

COMMENTARY

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REP-LECOTOX: an example of FP 6 INCO project to strengthen ecotoxicological research in WBC (Western Balkan countries)

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Abstract

REP-LECOTOX project - INCO-CT-2006-043559-REP-LECOTOX UNSPMF - Reinforcement of research potential of Laboratory for Ecotoxicology (LECOTOX) of University of Novi Sad Faculty of Sciences funded by EC FP6 (2007-2009) presented a crucial milestone for LECOTOX, both in terms of overall research performance and its visibility and presentation to international scientific community. The project enabled upgrading and renewal of S&T equipment, hiring new young researchers to reinforce human resources and it facilitated extensive networking - via workshops, exchange of scientific personnel and trainings of young scientists in some of the finest research institutions in the EU in the field of environmental research: Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany; School of Bioscience, University of Birmingham, UK and RECETOX, Masaryk University, Brno, Czech Republic. The project enabled LECOTOX, focusing mainly on two research topics, i.e. endocrine disruption/reproductive toxicity, and identification and characterisation of aquatic toxicity, to form a functional competitive research team ready for combining transcriptomics with the established conventional toxicity tests and traditional function-based biomarkers. LECOTOX became well-equipped and experienced research group confident enough to join any FP or otherwise funded ecotoxicological research project and network.

About LECOTOX - laboratory for ecotoxicology at UNSPMF

LECOTOX - Laboratory for Ecotoxicology at the Department of Biology and Ecology at University of Novi Sad Faculty of Sciences was formally established in 2006 as the result of intention to overcome the former fragmentation of the groups focusing on (eco) toxicological research since the mid 1990s. Relying on years of high-quality research in the field of animal physiology, specifically in reproductive endocrinology e.g. [1-9], the activities of LECOTOX researchers were (and still are) focused on toxic impact of persistent organic pollutants (POPs) on biota with special emphasis on specific sublethal types of toxicity - endocrine disruption, reproductive toxicity, and "dioxin-like" toxicity [10-16]. Bioanalyses based on molecular and cellular biomarkers of

effects, such as cytochrome P450 (CYP) enzyme induction and parameters and markers of oxidative stress and early intoxication have been applied in assessment of freshwater ecosystems e.g. [17]. In close co-operation with the Department of Chemistry, Biochemistry and Environmental Protection, the Laboratory of Ecotoxicology applies aquatic toxicity tests for effluent and ambient water quality assessment and environmental impact assessment for remediation activities on contaminated sediments and soil e.g. [18-20]. A big step forward related to ecotoxicological research was reflected in participation in the APOPSBAL project (Assessment of the selected POPs (PCBs, PCDD/Fs, POCPs) in the Atmosphere and Water Ecosystems from Waste Materials Generated by Warfare in the Area of Former Yugoslavia, FP5-ICA2-CT-2002-10007, 2002 - 2005). The combination of chemical and biological tools (micro-EROD analysis in the first place) was used to estimate the toxicity of sediment samples from three rivers located at Kragujevac hotspot (Serbia) [21], soil and sediment samples

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from the area of Zadar, Croatia [22], and soil from oil refineries and petrochemical plant in Vojvodina (Serbia) after the NATO campaign spills [23,24].

REP-LECOTOX project - reinforcement of research potential of LECOTOX

FP 6 INCO Programme was an ideal opportunity for the established but suboptimally equipped research groups from new, candidate and non-EU member countries to fully integrate into the international scientific community. The overall aim of the Capacities Programme is to enhance research and innovation throughout Europe by optimising research infrastructure, enhancing research potential of European convergence and outermost regions, and building strategic R&D partnerships with non-EU countries. A LECOTOX team, led by the head of laboratory, Prof. Dr. Radmila Kovačević, recognised that unique chance and prepared a project proposal for FP6 INCO-2005-C-WBC SSA call for reinforcement of the WBC research capacities launched in 2006. REP-LECOTOX is an acronym for the project INCO-2005-C-WBC 043559 - REP-LECOTOX "Reinforcement of Research Potential of the Laboratory for Ecotoxicology". Having recognised the great potential of "omic" methods in ecotoxicological research and risk assessment, LECOTOX team made an initial step towards application of genomics-based tools in ecotoxicology, aiming to combine transcriptomics with established conventional toxicity tests and traditional function-based biomarkers.

The overall aim of the project was to strengthen research capacities and potential of the Laboratory for Ecotoxicology at the University of Novi Sad Faculty of Sciences, Serbia and to facilitate introduction and application of genomics-based tools as a prerequisite for the laboratory's long-term strategic orientation towards one of the most promising fields of environmental research today - ecotoxicogenomics.

The major REP-LECOTOX objectives were to: (a) upgrade and renew equipment, (b) reinforce human potential and (c) expand international collaboration and networking. Four outstanding EU institutions have supported the project by offering their expertise and high-quality trainings for LECOTOX young researchers: (1) Centre for Environmental Research - UFZ, Leipzig, Germany; (2) RECETOX, Masaryk University, Brno, Czech Republic; (3) School of Biosciences, The University of Birmingham, Edgbaston, Birmingham, UK and (4) Department of Animal Physiological Ecology, Zoological Institute, University of Tübingen, Germany. The International Scientific Advisory Board (ISAB) consisted of the representatives of partnering institutions: Dr. Werner Brack (UFZ), Prof. Dr. James Kevin Chipman (The University of Birmingham), Prof. Dr. Ivan Holoubek (RECETOX) and Prof. Dr. Heinz-R. Koehler

(University of Tübingen). To facilitate efficient implementation of the project, the activities have been divided into five work packages: mobility and training, dissemination of information, organisation of the workshops, reinforcement of LECOTOX and Management.

Reinforcement of LECOTOX

The measurement of gene expression levels upon exposure to a chemical can be used both to provide mechanistic knowledge of the genetic basis of toxic effect and to form a sort of "genetic signature" for identification of toxic product. Real-time PCR allows accurate quantification of starting amounts of DNA, cDNA and RNA targets. This is in contrast with the end point detection in conventional PCR, which does not enable accurate quantification of nucleic acids. Therefore, the reinforcement of LECOTOX in terms of equipment included procurement of 7900HT FAST REAL-TIME PCR System consisting of standard hardware configuration (with a standard 96-well block) and computer with Sequence Detection Software, Primer Express Oligonucleotide Design Software and monitor. The upgrade of the system configuration consists of TaqMan low-density array, 384-well microfluidic cards designed for analysing gene expression patterns in many samples across a defined set of gene targets. For sample preparation, the system is accompanied by centrifuge supplied with rotor and adapter for fluidic cards. The laboratory was supplemented also with additional fast thermal cycler with a 96-well block module for parallel running of simple PCR when necessary (Figure 1).

Another important aspect of reinforcement was the selection of young research trainees to be hired for REP-LECOTOX project. Apart from three research trainees hired according to the plan, another three part-time research trainees - PhD students who had been granted the full scholarship by the Ministry of Science and Technological Development of the Republic of Serbia - were co-funded by the REP-LECOTOX project and included into all ongoing activities during the course of the project. All three full-time and three part-time research trainees progressed as planned during project implementation.

Mobility and trainings

During the project implementation, eleven trainings for young researchers were organised in three partnering institutions. Eleven short-term visits of LECOTOX senior and junior scientist to partnering institutions and ten return visits (researchers from partnering EU institutions to LECOTOX) were organised during REP-LECOTOX workshops.

Two Departments of UFZ Leipzig - Department of Effect-Directed Analysis (EDA) lead by Dr. Werner



Figure 1 REP-LECOTOX research trainees.

Brack and Department of Bioanalytical Ecotoxicology (former Department of Cell Toxicology) lead by Dr. Stefan Scholz - provided trainings for REP-LECOTOX research trainees and hosted LECOTOX senior and junior scientists during the project implementation.

Dr. Brack, with his EDA research group, focuses on developing tools for precise detection of key environmental (mainly aquatic) toxic pollutants, as well as methods and models for ecological risk assessment and environmental monitoring based on the observed biological effects. Therefore, the Department of EDA hosted both biologists and chemists from the UNSPMF, as building up a competent, coherent inter and multidisciplinary team is a prerequisite for implementation of a complex, tiered EDA approach, which, as the currently best recognised method in risk assessment of complex environmental matrices was the ultimate goal for LECOTOX. Since LECOTOX was already incapacitated and experienced in conducting a great variety of toxicity tests and bioassays applied in EDA, the trainings in UFZ included hands-on tool in fractionation of sediment

samples by pressurised liquid extraction and accelerated membrane-assisted cleanup [25], followed by an automated multistep online fractionation procedure on three coupled normal phase HPLC columns [26], automated online fractionation procedure on return phase HPLC and qualitative analysis of semi-volatile pollutants by means of gas chromatography-mass spectrometry operating in scan a SIM mode, in conjunction with appropriate data analysis applications and chemometric tools.

Dr. Scholz, with his research group within the Department of Bioanalytical Ecotoxicology, focuses on developing alternative ecotoxicological methods which would eventually replace whole animal testing (in line with the current trends) and ecotoxicogenomics; the group already has a respectful record of application of genomic-based tools (mainly transcriptomics) in researches on mode of action of a wide range of xenobiotics. The research interest of Dr. Scholz and his group directly corroborates with the main objectives of the REP-LECOTOX project, namely, the trainings organised there directly helped the introduction of ecotoxicogenomics into

laboratory practice of LECOTOX and diversification of *in vitro* methods on widely recognised but also new promising biological models. The aim of the trainings was to get familiar with fish embryo toxicity test (*DarT*) and gene-DarT test - alternatives to acute toxicity tests on fish, process of dechoriation of *Danio rerio* embryos (used for testing toxic substances whose action is blocked by chorion), process of microinjection (used to input specific substances in unicellular stage embryos) and development of *Danio rerio* testis tissue culture - as such system could be used for testing endocrine disrupting substances.

The ecotoxicological division of RECETOX represents one of the research units well-known in Europe for a high diversity of *in vivo* and *in vitro* ecotoxicological tests, routinely performed in research and for monitoring purposes. As one of the goals of REP-LECOTOX project was also to build up research potential by introduction of several new *in vivo* ecotoxicological test methods, the purpose of the trainings hosted by two research groups - AQUATOX (supervised by Dr. Ludek Blaha and Dr. Klara Hilschlerova) and SOILTOX (supervised by Dr. Jakub Hofman) - was to get know-how in several aquatic and terrestrial acute and chronic toxicity tests using *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Caenorhabditis elegans*, *Lactuca sativa*, *Folsomia candida* and *Enchytraeus albidus*.

The School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK - the research institution with sophisticated equipment and competent specialists (lead by Prof. Chipman) in the field of transcriptomics, techniques widely used in ecotoxicology and toxicology for measuring effects of stressors and understanding mechanism of their toxicity - was a perfect place to gain theoretical and practical knowledge, applicable to the planned research activities of LECOTOX. The trainings focused on gene expression analysis by qRT-PCR of some key responding genes of *Daphnia magna* after treatment with benzo (a) pyrene and sodium dichromate. As the first step, gene expression was checked upon using microarray, which is the main tool for global gene expression profiling of the response of an organism in a particular condition. However, expression levels of key responding genes of the microarray need to be validated by real-time PCR. This study was a part of a bigger project aiming at the development of 'omic'-based methods using *Daphnia magna* as a tool for monitoring the effects of toxic agents in the environment.

The exchange and mobility at the level of permanent, senior, and junior staff of LECOTOX and partnering institutions was very well balanced. In total, 11 visits of LECOTOX scientists to partnering institutions and ten return visits were organised during implementation of the projects. LECOTOX members' visits to partner

institutions were mainly aimed at detailed planning of the young research trainees, REP-LECOTOX workshop preparations and discussions on further co-operation opportunities. LECOTOX scientists were able to visit the research laboratories, get familiar with the state-of-the-art equipment, laboratory organisation and see and adopt the principles of good research management. Those visits also facilitated further networking and creation of very tight personal contacts with hosts. On the other hand, the return short-term visits of the researchers from partnering institutions to LECOTOX were mainly organised during two REP-LECOTOX workshops: the experts from UFZ (Dr. Werner Brack, Dr. Stefan Scholz and Dr. Mikhail Beketov), RECETOX (Dr. Ivan Holoubek, Dr. Ludek Blaha, Dr. Klara Hilschlerova and Dr. Jakub Hofman) and School of Biosciences (Dr. James Kevin Chipman) participated as keynote speakers and invited lecturers at both events. Moreover, the scientists from partnering institutions, particularly ISAB members, used the visits to inspect the progress of the REP-LECOTOX project, to visit research facilities at LECOTOX as well as other research groups at UNSPMF, meet other researchers and management structure and open the gate for potential further collaboration beyond REP-LECOTOX project.

REP-LECOTOX workshops

The main objective of the first REP-LECOTOX workshop: "Ecotoxicogenomics: the challenge of integrating genomics/proteomics/metabolomics into aquatic and terrestrial ecotoxicology", Novi Sad, June 15 to 18, 2008 was to bring together experts in the field of genomics, transcriptomics, proteomics, metabolomics and bioinformatics as invited speakers to the workshop in order to get a comprehensive overview of the state-of-the-art ecotoxicogenomics and implementation of omic technologies in the field of ecotoxicology. Workshop participants had the opportunity to learn about trends in ecotoxicogenomics from five plenary and six topic-specific presentations of recognised experts in each specific field (presentation available on LECOTOX Web pages). The last two days of the workshop were devoted to laboratory demonstrations of the 7900HT Fast Real-Time PCR system (Applied Biosystems) and 2100 Bioanalyzer (Agilent). The comprehensive workshop report is published in ESPR [27].

The second REP-LECOTOX workshop: "Trends in Ecological Risk Assessment", Novi Sad, September 21 to 23, 2009 was aimed at presenting comprehensive information about the current state, challenges and trends in the field of ecological risk assessment, with special reference to application into environmental management practice and providing local and regional professionals, decision makers, and different stakeholders (industry,

regulatory agencies, academia) with a broad overview of the current EU regulations and future trends regarding risk assessment of chemicals and ecological risk assessment (with special reference to REACH and WFD). The workshop scientific programme was structured into two large sessions: (1) risk assessment of chemicals with special attention to REACH (divided according to topics into three sub-sessions, (a) legislative aspect; (b) emerging substances and nanomaterials: analyses, environmental fate, effects, ERA; and (c) incorporating omic information into risk assessment and policy) and (2) ecosystem risk assessment (divided into three sub-sessions: (a) aquatic ecosystems, ERA in WFD and GD; (b) special ERA topic - upscaling and (c) terrestrial ecosystems). Two keynote lecturers and even 18 invited speakers from well-known European research institutions were invited to present extremely diverse ERA topics, ranging from case studies to conceptual frameworks and opinion statements (presentations available at project's homepage). To enable further exchange of experiences and information about the research potential and capacities of local (Serbian) and regional research institutions and teams, seven workshop participants were invited to present the results of their current work in the form of short oral presentations while others were invited to present posters. A comprehensive report is accepted for publication in ESEU [28].

Dissemination of information

The most important milestone in dissemination of information about the LECOTOX and REP-LECOTOX project was launching of the Web portal <http://www.lectox.net>. Other important activities included publications in scientific [27-29], and popular [30-32], journals, participation in various scientific meetings and other events and press releases. LECOTOX members promoted REP-LECOTOX project, as well as the results of recent and ongoing research projects of the group at the important international scientific conferences (SETAC EUROPE 2008, 2010; FEBS 2008; PRIMO 2009; IUBMB/FAOBMB 2008; ICCE 2009; SIL 2007; IAD 2008, 2010), workshops (MODELKEY 2009, RISKBASE 2009, COST Action 636 Xenobiotics in Urban Water Cycle 2007-2008, EuLemnos 2009) and round tables (SedNet 2009). Promotional material (flyers, leaflets - available for download from the home page as well as the project compendium - see Additional file 1) and all the above-mentioned events were used for further networking and promotion of LECOTOX as a suitable competitive and competent partner for future research projects within the FP and beyond. REP-LECOTOX project and other activities of LECOTOX were presented to a wide national public via press releases in printed (three articles in regional newspapers) and

electronic media (interviews of senior LECOTOX members for the radio and TV, talk show at regional TV, footage from the openings of the REP-LECOTOX workshops, Web resources).

REP-LECOTOX project outcomes and follow-up

The fully operational 7900HT Fast Real-Time PCR system has already yielded with results although its overall scientific output is only to be expected through activities of LECOTOX in ongoing and future research projects. Knowledge, new methods and know-how gained via trainings in partner institutions were transferred to LECOTOX and incorporated into individual research activities of young researchers in the form of MSc and PhD theses (completed and those in progress) and peer reviewed publications in scientific journals. Equally important, modern EDA and sediment TRIAD approach, new equipment, techniques, expertise and newly introduced *in vivo* and *in vitro* toxicity tests have already been applied directly into MSc and PhD level education at the Department of Biology and Ecology. Intensive networking with partnering institutions and individual researchers facilitated, in the first place, highly professional organisation of two workshops of outstanding scientific performance, while the workshops themselves were used not only for getting acquainted with state-of-the-art techniques in ecotoxicogenomics and risk assessment, but for further networking and scientific exchange. Besides its scientific and educational goals, the upgraded and strengthened LECOTOX will have an important task in the future, as well as the capability and capacity to offer its expertise and advice to policy and decision makers as well as to various stakeholders. One of such examples certainly is a project "Chemicals Risk Management in Serbia", funded by the Republic of Serbia, Ministry of Environment and Spatial Planning and Swedish Chemical Agency. (REACH, Part C Ecotoxicological methods, consultant I. Teodorovic, LECOTOX). I. Teodorovic serves currently as a member of the Commission in National Authority for Plant Protection Products registration; Republic of Serbia, Ministry of Agriculture, Forestry and Water Management and a member of the Commission in National Authority for Biocidal Products registration, Republic of Serbia, Ministry of Environment and Spatial Planning, Chemical Agency.

The project itself, as specified by the INCO programme, was not intended to be research, but was strictly capacity building-oriented. Therefore, apart from immediate impact and direct project outcomes presented above, the successful implementation of the project represented a qualitative step forward in research and educational performance of LECOTOX. In January 2006, LECOTOX launched a 5-year project focusing on EDCs

(BFRs, PCBs, PAHs and pesticides) and their effects on reproductive and thyroid function, environmental assessment and bioanalyses, (project no. 143058, funded by the Ministry of Science and Technological Development of the Republic of Serbia). The results of the project, thanks to newly introduced omic tools (transcriptomics) provided better insight into mechanisms of adverse effects of atrazine (Figure 2) [33,34], and BFRs [35] on peripubertal rat Leydig cell steroidogenesis.

As a follow-up of high-quality trainings, in close cooperation with UFZ, Leipzig, EDA approach, combining highly sophisticated chemical tools with specific biomarkers of exposure (CYP enzyme induction and cell proliferation) was successfully implemented (Figure 3) at

Pančevo hotspot (Serbia) for identification of key sediment toxic pollutants [36,37].

The know-how gained via trainings and cooperation with RECETOX, Brno, resulted in successful application of newly introduced tests in laboratory scale research [38] and complex sediment TRIAD assessment at Pančevo hotspot (Serbia) [39]. The results of the study that was carried out during the trainings organised at University of Birmingham, focusing on gene expression in neonate and adult daphnids in relation to relative susceptibility to genotoxicants, are in the final stage of preparation for publication [40]. The expertise gained via trainings in UFZ, Leipzig enabled LECOTOX to get sufficient financial support from the Secretariat for Science and Technological

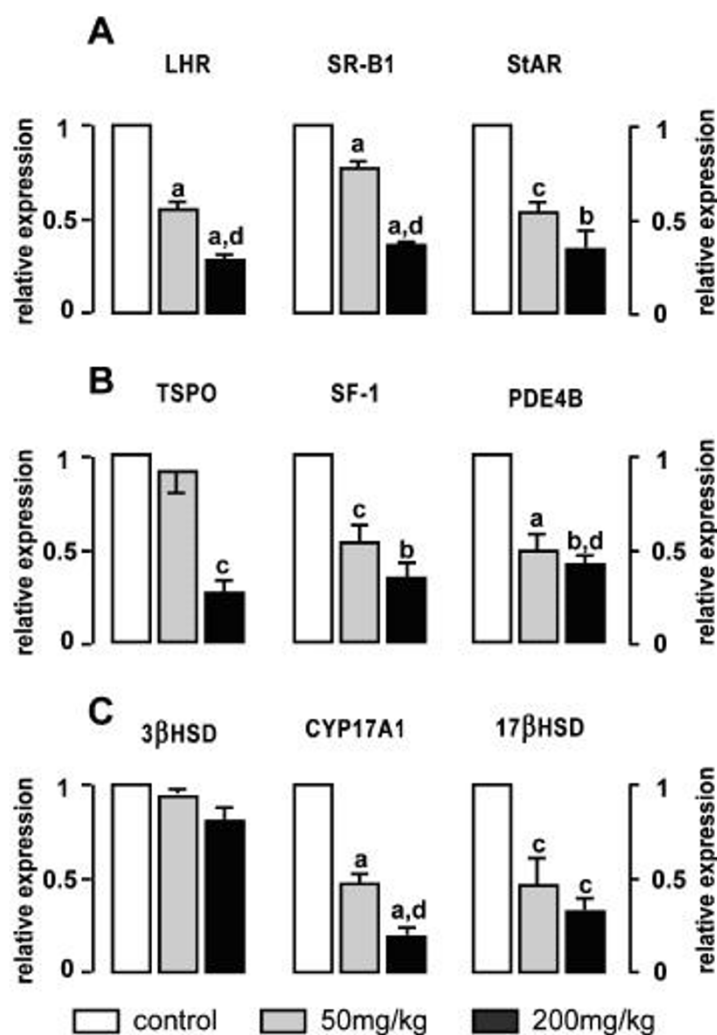


Figure 2 Effect of atrazine on gene expression. Effect on gene expression of luteinizing hormone receptor (LHR), scavenger receptor-B1 (SR-B1), steroidogenic acute regulatory protein (StAR), translocator protein (TSPO), steroidogenic factor-1 (SF-1), phosphodiesterase-4B (PDE4B), 3β-hydroxysteroiddehydrogenase (HSD), cytochrome P450 (CYP) 17A1, 17βHSD in Leydig cells of control and treated rats (50 and 200 mg/kg atrazine by gavage from postnatal day 23 to 50). The transcription of LHR gene in Leydig cells of atrazine-treated rats was downregulated in a dose-dependent manner, which could be the reason for reduction in cAMP level and expression of cAMP dependent genes. Reproduced with permission from [33].

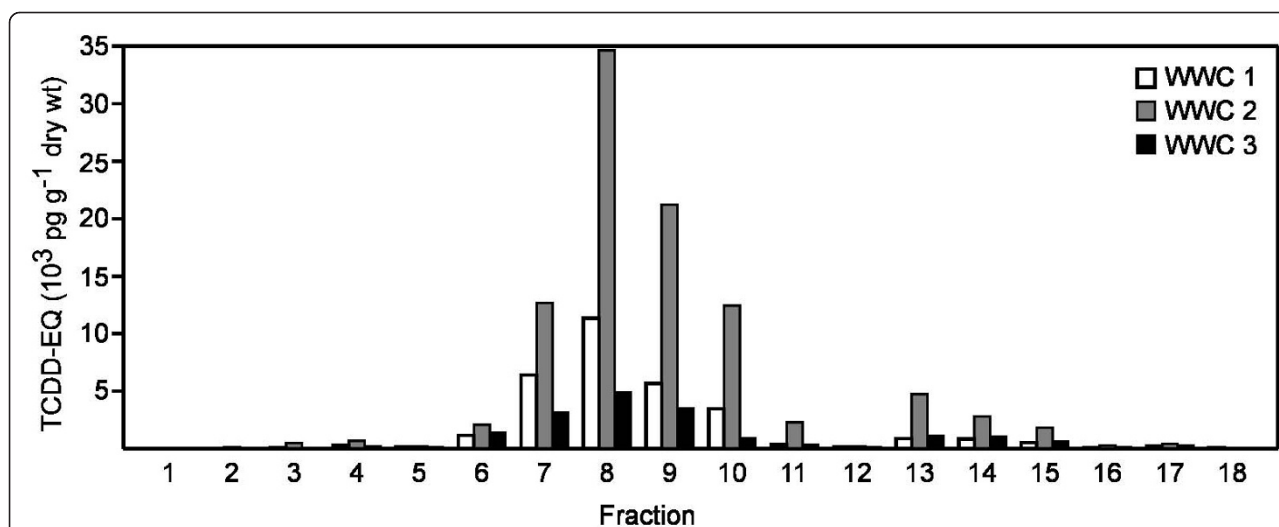


Figure 3 Bioanalytical results of EDA of contaminated sediment from the wastewater canal (WWC) in Pančevo industrial area, Serbia. The columns represent micro-EROD-derived TCDD equivalent concentrations (TCDD-EQ) of different fractions of the sediment from three different spots along the WWC (WWC1-WWC3). The 18 fractions were prepared by pressurised liquid extraction and accelerated membrane-assisted cleanup, followed by an automated multistep online fractionation procedure on three coupled normal phase HPLC columns. Each fraction contains different types of contaminants depending on their polarity, planarity and the of aromatic system, and the most active ones have been prioritised for further analysis aimed to identification and quantification of key pollutants [From [36], with permission].

Development of the autonomous province of Vojvodina to establish the laboratory facility for *Danio rerio* acute and chronic ecotoxicological tests, embryotoxicity DarT and, in the near future, to introduce Gene DarT test - the latest toxicity test on *D. rerio* based on gene expression of potential marker genes.

As a direct follow-up of increasing international networking and reinforcement facilitated by REP-LECOTOX, LECOTOX participates in the project "Establishing and developing of an ecotoxicology platform in Serbia and Croatia: a focus on zebra fish (*Danio rerio*)" with the University of Applied Sciences, School of Life Sciences, MuttENZ, Switzerland and Rudjer Bošković Institute Division for Marine and Environmental Research - Molecular Ecotoxicology, Zagreb, Croatia (2010-2012, funded by the Swiss National Scientific Foundation via SCOPES 2008-2012 programme). The project is directed to (a) the transfer of knowledge and expertise that will enable Serbian and Croatian partners to improve their overall research capacity, performing part of the research using zebra fish (*Danio rerio*) cell lines (PAC1 and ZFL) and (b) to better understanding of critical cellular, evolutionary conserved xenobiotic defence systems in aquatic organisms, using zebra fish as a model, specifically focused on the uptake of environmental chemicals and metabolites by specific transmembrane proteins, detoxification by phase I and II biotransformation enzyme systems; and finally, active elimination of xenobiotics and metabolites through specific transmembrane proteins (ABC transporters).

LECOTOX recently passed with two project proposals for the call launched by the Ministry of Science and Technological Development of the Republic of Serbia. The first one, submitted individually (Grant No. 173037), is intended to study reproductive, metabolic and developmental responses of selected model organisms and cell lines to some individual EDCs and their mixtures using different *in vivo* and *in vitro* biological models and elucidate further their mechanism of action applying newly established transcriptomics. The second proposal, submitted in co-operation with the Department of Chemistry, Biochemistry and Environmental Protection (Grant No. 172028), is focused on assessing the impact of chemical structure and different environmental matrices on bioavailability (and hence ecotoxicity) as well as efficiency of removal of selected xenobiotics and their mixtures in water treatment processes combining chemical, biological and *in silico* (mainly QSAR) tools. The implementation of the above mentioned projects will express the benefits of REP-LECOTOX project in a full scale. The latest proposal, which can be seen as the direct follow-up of REP-LECOTOX, is UNLOCKLECOTOX project proposal (Unlocking and developing the research potential of LECOTOX UNSPMF - Laboratory for Ecotoxicology, University of Novi Sad Faculty of Sciences, Serbia) has been submitted to FP 7-REGPOT-2011-1 call. If successful, the implementation of this proposal would prepare LECOTOX for some of the major challenges in the fields of ecotoxicology and chemical and ecological risk

assessment today: (a) application of integrated testing strategies by phasing out *in vivo* testing in line with 3R strategy (replace, reduce, refine), promoting omic and integrating chemical, *in vitro* and *in silico* methods, and (b) development of ecologically or ecosystem-relevant risk assessment approaches and methodologies.

Additional material

Additional file 1: REP LECOTOX project compendium. The project compendium contains comprehensive description of the FP 6 funded REP LECOTOX project and the profile of LECOTOX research team.

Authors' contributions

IT drafted the manuscript and participated in the design and coordination of the FP 6 funded project described herewith. RK designed and coordinated the FP 6 project described herewith and revised the manuscript. SK designed and performed EDA study. IP designed and performed TRIAD based study. KPM designed and performed the atrazine study. SF designed and performed the study with BFRs. VD participated in transcriptomic study with D. magna. JH and BG introduced D. rerio embryo test. All authors participated in the FP 6 funded project REP LECOTOX presented herewith, read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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