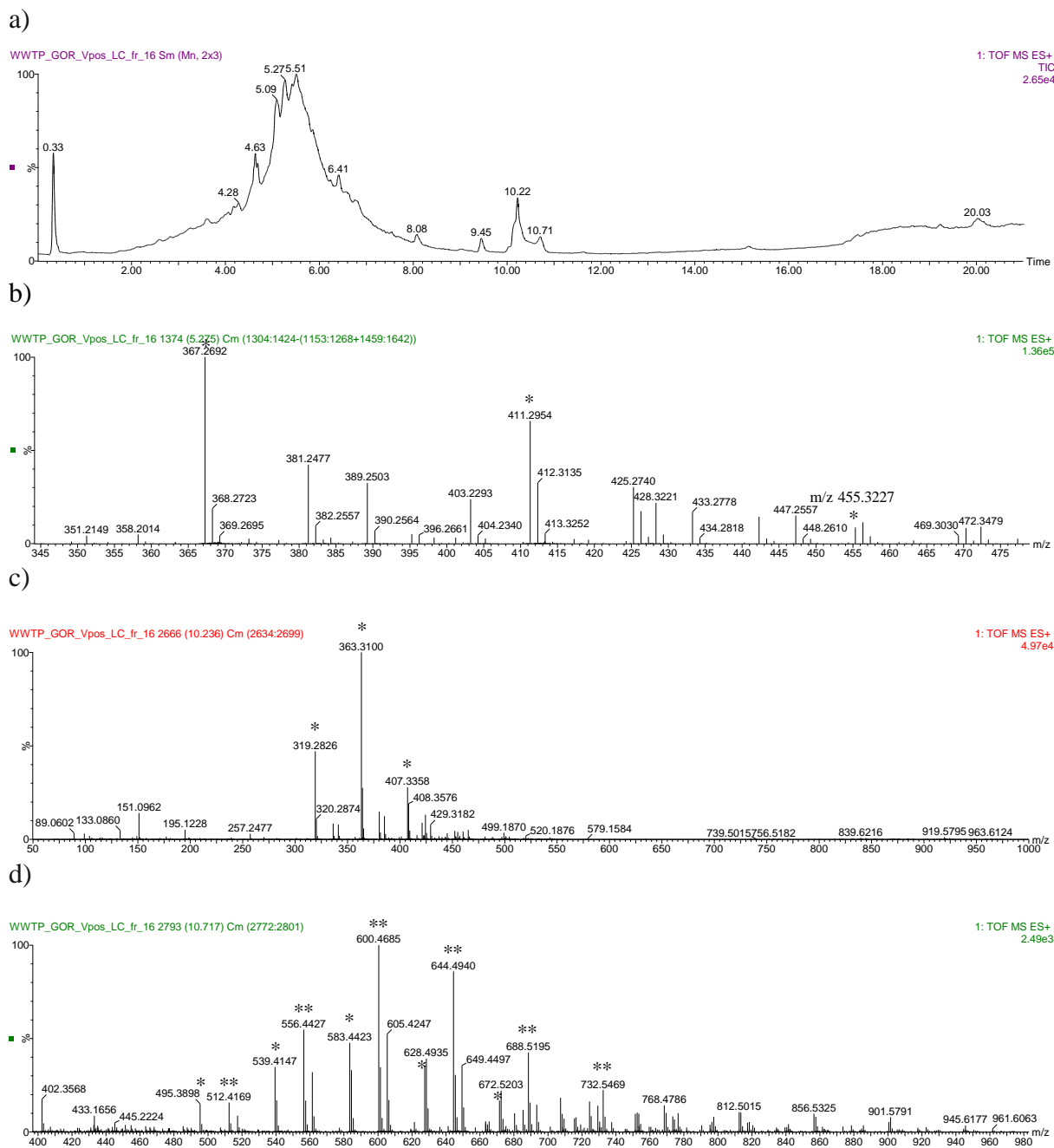


Figure 41. a) UHPLC-QTOFMS chromatogram of the WWTP VG fraction 16 in PI mode, showing total ion current chromatogram; b) mass spectrum of the peak at RT of 5.09-5.51 min showing C_6 AEOs (* $n=7-9$); c) mass spectrum of the peak at RT of 10.22 min showing protonated C_{12} AEOs (* $n=3-5$); d) mass spectrum of the peak at RT of 10.22 min (* protonated C_{12} AEOs, ** C_{12} AEOs adducts with ammonia).

In positive ionization mode, the most prominent peak in the TIC chromatogram revealed very complex mass spectrum with several main ions, including m/z 367.2692, m/z 411.2953 and 455.3227 (Figure 41b). The isotopic pattern displayed the presence of the non-ionic alcohol polyethoxylates surfactants having six carbon atoms in their alkyl chain with 7 - 9 ethoxy units. Peak at RT 10.22 min exhibited dodecyl alcohol polyethoxylates surfactants (C_{12} AEOs) having from 3-5 ethoxy units, while peak at RT of 10.71 min revealed C_{12} AEOs with higher ($n=7-12$) ethoxy units (Figure 41c-d). AEOs are the main non-ionic surfactants produced in Europe (e.g. 300,000 t/year just for household cleaning products).²²⁶ They are widely used in domestic and industrial applications (e.g. detergents, emulsifiers, wetting and dispersing agents, industrial cleaners, textile processing etc.). This is in part due to restrictions in use of alkylphenol polyethoxylates in household detergents since their metabolites are shown to be estrogenic.¹⁴⁴ Commercial AEOs consist of a mixture of several homologues of varying carbon chain length and degree of ethoxylation.

Detailed analysis of WWTP VG fraction 16 acquired in the NI mode showed strong predominance of anionic surfactants including C_{12} AS (RT of 6.28 min), C_{14} AS, C_{12} AES, C_{12} LAS, DOSS which coeluted at RT of 7.01-7.13 min (Figure 42).

a)

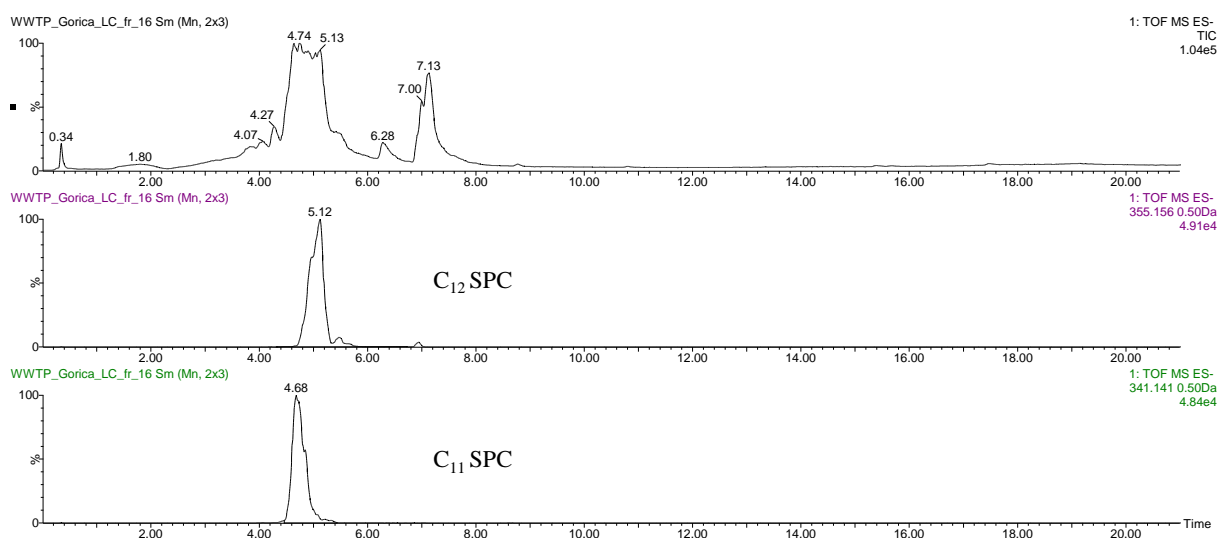


Figure 42. a) UHPLC-QTOFMS chromatogram of the WWTP VG fraction 16 in NI mode, showing total ion current chromatogram and extracted ion chromatograms reconstructed using accurate masses of deprotonated sulfophenyl carboxylic acid (SPC) homologues C_{11} , and C_{12} .

b)

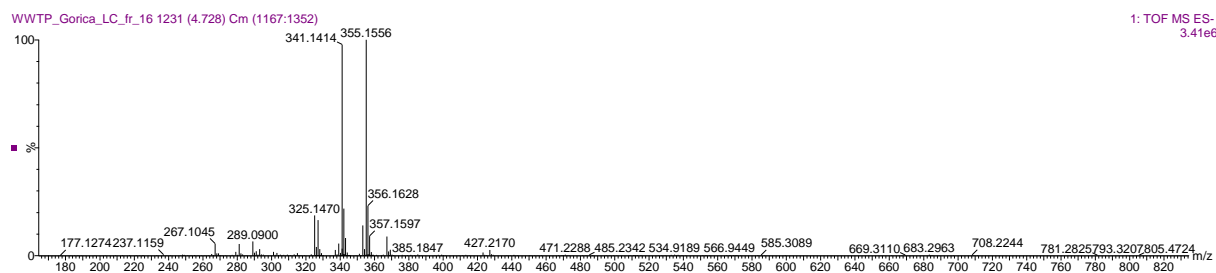


Figure 42. b) mass spectrum of the peak at RT of 4.74-5.13 min.

Peak at 4.74-5.13 min revealed the presence of sulfophenyl carboxylic acid (SPC) homologues C_{11} , and C_{12} with corresponding masses at m/z 341.1414 and m/z 355.1556, respectively (Figure 42).

Analysis of WWTP fraction 19 in positive ionization mode revealed very complex TIC chromatogram (Figure 43). Several peaks remained unidentified, however, some group of contaminants were successfully elucidated. The isotopic pattern of the highest peak in the UHPLC trace displayed the complex mass spectrum encompassed by the non-ionic alcohol polyethoxylates with 8 carbon atoms in their alkyl chain ($n=8-9$ ethoxy units). Peaks at RT 9.89-10.01 min and 11.60 min exhibited dodecyl alcohol polyethoxylates surfactants oligomers having from 4-6 ethoxy units. Peak at RT of 3.74 revealed ion at m/z 182.1900. The best match using MassLynx software was obtained for the formula $C_{12}H_{24}N$ which corresponded to protonated dicyclohexylamine (DCHA). Chemspider database offered this compound as the first hint. Dicyclohexylamine is a corrosion inhibitor present in certain semisynthetic metalworking fluids and is frequently found in WWTP effluents. DCHA also occurred in surface waters from urban and industrial locations.²²⁷ However, for the final confirmation the analysis of a reference standard has to be performed.

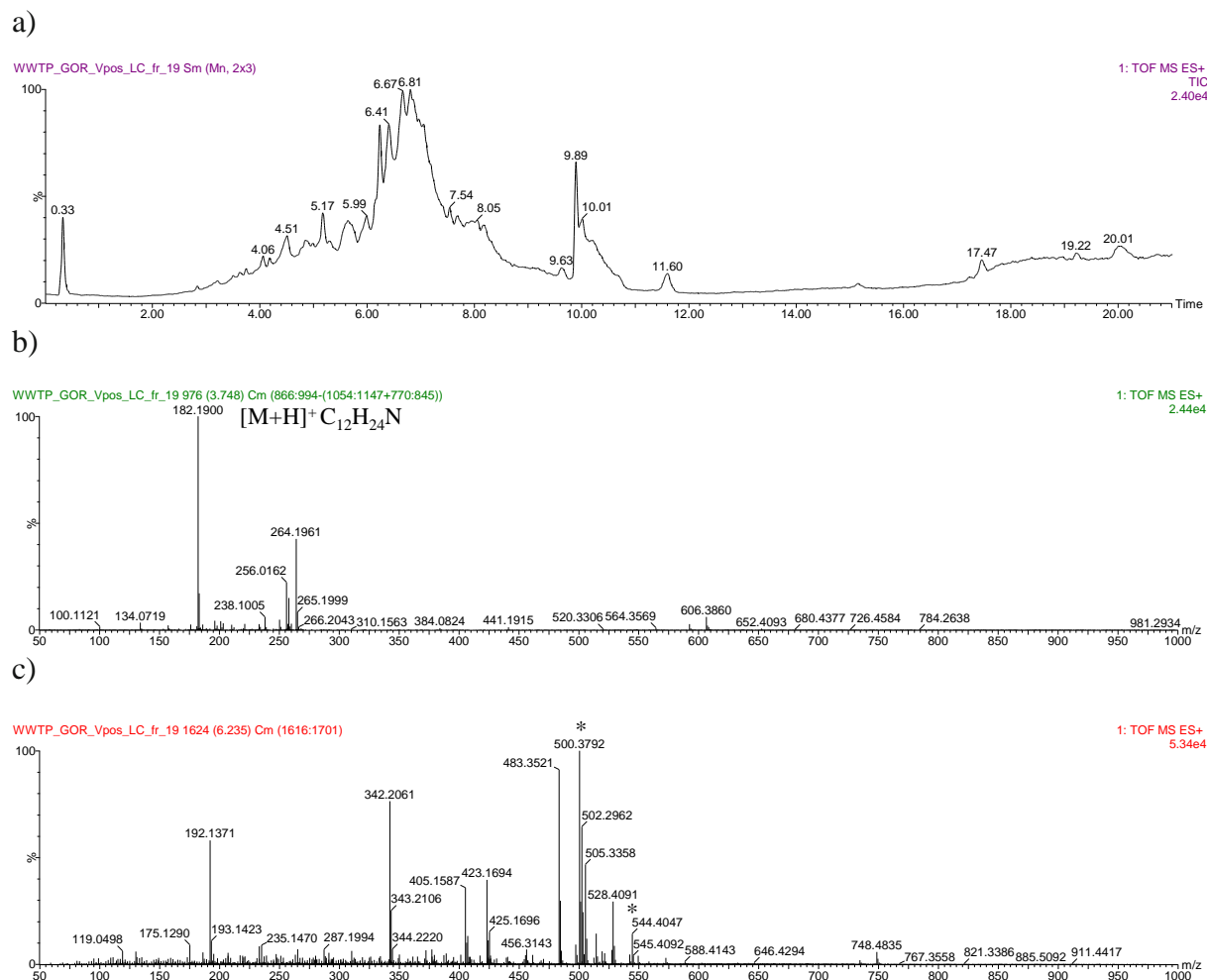


Figure 43. a) UHPLC-QTOFMS chromatogram of the WWTP VG fraction 19 in PI mode, showing total ion current chromatogram; b) mass spectrum of the peak at RT of 3.74 min; c) mass spectrum of the peak C₈ AEOs adducts with ammonia (*n=8-9).

Detailed analysis of the WWTP VG fraction 24 in PI mode revealed presence of non-ionic alcohol polyethoxylates having 10 carbon atoms in their alkyl chain as the most abundant contaminants in the fraction (Figure 44). The most prominent AEOs found in the fraction were those having 6-12 ethoxy units (Figure 44b). Peak at RT 9.65 exhibited rather complex mixtures of different oligomers, encompassed by C₁₀ AEOs with higher number of ethoxy units (n=16-18).

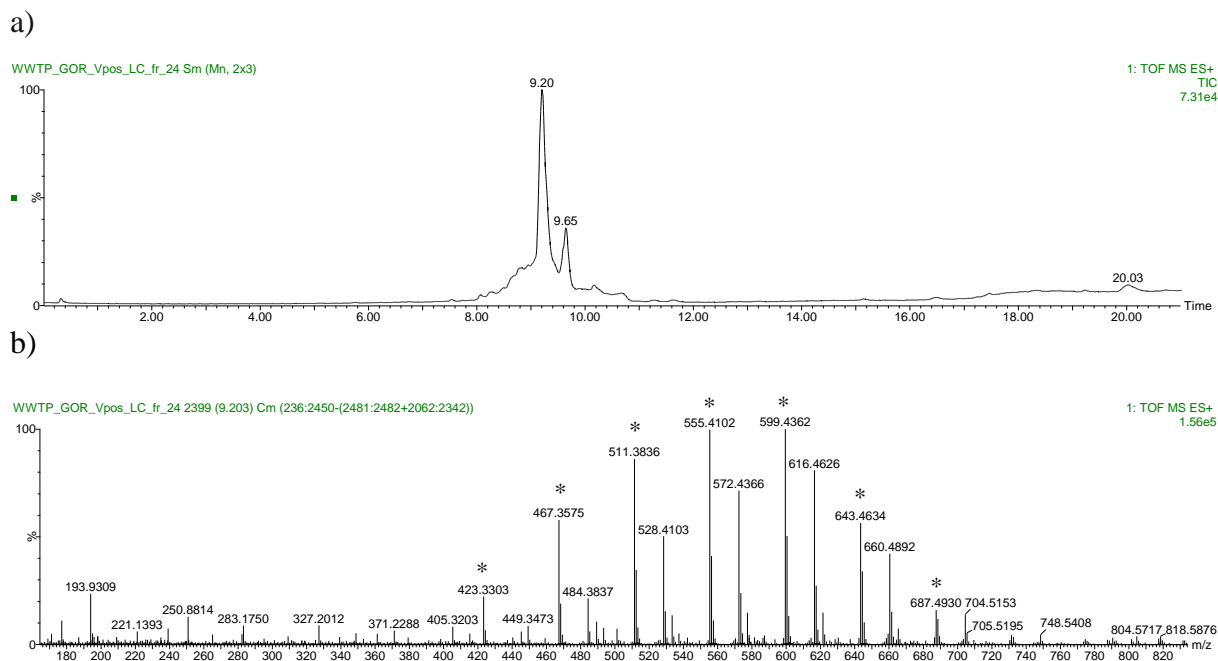


Figure 44. a) UHPLC-QTOFMS chromatogram of the WWTP Velika Gorica fraction 24 in PI mode showing total ion current chromatogram; b) mass spectrum of the peak at the RT 9.20 min showing C₁₀ AEOs (* 6-12 ethoxy units).

The anionic surfactants represented the most prominent peaks in the UHPLC chromatogram of the WWTP VG fraction 24 recorded in the NI mode (Figure 45). Total ion current chromatogram and extracted ion chromatograms reconstructed using accurate masses of identified anionic surfactants are given in Figure 45a. The peak at RT of 8.21 revealed ion at m/z 215.1657 (Figure 45b). The tentative elemental composition of the ion was calculated using MassLynx software, and the best match was obtained for the formula C₁₂H₂₃O₃ which corresponded to deprotonated 12-hydroxydodecanoic acid. However, for the final confirmation an additional MS-MS experiment or the analysis of a reference standard must be performed.

a)

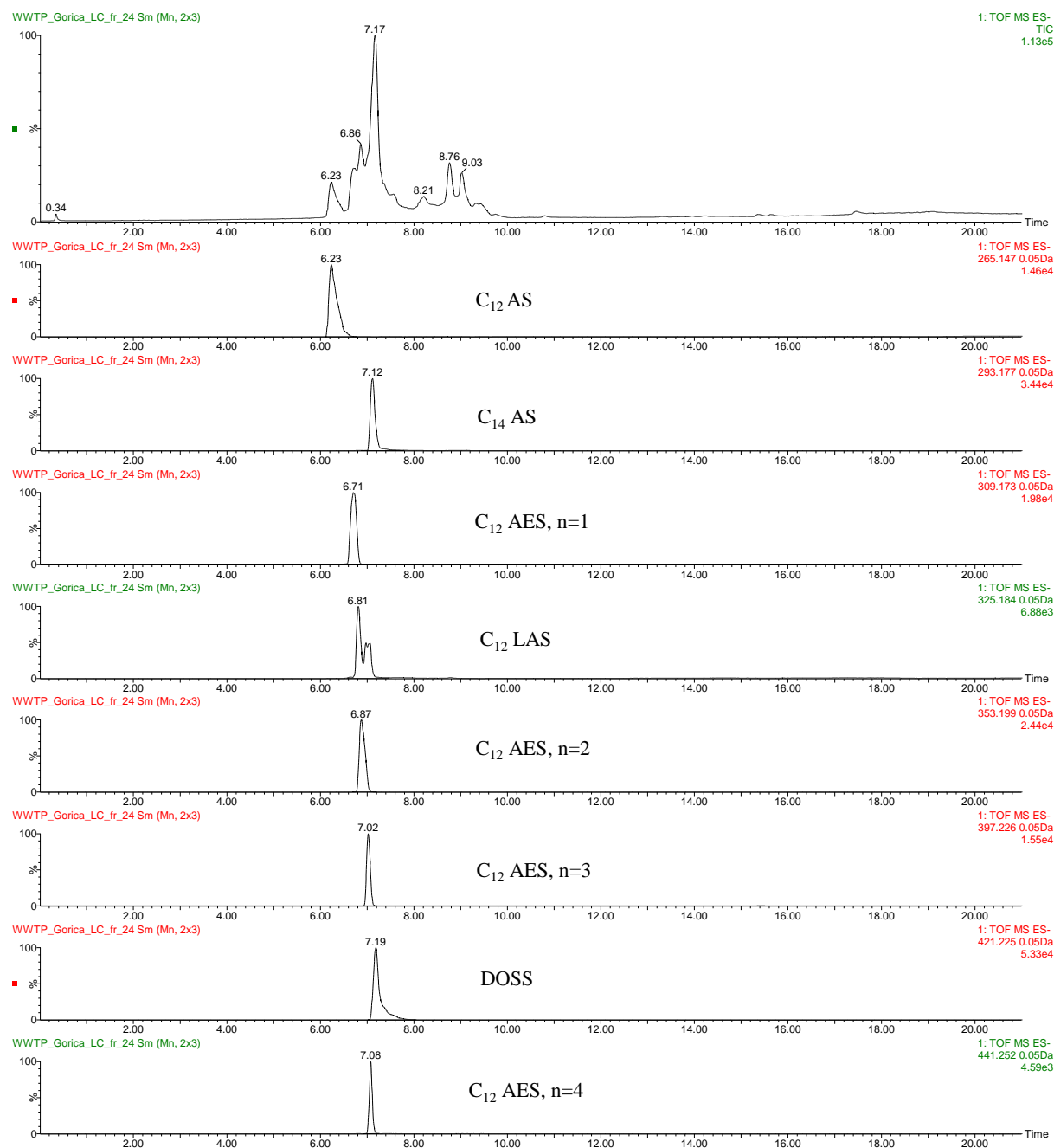


Figure 45. a) UHPLC-QTOFMS chromatogram of the WWTP Velika Gorica fraction 24 in NI mode showing total ion current chromatogram and extracted ion chromatograms reconstructed using accurate masses of deprotonated anionic surfactants.

b)

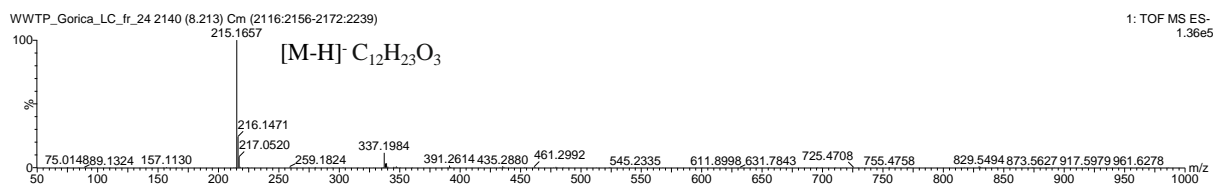
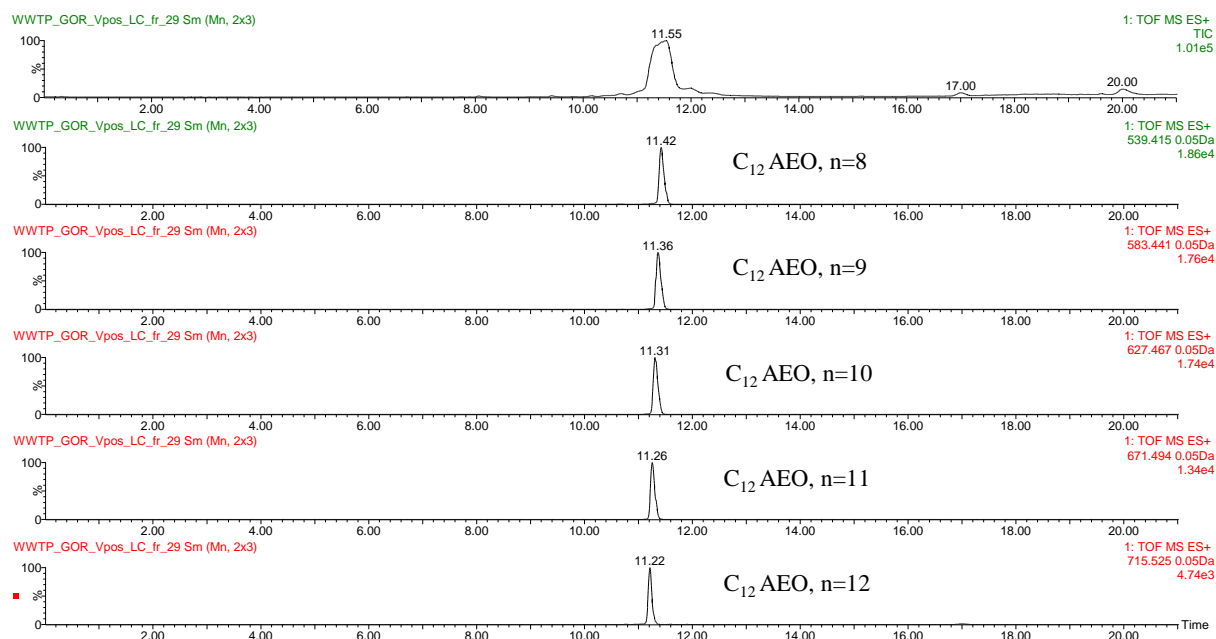


Figure 45. b) mass spectrum of the peak at the RT 8.21 min.

Prominent peaks eluting at the RT of 8.76 min and 9.03 min remained unidentified. The assignment of the molecular formulas to the measured accurate masses or linking the assumed elemental composition to a defined chemical structure for these peaks remained unresolved.

WWTP VG fraction 29 had an effect on survival of PLHC-1/wt cells in MTT test as well as on survival of unicellular green alga *Scenedesmus subspicatus* in AlgaeTox assay. A detailed Q-TOFMS analysis of WWTP VG fraction 29 in PI mode revealed the presence of non-ionic alcohol polyethoxylates surfactants having 12 carbon atoms in their alkyl chain predominant class of contaminants in the fraction (Figure 46). The most prominent AEOs found in the WWTP VG fraction 29 were those having 4-12 ethoxy units.

a)



b)

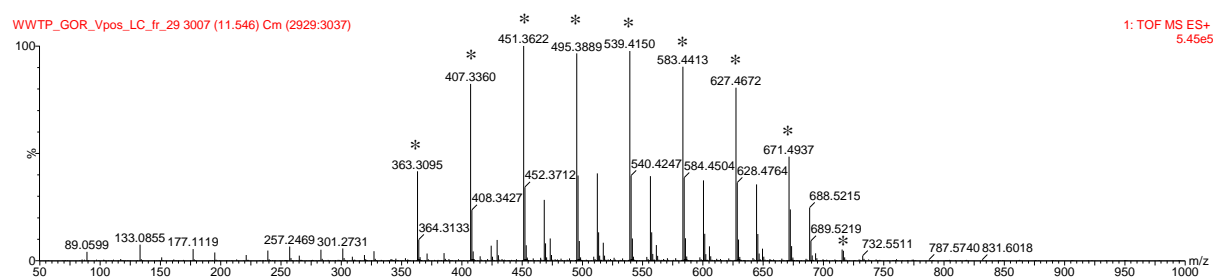


Figure 46. a) UHPLC-QTOFMS chromatogram of the WWTP Velika Gorica fraction 29 in PI mode, showing total ion current chromatogram and extracted ion chromatograms reconstructed using accurate masses of protonated C₁₂ AEOs oligomers; b) mass spectrum showing C₁₂ AEOs (* 4-12 ethoxy units).

The identification of the most prominent components of the highly active HPLC fractions indicated that surfactants represented the most prominent contaminant class in the analysed wastewater and river water extracts. This circumstantial evidence cannot be interpreted as a confirmation of surfactants as causative compounds responsible for the observed toxic effects. However, there is a very high probability that surfactants contribute to the overall toxicity either directly or indirectly by influencing transport of toxicant through the membranes or by some other synergistic effects.

§ 5. CONCLUSIONS

Despite the fact that EDA approach has already been applied in the literature for the characterization of organic contamination in environmental samples, the protocols developed so far are often used only for a limited range of chemical compounds, often lacking insight into the polar fraction. The enrichment and fractionation of complex contaminant mixtures typical of real systems represent a major challenge for the comprehensive EDA, which aims at establishing quantitative relationships between the observed effects and exposure concentration of causative toxicants.

In this work, improved procedures for the extraction and fractionation of an extended range of water contaminants were developed by combining different sorption mechanisms. The study indicated that use of the mixed-bed SPE, followed by sequential elution into neutral, basic and acidic fraction provides an attractive protocol for the simplified EDA. Such a preliminary fractionation of the total extract according to the charge is particularly suitable for the detailed characterization of polar contaminants, which often contain either positively or negatively charged moieties. It was shown that the optimal extraction/fractionation protocol for a simplified EDA approach should involve exhaustive extraction, employing polymeric sorbents, and fractionation of the total extract into well-defined cationic, anionic and neutral fractions, using silica-based ion-exchange sorbents.

The developed extraction and fractionation protocols were successfully applied for the assessment of toxic organic pollution in the Sava River during the European demonstration programme. The novel onsite LVSPE sampling device was successfully used for integrative effect-based and chemical monitoring purposes. The set of selected bioassays enabled detection of effects relevant for surface waters on an EU-wide scale. The most frequently observed effects were estrogenicity, zebrafish acute embryo toxicity and algal growth inhibition, while glucocorticoid activity was measured only at one sampling site. Some biological effects could not be explained by an extensive list of target compounds, therefore higher-tier EDA studies showed to be necessary to possibly close this gap for the bioactive samples.

The detailed EDA successfully demonstrated a large potential of effect-directed analysis for the characterization of contaminants in complex environmental mixtures. A

battery of selected ecotoxicological *in vitro* and *in vivo* bioassays that encompassed various toxicity end-points applied for the assessment of the collected Sava River and WW samples provided deeper insight into ecotoxicological potential of investigated samples. Fine HPLC fractionation was performed in order to facilitate identification of the most critical compounds contributed to a given endpoint. The data indicated the importance of detailed HPLC fractionation procedures for the prevention of masking effects, which often occur in complex environmental matrices. Despite well-known difficulties encountered during elucidation of chemical structures of unknown (non-target) compounds based on LC-MS analyses due to the lack of comprehensive searchable libraries of reference spectra, a large percentage of the major peaks in the total ion current chromatograms of the selected fractions was identified. Nevertheless, even after a detailed fractionation of the samples it was often impossible to identify individual contaminants, whose presence could explain some of the observed biological activity. However, detailed EDA allowed us to point out several compound classes, which are not included in the European and national monitoring strategies, thus creating a basis for region-specific pollutant prioritization and optimized water management. In particular, there is a need to include typical representatives of the polar organic contaminants such as surfactants and pharmaceuticals in the future region-specific monitoring activities. Their potential for being bioavailable in the aquatic environment is rather high compared to the classical hydrophobic pollutants.

This study emphasizes the importance of the effect-based monitoring approaches, which provide information on ecotoxicological risks of chemical mixtures, and confirms that further efforts need to be directed towards an identification of key pollutants, rather than focusing on relatively small number of previously selected individual compounds.

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§ 7. SUPPLEMENTARY MATERIALS

Table SI List of reference compounds used for the evaluation of extraction and fractionation procedures with their abbreviations, structural formulae, elemental composition and physicochemical properties.

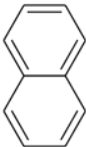
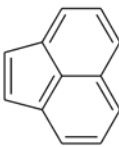
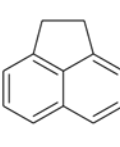
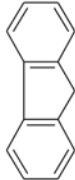
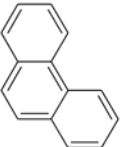
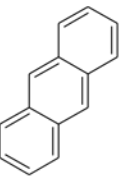
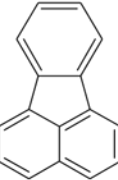
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K_{ow}
<i>Polycyclic aromatic hydrocarbons</i>						
Naphthalene	NA		C ₁₀ H ₈	128.062607	-	3.30 ^I
Acenaphthylene	ACN		C ₁₂ H ₈	152.062607	-	3.93 ^{II}
Acenaphthene	AC		C ₁₂ H ₁₀	154.078247	-	3.92 ^I
Fluorene	FL		C ₁₃ H ₁₀	166.078247	-	4.18 ^I
Phenanthrene	PHE		C ₁₄ H ₁₀	178.078247	-	4.46 ^I
Anthracene	AN		C ₁₄ H ₁₀	178.078247	-	4.45 ^I
Fluoranthene	FLU		C ₁₆ H ₁₀	202.078247	-	5.16 ^I

Table SI - continued.


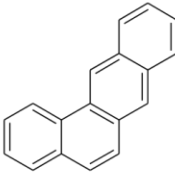
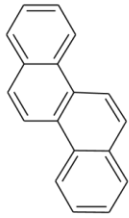
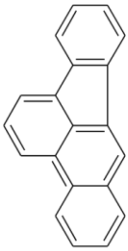
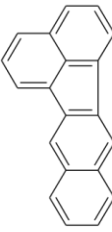

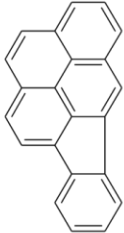
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
Pyrene	PY		C ₁₆ H ₁₀	202.078247	-	4.88 ^I
Benz[a]anthracene	B(a)A		C ₁₈ H ₁₂	228.093903	-	5.76 ^{III}
Chrysene	CHR		C ₁₈ H ₁₂	228.093903	-	5.73 ^I
Benzo[b]fluoranthene	B[b]F		C ₂₀ H ₁₂	252.093903	-	5.78 ^{III}
Benzo[k]fluoranthene	B[k]F		C ₂₀ H ₁₂	252.093903	-	6.11 ^{IV}
Benzo[a]pyrene	B[a]P		C ₂₀ H ₁₂	252.093903	-	6.13 ^{IV}
Indeno[1,2,3-cd]pyrene	I[c]P		C ₂₂ H ₁₂	276.093903	-	6.70 ^V

Table SI - continued.

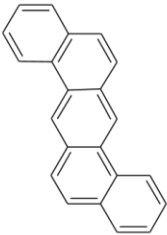
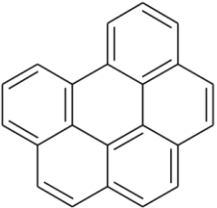
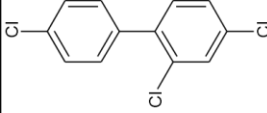
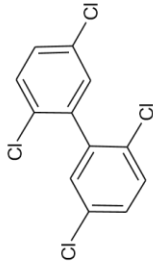
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
Dibenz[a,h]anthracene	D[a,h]A		C ₂₂ H ₁₄	278.109558	-	6.50 ¹
Benzo[g,h,i]perylene	B[g,h,i]P		C ₂₂ H ₁₂	276.093903	-	6.63 ¹
<i>Polychlorinated biphenyls</i>						
2,4,4'-Trichlorobiphenyl	PCB 28		C ₁₂ H ₇ Cl ₃	255.961334	-	5.62 ¹
2,2',5,5'-Tetrachlorobiphenyl	PCB 52		C ₁₂ H ₆ Cl ₄	289.922363	-	6.09 ¹

Table SI - continued.

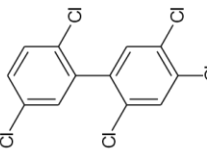
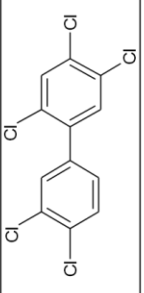
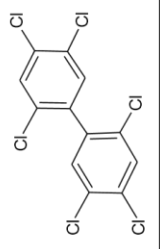
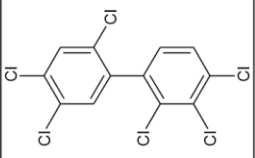
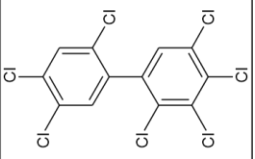
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
2,2',4,5,5'-Pentachlorobiphenyl	PCB 101		C ₁₂ H ₅ Cl ₅	323.883392	-	6.80 ^I
2,3',4,4',5-Pentachlorobiphenyl	PCB 118		C ₁₂ H ₅ Cl ₅	323.883392	-	7.12 ^I
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB 153		C ₁₂ H ₄ Cl ₆	357.844421	-	7.7 ^I
2,2',3,4,4',5'-Hexachlorobiphenyl	PCB 138		C ₁₂ H ₄ Cl ₆	357.844421	-	7.74 ^{VI}
2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB 180		C ₁₂ H ₃ Cl ₇	391.805450	-	8.27 ^{VII}

Table SI - continued.

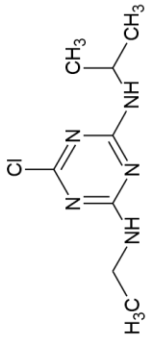
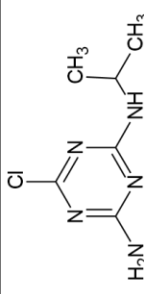
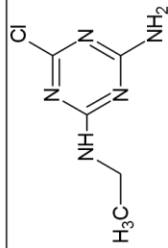
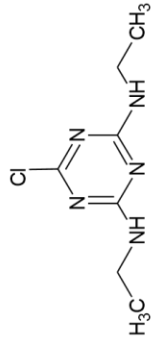
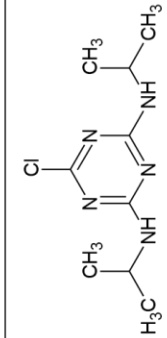
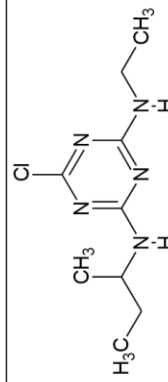
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log Kow
<i>Pesticides and their TPs</i>						
Atrazine	ATZ		C ₈ H ₁₄ ClN ₅	215.093781	1.60 ^{VIII}	2.61 ^I
Atrazine-desethyl	DEA		C ₆ H ₁₀ ClN ₅	187.062469	-	1.51 ^{IX}
Atrazine desisopropyl	DIA		C ₃ H ₈ ClN ₅	173.046829	-	1.15 ^{IX}
Simazine	SIM		C ₇ H ₁₂ ClN ₅	201.078125	1.62 ^X	2.18 ^I
Propazine	PROP		C ₉ H ₁₆ ClN ₅	229.109421	1.70 ^X	2.93 ^I
Sebuthylazine	SEB		C ₉ H ₁₆ ClN ₅	229.109421	-	3.31 ^{VII}

Table SI - continued.

Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log <i>K_{ow}</i>
Terbutylazine	TERB		C ₉ H ₁₆ ClN ₅	229.109421	2 ^{VIII}	3.40 ^{VIII}
Diuron	DIU		C ₉ H ₁₀ Cl ₂ N ₂ O	232.017014	-	2.68 ^I
Isoproturon	IPU		C ₁₂ H ₁₈ N ₂ O	206.141907	-	2.87 ^I
Chlorfenvinphos	CVP		C ₁₂ H ₁₄ Cl ₃ O ₄ P	357.969513	-	3.81 ^{XI}
Pentachlorophenol	PCP		C ₆ Cl ₅ OH	263.847015	4.70 ^{XII}	5.12 ^I

Table SI - continued.

Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
<i>Pharmaceuticals and their TPs</i>						
Zolpidem	ZOL		C ₁₉ H ₂₁ N ₃ O	307.168457	6.20 ^{XIII}	3.85 ^{VII}
Warfarin	WAR		C ₁₉ H ₁₆ O ₄	308.104858	5.87 ^{XIV}	2.70 ^I
Torseamide	TOR		C ₁₆ H ₂₀ N ₄ O ₃ S	348.125610	7.10 ^{XV}	1.95 ^{VII}
Terbinafine	TER		C ₂₁ H ₂₅ N	291.198700	-	5.81 ^{VII}

Table SI - continued.

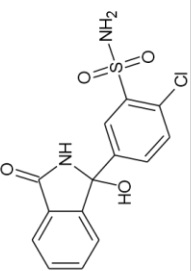
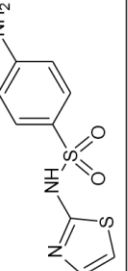
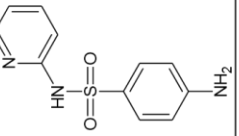
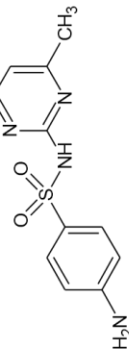
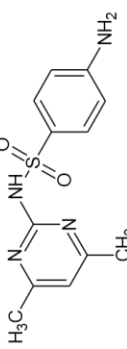
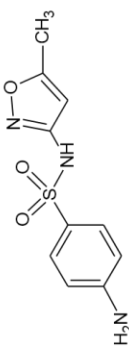
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log Kow
Chlorthalidone	CHTD		C ₁₄ H ₁₁ ClN ₂ O ₄ S	338.012817	-	0.85 ^{XVI}
Sulfathiazole	STZ		C ₉ H ₆ N ₃ O ₂ S ₂	255.013611	2; 7.1 ^{XVII}	0 ^{XVIII}
Sulfapyridine	SPY		C ₁₁ H ₁₁ N ₃ O ₂ S	249.057190	2.6; 8.4 ^{XIX}	0.35 ^I
Sulfamerazine	SMR		C ₁₁ H ₁₂ N ₄ O ₂ S	264.068085	2.1; 6.9 ^{XVII}	0.14 ^I
Sulfamethazine	SMZ		C ₁₂ H ₁₄ N ₄ O ₂ S	278.083740	2.1; 7.5 ^{XVII}	0.3 ^{XVIII}
Sulfamethoxazole	SMX		C ₁₀ H ₁₁ N ₃ O ₃ S	253.052109	1.9; 5.6 ^{XVII}	0.89 ^I

Table SI - continued.

Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
N-acetyl sulfamethoxazole	N-acetyl-SMX		C ₁₂ H ₁₃ N ₃ O ₄ S	295.062683	-	1.21 ^{VII}
Trimethoprim	TMP		C ₁₄ H ₁₈ N ₄ O ₃	290.137878	3.2; 6.8 ^{XVII}	0.91 ^I
Norfloxacin	NOR		C ₁₆ H ₁₈ FN ₃ O ₃	319.133209	3.1; 8.6 ^{XVII}	-1.0 ^{XX}
Ciprofloxacin	CIP		C ₁₇ H ₁₈ FN ₃ O ₃	331.133209	3; 8.7 ^{XVII}	0.3 ^{XX}
Enrofloxacin	ENR		C ₁₉ H ₂₂ FN ₃ O ₃	359.164520	3.9; 7.6 ^{XVII}	0.9 ^{XXI}

Table SI - continued.

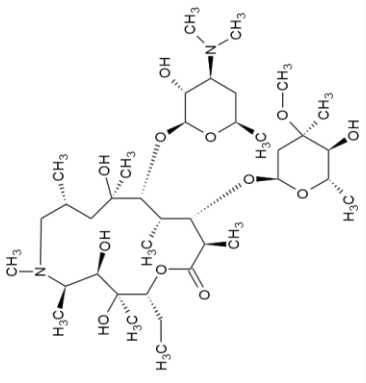
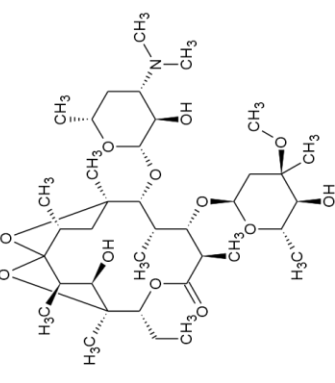
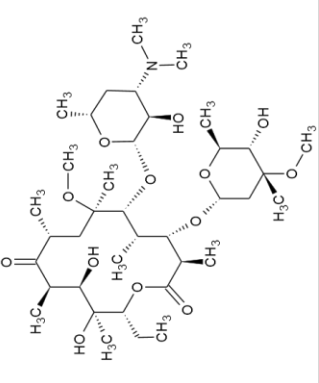
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log Kow
Azithromycin	AZY		$C_{38}H_{72}N_2O_{12}$	748.508545	8.7; 9.5 ^{XIX}	4 ^{XXII}
Erythromycin – H ₂ O	ERY-H ₂ O		$C_{37}H_{65}NO_{12}$	715.450684	-	3.93 ^{XXIII}
Clarithromycin	CLA		$C_{38}H_{69}NO_{13}$	747.476868	8.9 ^{XIX}	1.8 ^{XXIV}

Table SI - continued.

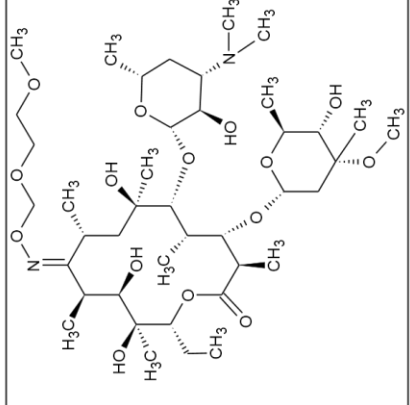
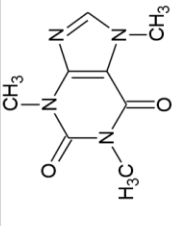
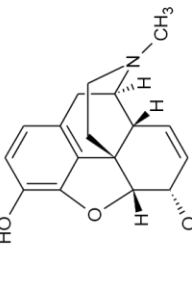
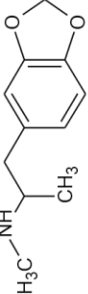
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K_{ow}
Roxithromycin	ROX		$C_{41}H_{76}N_2O_{15}$	836.524597	9.2 ^{XXVII}	-
Caffeine	CAF		$C_8H_{10}N_4O_2$	194.080383	10.4 ^{XXV}	-0.07 ^I
<i>Illicit drugs and their metabolites</i>						
6-acetylmorphine	6-AM		$C_{19}H_{21}NO_4$	327.147064	9.6 ^{XXVI}	1.7 ^{XXVII}
3,4-methylenedioxymethamphetamine	MDMA		$C_{11}H_{15}NO_2$	193.110275	8.7 ^{XXVII}	2.28 ^{XXVII}

Table SI - continued.

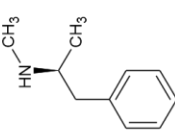
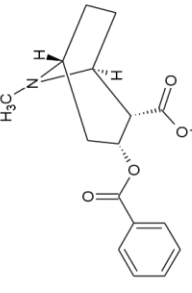
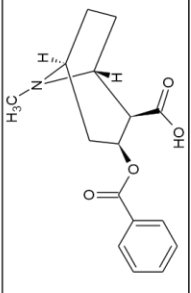
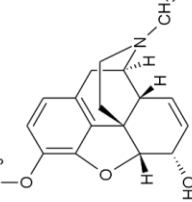
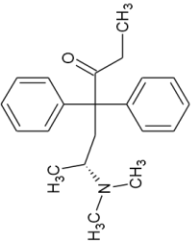
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log <i>K_{ow}</i>
Methamphetamine	MAMP		C ₁₀ H ₁₅ N	149.120453	9.9 ^{XXVII}	1.64 ^{XXVII}
Cocaine	COC		C ₁₇ H ₂₁ NO ₄	303.147064	8.6 ^{XXVII}	2.30 ^{XXVII}
Benzoylcegonine	BE		C ₁₆ H ₁₉ NO ₄	289.131409	10.8; 3.3 ^{XXVIII}	< 0 ^{XXVII}
Codeine	COD		C ₁₈ H ₂₁ NO ₃	299.152130	8.2 ^{XXVII}	1.19 ^{XXVII}
Methadone	MTHD		C ₂₁ H ₂₇ NO	309.209259	8.6 ^{XXVII}	3.39 ^{XXVII}

Table SI - continued.

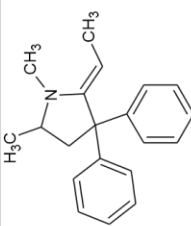
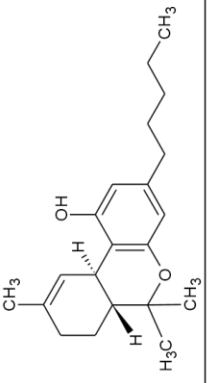
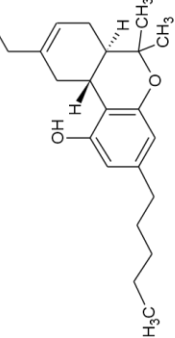
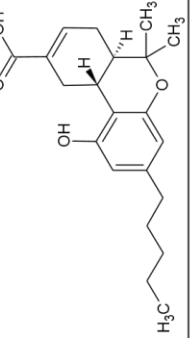
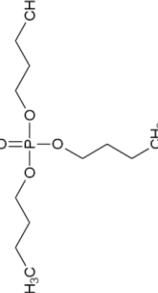
Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log K _{ow}
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP		C ₂₀ H ₂₃ N	277.183044	8.4 ^{XXVIII}	2.97 ^{XXVII}
Tetrahydrocannabinol	THC		C ₂₁ H ₃₀ O ₂	314.224579	10.6 ^{XXIX}	6.97 ^{XXX}
11-Hydroxy-Δ ⁹ -tetrahydrocannabinol	THC-OH		C ₂₁ H ₃₀ O ₃	330.219482	3.7 ^{XXVI}	5.33 ^{XXVII}
Δ ⁹ -Tetrahydrocannabinol carboxylic acid	THC-COOH		C ₂₁ H ₂₈ O ₄	344.198761	4.1 ^{XXVI}	6.57 ^{XXVII}
Plasticizers and flame retardants						
Tributyl phosphate	TPP		C ₁₂ H ₂₇ O ₄ P	266.164703	-	4.00 ^I

Table SI - continued.

Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log <i>K_{ow}</i>
Triphenyl phosphate	TPP		C ₁₈ H ₁₅ O ₄ P	326.070801	-	4.59 ^I
Bisphenol A	BPA		C ₁₅ H ₁₆ O ₂	228.115036	9.6 ^{XXXI}	3.32 ^I
Surfactants and their TPs						
Benzyltrimethyl decylammonium chloride	ADBAC, C ₁₀		C ₁₉ H ₃₄ ClN	311.237976	-	-
Benzyltrimethyl dodecylammonium chloride	ADBAC, C ₁₂		C ₂₁ H ₃₈ ClN	339.269287	-	-
Benzyltrimethyl tetradecylammonium chloride	ADBAC, C ₁₄		C ₂₃ H ₄₂ ClN	367.300568	-	3.91 ^V
Benzyltrimethyl hexadecylammonium chloride	ADBAC, C ₁₆		C ₂₅ H ₄₆ ClN	395.331879	-	-
Polypropylene glycols	PPG (n=8-14)		HO-(C ₃ H ₆ O) _n -H	482.345483- 830.596672	-	-

Table SI - continued.

Compound name	Abbreviation	Structure	Elemental composition	Monoisotopic mass/ Da	pKa	Log <i>K_{ow}</i>
Polyethylene glycols	PEG (n=8-16)		HO-(C ₂ H ₄ O) _n -H	370.220283- 722.430001	-	-
Alcohol polyethoxylates	AEO (n=5;6)		C ₁₂ H ₂₅ - (C ₂ H ₄ O) _n -OH	406.329439; 450.355654	-	-
Nonylphenol polyethoxylates	NPnEO (n=10;12)		C ₁₅ H ₂₃ O- (C ₂ H ₄ O) _n -H	660.444863; 748.497292	-	4.06 ^{XXXII}
Nonylphenol	NP		C ₁₅ H ₂₄ O	220.182709	-	4.48 ^{XXXII}
Nonylphenoxyacetic acid	NPiEC		C ₁₇ H ₂₆ O ₃	278.188202	-	5.80 ^{VII}
Nonylphenoxyethoxyacetic acid	NP2EC		C ₁₉ H ₃₀ O ₄	322.214417	-	5.53 ^{VII}
Octylphenol	OP		C ₁₄ H ₂₂ O	206.167068	-	4.12 ^{XXXII}
Octylphenoxyacetic acid	OPIEC		C ₁₆ H ₂₄ O ₃	264.172546	-	5.31 ^{VII}

Table SII Results of receptor-mediated *in vitro* and *in vivo* bioassays shown for the neutral (HR-X) sorbent, anionic (HR-XAW) and cationic (HR-XCW) resins.

		SO			PO			OB			SC		
		HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW
GR	Glucocorticoid activity	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.32	<LOD	<LOD	<LOD	<LOD	<LOD
	Dex-EQ [ng/L]	<80	>100	>100	>100	>100	>100	<100	>100	>100	>100	>100	>100
CALUX	Cytotoxicity	<80	>100	>100	>100	>100	>100	<100	>100	>100	>100	>100	>100
	LOEC [REF]	<80	>100	>100	>100	>100	>100	<100	>100	>100	>100	>100	>100
Algal growth inhibition	Growth rate inhibition	259	NA	NA	94.6	NA	NA	132	NA	NA	NA	NA	NA
	EC50 [REF]	259	NA	NA	94.6	NA	NA	132	NA	NA	NA	NA	NA
ER-mediated activity	Growth rate inhibition	100	>100	>100	33	>100	>100	33	>100	>100	>100	>100	>100
	LOEC [REF]	100	>100	>100	33	>100	>100	33	>100	>100	>100	>100	>100
ER-mediated activity	Estrogenicity	0.16	<LOQ	<LOQ	0.24	<LOQ	<LOQ	0.42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	E2-EQ [ng/L]	0.16	<LOQ	<LOQ	0.24	<LOQ	<LOQ	0.42	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
MELN cells	Anti-estrogenicity	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	OH-Tam-EQ [ng/L]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
AR-mediated activity	Cytotoxicity	100	>100	>100	100	>100	>100	100	>100	>100	>100	>100	>100
	LOEC [REF]	100	>100	>100	100	>100	>100	100	>100	>100	>100	>100	>100
AR-mediated activity	Androgenicity	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	- DHT-EQ [ng/L]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

Table SII – continued.

	SO			PO			OB			SC		
	HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW	HR-X	HR-XAW	HR-XCW
MDA-kb2 cells	Anti-androgenicity Flu-EQ [ng/L]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Glucocorticoid activity Dex-EQ [ng/L]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Cytotoxicity LOEC [REF]	100	>100	>100	100	>100	>100	100	>100	>100	>100	>100
	AChE inhibition IC50 [REF]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
AChE inhibition	AChE inhibition LOEC [REF]	>250	>500	500	>250	>500	500	>250	>500	500	>250	>500
	Survival LC50 [REF]	19.1	NA	NA	72	NA	NA	23.3	NA	NA	NA	NA
Zebrafish embryo acute toxicity	LOEC [REF]	12.5	>100	>100	25	>100	>100	25	>100	>100	>100	>100
	Sublethal endpoints - EC50 [REF]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	LOEC [REF]	NA	>100	>100	NA	>100	>100	NA	>100	>100	>100	>100

EC50 - concentration at which the effect reaches 50% of the effect in untreated control; Dex-EQ - dexamethasone equivalent; Flu-EQ - flutamide equivalent;

LOEC - lowest observed effect concentration; OH-Tam-EQ - hydroxytamoxifen equivalent; REF - relative enrichment factor.

Table SIII List of all target compounds investigated in the Sava River samples during the detailed EDA.

Compound	Compound class/Usage pattern
10,11-Dihydro-10,11-dihydroxycarbamazepine	pharmaceutical TP
17-alpha ethinylestradiol	hormone, estrogen
17-beta-estradiol	hormone, estrogen, WFD watch list
2-(2-(Chlorophenyl)amino)benzaldehyde	pharmaceutical TP
2,4-Dichlorophenoxyacetic acid	herbicide/biocide
2,4-Dinitrophenol	herbicide, TP
2-aminobenzimidazole	fungicide TP
2-Hydroxycarbamazepine	pharmaceutical TP
2-Isopropylthioxanthone	industrial chemical
2-Methylbenzothiazole	aroma constituent of tea leaves
2-Naphthalenesulfonic acid	industrial chemical
2-Octyl-4-isothiazolin-3-one	biocide
2-Phenylphenol	fungicide
3,4-dichlorophenylurea	herbicide TP
3,5,6-Trichloro-2-pyridinol	insecticide TP
3-Cl-2-hydroxypropyltrimethylammonium	industrial chemical
4-Androstene-3,17-dione	steroid
4-Androstenedione	hormone, androgen
4-Bromophenol	flame retardant
4-Formyl-antipyrine	pharmaceutical TP
4-Hydroxybenzotriazole	corrosion inhibitor TP
4-Hydroxytamoxifen	pharmaceutical, anti-estrogen
4-Methylcoumarin	industrial chemical
4-n-Nonylphenol	surfactant transformation product, WFD priority, xenoestrogen
7-Hydroxy-4-methylcoumarin	pharmaceutical, antispasmodic drug
Acetamiprid	insecticide
Acetyl-Sulfamethoxazole	pharmaceutical TP
Acid orange 7	dye
Albendazole	pharmaceutical, anthelmintic drug
Ametryn	legacy herbicide
Androsterone	hormone, androgen
Anastrozole	pharmaceutical, anti-estrogen
Atrazine	legacy herbicide
Azinphos-methyl	insecticide
Azoxystrobin	fungicide
Bendiocarb	insecticide
Benalaxyl	fungicide
Bentazone	herbicide

Table SIII – continued.

Compound	Compound class/Usage pattern
Benzethonium	quat. ammonium surfactant/biocide
Benzophenone-4	UV filter
Benzyltrimethylammonium	quat. ammonium surfactant/biocide
Benzothiazole	industrial chemical
Benzyltrimethylhexadecylammonium	quat. ammonium surfactant/biocide
Bezafibrate	pharmaceutical
Bicalutamide	pharmaceutical/hormone
Bifenox	herbicide, WFD priority
Bisphenol A	plasticizer, xenoestrogen
Bisphenol A-(2,3-dihydroxypropylether)	plasticizer
Bisphenol F	plasticizer, xenoestrogen
Bisphenol S	industrial chemical
Boscalid	fungicide
Bromoxynil	herbicide
Butylparaben	preservative
Caffeine	stimulant in beverages, marker compound
Canrenone	steroid
Carbamazepine	pharmaceutical
Carbaryl	insecticide
Carbendazim	fungicide
Carfentrazone-ethyl	herbicide
Cetirizine	pharmaceutical
Chloramphenicol	legacy pharmaceutical
Chlorfenvinphos	insecticide, WFD priority
Chloridazon	herbicide
Chlorophene	biocide/preservative
Chlorotoluron	herbicide
Chloroxuron	herbicide
Chlorpropham	herbicide
Cholic acid	steroid TP
Citalopram	pharmaceutical
Clarithromycin	pharmaceutical
Clofibric acid	pharmaceutical TP
Clomazone	herbicide
Clopidogrel	pharmaceutical, anti-coagulant
Clotrimazole	pharmaceutical
Clozapine	pharmaceutical, anti-psychotic
Cortisone	hormone, glucocorticoid
Cotinine	nicotine TP
Crotamiton	pharmaceutical
Cyclophosphamide	pharmaceutical
Cyproterone	pharmaceutical, anti-androgen
Daidzein	natural food isoflavone

Table SIII – continued.

Compound	Compound class/Usage pattern
DEET	insect repellent
Denatonium	bittering compound
Desethylatrazine	herbicide TP
Desethylterbuthylazine	herbicide TP
Desisopropylatrazine	herbicide TP
Dexamethasone	pharmaceutical, glucocorticoid
Diazepam	pharmaceutical
Diazinon	insecticide
Dichlorvos	insecticide, WFD priority
Diclofenac	pharmaceutical, WFD watch list
Didecyldimethylammonium	quat. ammonium surfactant/biocide
Diflufenican	herbicide
Dihydrotestosterone	hormone, androgen
Dimethoate	insecticide
Diphenhydramine	pharmaceutical
Diphenyl phosphate	industrial chemical
Diuron	herbicide, WFD priority
Enalapril	pharmaceutical
Epi-Androsterone	hormone, androgen
Erythromycin	pharmaceutical
Estriol	hormone, estrogen
Estrone	hormone, estrogen
Ethion	insecticide
Ethofumesate	herbicide
Ethyl-azinphos	insecticide
Ethylparaben	preservative
Exemestane	pharmaceutical, androgen
Fenpropimorph	fungicide/biocide
Fenuron	herbicide
Finasteride	pharmaceutical
Fipronil	insecticide
Flumequine	pharmaceutical
Flurtamone	herbicide
Flusilazole	fungicide
Flutamide	pharmaceutical
Furosemide	pharmaceutical
Gemfibrozil	pharmaceutical
Genistein	nrtural food isoflavone
Gestoden	pharmaceutical, progesterone
Hexadecyltrimethylammonium	quat. ammonium surfactant/biocide
Hydrocortisone	hormone, glucocorticoid
Ibuprofen	pharmaceutical
Icaridin	insect repellent

Table SIII – continued.

Compound	Compound class/Usage pattern
Imazalil	biocide/preservative
Irgarol	biocide
Isoproturon	herbicide
Ketoconazole	pharmaceutical
Ketoprofen	pharmaceutical
Lauric isopropanolamide	surfactant
Lauryl diethanolamide	surfactant
Lenacil	herbicide
Levo-norgestrel	pharmaceutical, progesterone
Lidocaine	pharmaceutical
Lincomycin	pharmaceutical
Linuron	herbicide
Lorazepam	pharmaceutical
MCPA	herbicide
Mebendazole	nematicide
Mecoprop	herbicide
Medroxyprogesterone	pharmaceutical, progesterone
Mefenamic acid	pharmaceutical
Metamitron	herbicide
Metazachlor	herbicide
Metformin	pharmaceutical
Methiocarb	insecticide
Metolachlor	herbicide
Metolachlor ESA	herbicide TP
Metoprolol	pharmaceutical
Monensin	pharmaceutical
Monobenzyl-phthalate	plasticizer TP
Monoethyl-phthalate	plasticizer TP
N,N-Dimethyldodecylamine-N-oxide	surfactant
N-Acetyl-4-aminoantipyrine	pharmaceutical TP
N-Formyl-4-aminoantipyrine	pharmaceutical TP
Norethindrone	steroid
Norgestimate	steroid
Naproxen	pharmaceutical
Pantoprazole	pharmaceutical
Paroxetine	pharmaceutical
p-Chlorocresol	biocide
Pendimethalin	herbicide
Penicillin G	pharmaceutical
Pentobarbital	pharmaceutical
Perfluoroheptanoic acid	perfluorinated compound
Perfluorohexanoic acid	perfluorinated compound
Perfluorooctanoic acid	perfluorinated compound

Table SIII – continued.

Compound	Compound class/Usage pattern
Pethoxamid	herbicide
Phenazone	pharmaceutical
Picolinafen	herbicide
Pindolol	pharmaceutical
Pirimicarb	insecticide
Pirimiphos-methyl	insecticide
p-Nitrophenol	industrial chemical/TP
Pravastatin	pharmaceutical
Prednisolone	glucocorticoid
Prednisone	pharmaceutical, glucocorticoid
Primidone	pharmaceutical
Prochloraz	herbicide
Progesterone	steroid
Prometryn	legacy herbicide
Propiconazole	fungicide
Propoxycarbazone	herbicide
Propranolol	pharmaceutical
Propylparaben	preservative
Propyphenazone	pharmaceutical
Prosulfocarb	herbicide
Prothioconazole-desthio	fungicide TP
Pyrantel	nematicide
Pyrazophos	insecticide
Quinoxifen	fungicide, WFD priority
Raloxifene	pharmaceutical, anti-/estrogen
Roxithromycin	pharmaceutical
Simazine	herbicide, WFD priority
Simetryn	legacy herbicide
Spiroxamine	fungicide
Sulfamethazine	pharmaceutical
Sulfamethoxazole	pharmaceutical
Sulfapyridine	pharmaceutical
Tamoxifen	pharmaceutical, anti-estrogen
Tebuconazole	fungicide
Terbinafine	pharmaceutical
Terbuthylazine	herbicide
Terbuthylazine-2-hydroxy	herbicide TP
Testosterone	steroid
Tetrabromobisphenol A	flame retardant
Tetrachlorosalicylanilide	biocide
Thiabendazole	biocide/preservative
Thiacloprid	insecticide

Table SIII – continued.

Compound	Compound class/Usage pattern
Tramadol	pharmaceutical
Trenbolone	steroid
Tri(butoxyethyl)phosphate	plasticizer
Triamcinolone	pharmaceutical, corticosteroid
Triclocarban	bactericide/personal-care product
Triclosan	biocide/preservative
Triethyl citrate	plasticizer
Trifloxystrobin	fungicide
Tri-isobutylphosphate	flame retardant
Trimethoprim	pharmaceutical
Trimethyloctylammonium	surfactant
Triphenyl phosphate	plasticizer, flame retardant
Tris(1,3-dichloro-2-propyl)phosphate	flame retardant/plasticizer
Tris(2-chloroethyl)phosphate	flame retardant, plasticizer
Verapamil	pharmaceutical
Warfarin	pharmaceutical

TP- transformation product; WFD- Water Framework Directive.

Table SIV List of compounds identified in selected WWTP Velika Gorica and Oborovo fractions.

Compound	Abbreviation	Retention time (min)	Elemental composition	Theoretical mass (m/z)	Experimental mass (m/z)	Error	
						mDa	ppm
<i>PI mode</i>							
Polyethylene glycols							
	PEG						
	n=9	3.05	[M + NH ₄] ⁺ C ₁₈ H ₄₂ NO ₁₀	432.2809	432.2796	-0.1	-3.0
	n=10	3.24	[M + NH ₄] ⁺ C ₂₀ H ₄₆ NO ₁₁	476.3071	476.3064	-0.1	-1.5
	n=11	3.40	[M + NH ₄] ⁺ C ₂₂ H ₅₀ NO ₁₂	520.3333	520.3333	0	0
	n=12	3.55	[M + NH ₄] ⁺ C ₂₄ H ₅₄ NO ₁₃	564.3595	564.3593	0.07	-0.4
	n=13	3.68	[M + NH ₄] ⁺ C ₂₆ H ₅₈ NO ₁₄	608.3857	608.3851	-0.1	-1.0
	n=14	3.80	[M + NH ₄] ⁺ C ₂₈ H ₆₂ NO ₁₅	652.4119	652.4114	0.05	-0.8
	n=15	3.92	[M + NH ₄] ⁺ C ₃₀ H ₆₆ NO ₁₆	696.4382	696.4375	-0.1	-1.0
Polyethylene glycol dimethyl ethers							
	PEGDME						
	n=15	3.92	[M + NH ₄] ⁺ C ₃₂ H ₇₀ NO ₁₆	724.4695	724.4706	0.1	1.5
	n=16	4.02	[M + NH ₄] ⁺ C ₃₄ H ₇₄ NO ₁₇	768.4957	768.4965	0.1	1.0
	n=28	4.89	[M + 2NH ₄] ⁺ C ₅₈ H ₁₂₅ N ₂ O ₂₉	657.9262	657.3976	-52.9	-803.4
	n=29	4.94	[M + 2NH ₄] ⁺ C ₆₀ H ₁₂₉ N ₂ O ₃₀	679.9393	679.4164	-52.3	-769.0
	n=30	4.99	[M + 2NH ₄] ⁺ C ₆₂ H ₁₃₃ N ₂ O ₃₁	701.9524	701.4289	-52.4	-745.8
	n=31	5.03	[M + 2NH ₄] ⁺ C ₆₄ H ₁₃₇ N ₂ O ₃₂	723.9655	723.4356	-53.0	-731.9
Alcohol polyethoxylates							
	AEO						
	C ₆ , n=6	5.09	[M + H] ⁺ C ₁₈ H ₃₉ O ₇	367.2695	367.2692	0.03	-0.8
	C ₆ , n=7	5.25	[M + H] ⁺ C ₂₀ H ₄₃ O ₈	411.2958	411.2954	0.04	-1.0
	C ₆ , n=8	5.27	[M + H] ⁺ C ₂₂ H ₄₇ O ₉	455.3220	455.3227	0.1	1.5
	C ₈ , n=8	6.42	[M + NH ₄] ⁺ C ₂₄ H ₅₄ NO ₉	500.3798	500.3792	-0.1	-1.2
	C ₈ , n=9	6.49	[M + NH ₄] ⁺ C ₂₆ H ₅₈ NO ₁₀	544.4061	544.4047	-0.1	-2.6
	C ₁₀ , n=6	9.25	[M + H] ⁺ C ₂₂ H ₄₇ O ₇	423.3322	423.3303	-0.2	-4.5
	C ₁₀ , n=7	9.23	[M + H] ⁺ C ₂₄ H ₅₁ O ₈	467.3584	467.3535	-0.5	-10.5
	C ₁₀ , n=8	9.23	[M + H] ⁺ C ₂₆ H ₅₅ O ₉	511.3846	511.3836	-0.1	-2.0
	C ₁₀ , n=9	9.20	[M + H] ⁺ C ₂₈ H ₅₉ O ₁₀	555.4108	555.4102	-0.1	-1.1

Table SIV - continued.

Compound	Abbreviation	Retention time (min)	Elemental composition	Theoretical mass (m/z)	Experimental mass (m/z)	Error	
						mDa	ppm
	C ₁₀ , n=10	9.18	[M + H] ⁺ C ₃₀ H ₆₃ O ₁₁	599.4370	599.4362	-0.1	-1.3
	C ₁₀ , n=11	9.18	[M + H] ⁺ C ₃₂ H ₆₇ O ₁₂	643.4633	643.4634	0.01	0.2
	C ₁₀ , n=12	9.18	[M + H] ⁺ C ₃₄ H ₇₁ O ₁₃	687.4895	687.4930	0.4	5.1
	C ₁₀ , n=16	9.67	[M + H] ⁺ C ₄₂ H ₈₇ O ₁₇	863.5943	863.5953	0.1	1.2
	C ₁₀ , n=17	9.66	[M + H] ⁺ C ₄₄ H ₉₁ O ₁₈	907.6205	907.6216	0.1	1.2
	C ₁₀ , n=18	9.64	[M + H] ⁺ C ₄₆ H ₉₅ O ₁₉	951.6467	951.6494	0.3	2.8
	C ₁₂ , n=3	11.64	[M + H] ⁺ C ₁₈ H ₃₉ O ₄	319.2848	319.2840	-0.1	-2.5
	C ₁₂ , n=4	11.62	[M + H] ⁺ C ₂₀ H ₄₃ O ₅	363.3110	363.3095	-0.1	-4.1
	C ₁₂ , n=5	11.58	[M + H] ⁺ C ₂₂ H ₄₇ O ₆	407.3373	407.3360	-0.1	-3.2
	C ₁₂ , n=6	11.54	[M + H] ⁺ C ₂₄ H ₅₁ O ₇	451.3635	451.3622	-0.1	-2.9
	C ₁₂ , n=7	11.48	[M + H] ⁺ C ₂₆ H ₅₅ O ₈	495.3897	495.3889	-0.1	-1.6
	C ₁₂ , n=8	11.42	[M + H] ⁺ C ₂₈ H ₅₉ O ₉	539.4159	539.4150	-0.1	-1.7
	C ₁₂ , n=9	11.36	[M + H] ⁺ C ₃₀ H ₆₃ O ₁₀	583.4421	583.4413	-0.1	-1.4
	C ₁₂ , n=10	11.31	[M + H] ⁺ C ₃₂ H ₆₇ O ₁₁	627.4683	627.4672	-0.1	-1.8
	C ₁₂ , n=11	11.26	[M + H] ⁺ C ₃₄ H ₇₁ O ₁₂	671.4946	671.4937	-0.1	-1.3
	C ₁₂ , n=12	11.22	[M + H] ⁺ C ₃₆ H ₇₅ O ₁₃	715.5208	715.5249	0.4	5.7
Dicyclohexylamine	DCHA	3.74	[M + H] ⁺ C ₁₂ H ₂₄ N	182.1909	182.1900	-0.1	-4.9
Dibutyl phthalate	DBP	10.39	[M + H] ⁺ C ₁₆ H ₂₃ O ₄	279.1596	279.1584	-0.1	-4.3
<i>NI mode</i>							
Linear alkylbenzene sulfonates	C ₁₂ LAS	6.81-7.07	[M - H] ⁻ C ₁₈ H ₂₉ O ₃ S	325.1837	325.1812	-0.2	-7.7
Sulfophenyl carboxylic acid	SPC						
	C ₈	3.50	[M - H] ⁻ C ₁₄ H ₁₉ O ₅ S	299.0953	299.0916	-0.4	-12.4
	C ₉	3.87	[M - H] ⁻ C ₁₅ H ₂₁ O ₅ S	313.1110	313.1065	-0.5	-14.4
	C ₁₁	4.68	[M - H] ⁻ C ₁₇ H ₂₅ O ₅ S	341.1423	341.1414	-0.1	-2.6
	C ₁₂	5.12	[M - H] ⁻ C ₁₈ H ₂₇ O ₅ S	355.1579	355.1556	-0.2	-6.5

Table SIV – continued.

Compound	Abbreviation	Retention time (min)	Elemental composition	Theoretical mass (m/z)	Experimental mass (m/z)	Error	
						mDa	ppm
Alkylsulfate	AS						
	C ₁₂ AS	6.23	[M - H] ⁻ C ₁₂ H ₂₅ O ₄ S	265.1474	265.1458	-0.2	-6.0
Alkyether sulfates	C ₁₄ AS	7.12	[M - H] ⁻ C ₁₄ H ₂₉ O ₄ S	293.1787	293.1784	0.03	-1.0
	AES						
	C ₁₂ , n=1	6.71	[M - H] ⁻ C ₁₄ H ₂₉ O ₅ S	309.1736	309.1729	-0.1	-2.3
	C ₁₂ , n=2	6.87	[M - H] ⁻ C ₁₆ H ₃₃ O ₆ S	353.1998	353.1989	-0.1	-2.5
	C ₁₂ , n=3	7.02	[M - H] ⁻ C ₁₈ H ₃₇ O ₇ S	397.2260	397.2280	0.2	5.0
C ₁₂ , n=4	7.08	[M - H] ⁻ C ₂₀ H ₄₁ O ₈ S	441.2522	441.2526	0.04	0.9	
1,4-Bis(2-ethylhexyl) sulfosuccinate	DOSS	7.19	[M - H] ⁻ C ₂₀ H ₃₇ O ₇ S	421.2260	421.2218	-0.4	-10.0
12-hydroxydodecanoic acid	-	8.21	[M - H] ⁻ C ₁₂ H ₂₃ O ₃	215.1647	215.1657	0.1	4.6

^a PEG oligomers with n=5-15 were detected, however only the most abundant PEGs are listed in the Table; n - number of ethoxy units.

Anex I: Biological analyses

The effective concentrations of surface water samples were expressed in relative enrichment factors (REFs) as proposed by Escher et al.^{XXXIII} For active samples in the receptor-mediated assays, effect equivalents of standard agonists or antagonists were calculated. Prior to testing, the extracts of all sample fractions were reconstituted in a solvent and mixed with the test media to reach safe solvent concentration related to the bioassay used and final REFs from 1 to 100, meaning that the tested range covered the original river concentrations (REF=1) as well as concentrations up to 100-times higher (REF=100). In exceptional cases, REF 500 were used for the upper limit (e.g., in the AChE inhibition assay, where no effects were observed up to REF 100). *In vivo* thyroid activity assay with *Xenopus* was performed with whole raw water samples. Blank samples, solvent control and positive control with standard reference compounds were tested in parallel with the samples in each bioassay. The results of bioassays were evaluated using non-linear regression models to derive the effective concentrations (EC50 or EC20) in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, USA) or freely available RegTox Microsoft Excel™ Macro (http://www.normalesup.org/~vindimian/fr_index.html).

ER-mediated activity (MELN cells) assay

The estrogen receptor mediated bioassay based on human breast cancer cell line (MELN cells transfected with a promoter containing estrogen responsive elements driving expression of luciferase^{XXXIV} was used for assessment of estrogenicity and antiestrogenicity of the sample. The receptor mediated activity of a sample was assessed in 96-well plates by measurement of luminescence after 16 hours of exposure at 37°C. Effective concentrations and equivalents of estrogenic potency (using the reference estrogen estradiol, E2) and anti-estrogenic potency (with OH-tamoxifen as the reference) were determined as previously reported.^{XXXV}

AR-mediated activity (MDA-kb2 cells) assay

The androgen receptor mediated bioassay based on human breast cancer cell line (MDA-kb2 cells transfected with a promoter containing androgen responsive elements driving expression

of luciferase^{XXXVI} was used for assessment of androgenicity and antiandrogenicity of the sample. The receptor mediated activity of the sample was assessed in 96-well plates by measurement of luminescence after 16 hours of exposure at 37°C. Potencies were expressed as equivalents (EQ) of standard reference compounds, i.e., DHT-EQ for androgenicity and flutamide-EQ for anti-androgenicity as described previously.^{XXXVII} The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, based on the color change of tetrazolium dye induced by oxidoreductase enzymes of viable cells as previously described by Creusot et al.^{XXXVIII} was used to assess cytotoxicity of the samples in the cell-based bioassays (MELN and MDA-kb2 cells).

GR- CALUX® assay

GR-CALUX is a patented glucocorticoid receptor mediated bioassay based on human osteoblastic cells (U2-OS) transfected with a promoter containing glucocorticoid responsive elements driving expression of luciferase. Glucocorticoid activity of the samples was assessed in 96-well plates by measurement of luminescence after 24 hours of exposure at 37°C. Potencies were expressed as equivalents (EQ) of standard reference compound dexamethasone Dex-EQ as previously described in Macikova et al.^{XXXIX}

Zebrafish embryo acute toxicity assay (FET)

Fish embryo acute toxicity was assessed in zebrafish (*Danio rerio*) according to the OECD guideline 236.^{XL} Freshly fertilized zebrafish eggs were exposed to six sample concentrations in 24-well plates for 96 hours, at 26°C and 14 h light: 10 h dark cycle. Four apical endpoints were observed daily as indicators of lethality: embryo coagulation, lack of somite formation, non-detachment of the tail and absence of heartbeat. Additionally, the occurrence of sublethal morphological effects was also recorded. Effects are described either as the occurrence of lethality, or as the cumulative occurrence of any endpoint for lethality or sublethal toxicity (i.e., any lethal or sublethal effect). In each test, ten embryos were exposed per exposure concentration. 3,4-dichloroaniline was used as the standard reference compound.

Algal growth inhibition assay

Growth of a population of unicellular green alga (*Raphidocelis subcapitata*) was assessed after 72 h of exposure to the sample by measuring absorbance at 680 nm. The assay was performed in transparent 96-well plates, at 24°C and under permanent illumination (1600 lux). Potassium dichromate was used as the standard reference compound. The method was based on OECD guideline 201^{XLI} and modified according to Rojíčková et al.^{XLII}

In vivo thyroid activity assay

Tadpoles of a stable line of transgenic *Xenopus* (*Xenopus laevis*), harboring the TH/bZIP-eGFP genetic construct (green fluorescent protein coding sequence under the control of thyroid hormone responsive region of the TH/bZIP-eGFP promoter) were exposed to the sample for 72 h to assess thyroid axis activity by fluorescence microscopy.^{XLIII} The assay was performed with unfiltered whole water samples.

Acetylcholine esterase (AChE) inhibition assay

Changes in the activity of the AChE were determined after 1 hour of exposure at room temperature using the color change of dithiobisnitrobenzoate to 5-thio-2-nitrobenzoic acid, which is a direct measure of hydrolysis catalyzed by AChE.^{XLIV,XLV} The assay was performed in 96-well plates, using an automated liquid handling system. Dichlorvos was used as a standard reference compound.

AMES fluctuation assay

Mutagenic potential of a sample was assessed using histidine-deficient mutants of bacteria *Salmonella typhimurium* of two strains (TA98 with frameshift mutation and TA100 with base pair substitution). Growth of bacteria in histidine-free medium, which is a marker of back mutation induced by mutagenic agents in the sample, was quantified using bromocresol purple as an indicator of acidification by growing bacteria. Addition of S9 fraction (rat liver microsomal activation system) was applied to detect promutagenic agents. The 100-minute-

exposure was carried out in 24-well plates, at 37°C and six concentrations were tested to derive the effective concentrations. Nitrofurantoin, 4-nitro-o-phenylenediamine and 2-aminoantracene were used as standard reference compounds. The method was based on ISO 11350:2012.^{XLVI}

AlgaeTox test

The chronic toxicity of biological samples was evaluated using ISO validated freshwater algal growth inhibition test with the unicellular green alga *Scenedesmus subspicatus* Chodat. (alternative name: *Desmodesmus subspicatus*), 89.81 SAG (Sammlung von Algenkulturen/Culture Collection of Algae).^{XLVII} Samples were tested for their potential to inhibit the growth rate of *Scenedesmus subspicatus*.

Alga was harvested from the liquid stock culture and 100 µL of 2×10^4 cells/well was inoculated in 96-well microplates. Serial dilutions of test samples (1:100–1:100000) or HPLC fractions (1:100–1:5000) were prepared in algal growth medium and 100 µL/well of each dilution was added in duplicate making the total reaction volume of 200 µL. Deionized water was used as a negative control and potassium dichromate ($K_2Cr_2O_7$) was used as a positive control. Cell density of algal inoculum was determined at zero time point by measuring fluorescence of chlorophyll a pigment ($\lambda_{ex}/\lambda_{em}=440/680$ nm) by a microplate reader. Test algal samples were incubated for 96 ± 2 h after which the cell density was measured again. Inhibition was measured as decrease in growth rate of the alga exposed to biological extracts relative to control cultures grown under identical conditions. Dose - response curve of $K_2Cr_2O_7$ as a reference standard was included in all experiments and the data analyses were done in GraphPad Prism 5.0 software.

MTT test

The acute toxicity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay for determining cell viability adapted according to Mosmann's procedure.^{XLVIII} Potential acute toxicity of water samples was assessed by measuring the inhibition of PLHC-1/wt cell viability.

PLHC-1/wt cell culture and experimental procedure: Fish hepatoma cell line derived from topminnow (*Poeciliopsis lucida*) was used for *in vitro* acute toxicity assessment. The PLHC- 1/wt (wild type) cells were grown in a cell incubator at 28°C in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 HAM (DMEM F-12), pH=7.4 medium containing L-glutamine, 15 mM HEPES and 5% fetal bovine serum (FBS).

Cells were seeded in 96-well plates with 25×10^4 cells/mL cultivation density and 200 μ L cells/well seeding volume. Following the 24 h incubation, the cells were exposed to serial dilutions of samples (1:100–1:100000) or HPLC fractions (1:100–1:5000) that were made in DMEM F-12 medium without FBS. DMEM F-12 was used as a negative control and Cyclosporin A (CYC) was used as a positive control.

PLHC-1/wt cells were exposed 72 h to sample concentrations after which the medium was removed from the wells and cells were washed with phosphate-buffered saline (PBS). Five mg/mL stock solution of MTT in PBS was made, then diluted in DMEM F-12 medium and 100 μ L was added to the wells. Incubation period was 3 h, at which point MTT was removed from the wells and isopropanol was added to the cells to dissolve formed formazan salts. Plates were shaken and then absorbance was measured using microplate reader at 570 nm with reference filter set to 750 nm. Viability was expressed as the percentage of the product formazan formed by cellular mitochondrial activity relative to that of the control (untreated) cells.

Phase I - EROD biotest

This bioassay was conducted using the PLHC-1/wt cell line described above. The assay was performed according to the protocol previously described by Hahn et al.^{XLIX} Potential mechanism of toxicity of water samples was determined by measuring induction of CYP1A1 monooxygenase activity indicative for the phase I of the cellular detoxification mechanism.

PLHC-1/wt cells were seeded into 96-well plates 24 h before performing the experiment. Cells were exposed to serial dilutions of samples (1:100–1:100000) or HPLC fractions (1:100–1:5000) that were prepared in DMEM F-12 growth medium (pH 7.4) without FBS. Exposure of cells to potential CYP1A1 inducers lasted for 24 h at 28°C after which EROD measurement proceeded. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was used as a positive control and DMEM F-12 medium was used as a negative control. Phosphate buffer

(Na₂HPO₄, 50 mM) was used to set pH to 8. The medium was removed from the plate and the cells were washed once with 100 µL of PBS. Six mM stock of 7-ethoxyresorufin was diluted 3000 times in phosphate buffer to gain the final concentration of 2 µM. The reaction was initiated by adding 100 µL/well of the substrate, after which the plate was immediately put into the microplate reader. Conversion of 7-ethoxyresorufin to resorufin was monitored fluorometrically at specific wavelengths (535/590 nm) for 10 kinetic cycles with 1 min time interval. After 10 min of kinetic measurement plates were kept for further protein measurement and frozen at -20°C. Using the reactive compound fluorescamine, protein content was measured for the normalization of the EROD activity.^L After the last measurement, plates were frozen at 20°C for protein determination. Prior to protein quantification, 10 mg/mL of bovine serum albumin (BSA) solution was made and serial dilutions were prepared in phosphate buffer (50 mM, pH 8) to obtain the protein standard curve. Fluorescamine stock was prepared in acetone (1.5 mg/mL) and further diluted 10 times in ACN to gain the final concentration of 0.15 mg/mL. Hundred µL/well of fluorescamine was added to the plate and incubated for 3-5 min. Plates were shaken for 5 sec and fluorescence intensity was measured at specific wavelengths (390/465nm).

ChgH-gfp medaka assay

All experiments were carried out with fry from generation F6 or greater of the WatchFrog chgh-gfp line. This line harbours a genetic construct consisting of 2047 bp of the medaka chgh gene immediately upstream from the translation start site driving expression of gfp, as described by Spirhanzlova et.al.^{L1} The line has a CarBio genetic background and displays a non-inducible basal fluorescence in cardiac muscle fibres and in certain cells around the mouth and eyes, allowing transgenic fry to be selected prior to exposure to potential endocrine disrupting compounds. Extracts were made up in DMSO and added directly to medaka medium in 6-well plates. Final DMSO concentration was 0.4% in all wells. Eight newly hatched (day post hatch zero; dph 0) medaka fry were added to each well. Three replicate experiments were read after 24 h.

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§ 8. CURRICULUM VITAE

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 Faculty of Technology

Additional training:

- 13th July-1st of August 2015, advanced training at The Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany
- 4th of December 2014, Local Course LC3: Monitoring and Assessment in the context of the Water Framework Directive, and Science-based policy support with regard to emerging pollutants, Istituto Superiore di Sanità (ISS), Rome, Italy
- 2-3rd of July 2014, Advanced course on preparative and analytical chromatography in effect-directed analysis, Institute Rudjer Boskovic, Zagreb, Croatia
- 18 March 2014, Specialised course on Biotechnology for environmental issues, Watchfrog, Evry, France
- 21-22nd of January 2014, Special course in Hyphenation of cell-based assays with

- microfractionation procedures, VU-IVM in Amsterdam, Netherlands
- 20th of January 2014, Special course on the water cycle and human health, KWR, Nieuwegein, Netherlands
 - 2-3rd of July 2013 Short Course, SC5: Fate of emerging pollutants in the aquatic water cycle, Eawag, Switzerland
 - 22-24th of May 2013 Training workshop, SC 3: A specialized training course on chemical screening, prioritization of environmental pollutants and data storage in European databases, organized by the Environmental Institute in Slovakia, Jasna, Slovak Republic
 - 22-24th of April 2013 Valorization Grant Workshop; Project title „Establishing and developing of an ecotoxicology platform in Serbia and Croatia: A focus on zebrafish (*Danio rerio*)“, Faculty of Biology, University of Novi Sad, Serbia.
 - 30-31th of January 2013 Training Workshop SC2: Bioassays for EDCs, A training workshop with a specialized course focusing on in vitro and in vivo bioassays, INERIS, Verneuil-en-Halatte, France
 - 23-25th of October 2012 Training workshop: SC6 and LC1: A training workshop with a specialized course focusing on advanced LC-MS methods and a local course on multivariate statistics, The Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany
 - 25-29th of June 2012 Summer School, Helmholtz Center UFZ, Leipzig, Germany
 - 7-11th of May 2012 - Fifth CEFSER training course "Analysis of chemical contaminants in food and the environment", Faculty of Technology, Novi Sad
 - 14-15th of November 2011 - Fourth CEFSER training course "Persistent organic pollutants in food and environment: Risk assessment", Faculty of Technology, Novi Sad
 - 8-10th September 2011 - IPA HU-SRB BIOXEN Seminar “Novel approaches for environmental protection”, Faculty of Technology, Novi Sad
 - 8-10th of September 2011 - Second CEFSER workshop "Persistent organic pollutants in food and environment", Faculty of Technology, Novi Sad
 - 1-3rd of June 2011 - IPA HU-SRB BIOXEN training course “High resolution mass spectrometry of xenobiotics”, Faculty of Technology, Novi Sad
 - 29-11.-11.12.2010.- International school: "Monitoring, fate and toxicity of toxic compounds in the terrestrial environment", Nova Gorica, Slovenia

- 7-8th of October 2010 - Course "Liquid Chromatography and Mass Spectrometry", Faculty of Technology and metallurgy, Belgrade
- 16-17th September 2010 - Third CEFSER training course "High resolution mass spectrometry in quantitative analysis and screening of organic contaminants in food and environment", Faculty of Technology, Novi Sad
- 14-15th September 2010 - First CEFSER workshop "Regional perspectives in food safety", Faculty of Technology, Novi Sad
- 9th April 2010 - Second CEFSER training course "Quality Assurance (QA) and Quality Control (QC) Procedures in Analysis of Contaminants and Pharmaceutical Compounds in Food and the Environment", Faculty of Technology, Novi Sad
- 6-8th April 2010 - First CEFSER training course "Capabilities of UPLC-MS/MS in Analysis of Contaminants and Pharmaceutical Compounds in Food and the Environment", Faculty of Technology, Novi Sad
- 30th of November 2009 - CEFSER Symposium "Communicating science and risks", Faculty of Technology, Novi Sad

Awards:

Second award for the poster presentation „The occurrence of Fusarium mycotoxins in Serbian wheat“, 12th Danube-Kris-Mures-Tisa (DKMT) Euroregion Conference on Food, Environment and Health, September 2010, Faculty of Technology, Novi Sad.

Scientific Publications:

1. T. Schulze, M. Ahel, J. Ahlheim, S. Aït-Aïssa, F. Brion, C. Di Paolo, J. Froment, A.O. Hidasi, J. Hollender, H. Hollert, M. Hu, A. Kloß, S. Koprivica, M. Krauss, M. Muz, P. Oswald, M. Petre, J.E. Schollée, T.B. Seiler, Y. Shao, J. Slobodnik, M. Sonavane, M.J. Suter, K.E. Tollefsen, Z. Tousova, K.H. Walz and W. Brack. Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis, *Sci. Total Environ.* **581-582** (2017) 350-358.

2. Z. Tousova, P. Oswald, J. Slobodnik, L. Blaha, M. Muz, M. Hu, W. Brack, M. Krauss, C. Di Paolo, Z. Tarcai, T.-B. Seiler, H. Hollert, S. Koprivica, M. Ahel, J. E. Schollée, J. Hollender, M. J.-F. Suter, A. O. Hidasi, K. Schirmer, M. Sonavane, S. Ait-Aissa, N. Creusot, F. Brion, J. Froment, A. C. Almeida, K. Thomas, Knut Erik Tollefsen, Sara Tufi, Xiyu Ouyang, P. Leonards, M. Lamoree, V. Osorio Torrens, A. Kolkman, M. Schriks, P. Spirhanzlova, A. Tindall and T. Schulze. European demonstration program on the effect-based and chemical identification and monitoring of organic pollutants in European surface waters, *Sci. Total Environ.* **601-602** (2017) 1849-1868.
3. W. Brack, S. Govender, T. Schulze, M. Krauss, M. Hu, M. Muz, J. Hollender, K. Schirmer, J. Schollee, A. Hidasi, J. Slobodnik, Z. Rabova, S. Ait-Aissa, M. Sonavane, M. Carere, M. Lamoree, P. Leonards, S. Tufi, X. Ouyang, M. Schriks, K. Thomas, A. C. de Almeida, J. Froment, M. Hammers-Wirtz, M. Ahel, S. Koprivica, H. Hollert, T.-B. Seiler, C. Di Paolo, A. Tindall and P. Spirhanzlova. EDA-EMERGE: an FP7 initial training network to equip the next generation of young scientists with the skills to address the complexity of environmental contamination with emerging pollutants, *Environ. Sci. Europe.* **25** (2013) 1-7.
4. B. Škrbić, S. Koprivica and M. Godula. Validation of a method for determination of mycotoxins subjected to the EU regulations in spices: The UHPLC–HESI–MS/MS analysis of the crude extracts, *Food Control.* **31** (2013) 461-466.