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Body burden of pesticides and wastewater-derived pollutants on freshwater invertebrates: Method development and application in the Danube River☆

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ABSTRACT

While environmental risk assessment is typically based on toxicant concentrations in water and/or sediment, awareness is increasing that internal concentrations or body burdens are the key to understand adverse effects in organisms. In order to link environmental micropollutants as causes of observed effects, there is an increasing demand for methods to analyse these chemicals in organisms. Here, a multi-target screening method based on pulverised liquid extraction (PuLE) and a modified QuEChERS approach with an additional hexane phase was developed. It is capable to extract and quantify organic micropollutants of diverse chemical classes in freshwater invertebrates. The method was tested on gammarids from the Danube River (within the Joint Danube Survey 3) and target compounds were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Furthermore, a non-target screening using high resolution-tandem mass spectrometry (LC-HRMS/MS) was conducted. A total of 17 pollutants were detected and/or quantified in gammarids at low concentrations. Pesticide concentrations ranged from 0.1 to 6.52 ng g^{-1} (wet weight), those of wastewater-derived pollutants from 0.1 to 2.83 ng g^{-1} (wet weight). The presence of wastewater-derived pollutants was prominent at all spots sampled. Using non-target screening, we could successfully identify several chlorinated compounds. These results demonstrate for the first time the presence of pesticides and wastewater-derived pollutants in invertebrates of the Danube River.

1. Introduction

Organic micropollutants such as pesticides, biocides, pharmaceuticals, personal care products, or industrial chemicals are ubiquitous in the aquatic environment (Schwarzenbach et al., 2006). These synthetic compounds enter surface water bodies through various pathways including wastewater treatment plant effluents, untreated wastewater, urban runoff and leaching from agricultural lands. Depending on their hydrophobicity and volatility, these compounds partition between sediments, water and the atmosphere. Many of these chemicals may persist in the environment or may show a permanent exposure if losses by environmental transformation and degradation are continuously replaced by new emissions. If these chemicals are taken up by aquatic organisms and bind to biological receptors they may cause adverse effects and pose a risk to freshwater ecosystems (Beketov et al., 2013; Malaj et al., 2014).

While risk assessment is typically based on external toxicant concentrations in water and sediment, awareness is increasing that the internal chemical environment is the key to adverse effects in organisms (Escher and Hermens, 2002). The concept of the internal exposome has been set up for humans (Rappaport and Smith, 2010) but can be easily transferred to other organisms (Simon et al., 2013).

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In aquatic ecosystems, invertebrates play a key role in food webs and for ecosystem functions (e.g., litter degradation). They have relatively long life cycles and may integrate over environmental conditions, including contamination, for a longer time. Invertebrate communities represent one of the Biological Quality Elements (BQEs) according to the European Union Water Framework Directive (EU WFD) and they are extensively used as biological indicators to assess water quality (Birk and Hering, 2006; Metcalfe-Schmith, 1994). Macroinvertebrates are known to be highly sensitive to insecticides but may be also affected by a large range of contaminants. For linking environmental micropollutants as causes to observed effects, there is an increasing demand for methods to analyse these chemicals in the organism focusing on bioavailable and bioaccumulating pollutants. Hydrophobic organic chemicals (log $K_{OW} > 3$) are typically accumulated in lipids in organisms. However, also interaction of less hydrophobic chemicals with proteins and other biomolecules and thus accumulation in biota tissues have been observed (Berlioz-Barbier et al., 2014). Thus, multi-target screening tools with a broad chemical domain are required to determine body burdens in macroinvertebrates.

Gammarids, a family of amphipods, are ubiquitous benthic macroinvertebrates in European inland water courses (Jażdżewski, 1980). They play a prominent function in the freshwater ecosystems breaking down coarse particulate organic matter and linking lower trophic levels to higher-level consumers as prey to fish (Friberg et al., 1994). They spend much of their life in contact with sediments, providing a continuous exposure to both hydrophilic water- and hydrophobic sediment contaminants (Ashauer et al., 2012; Tlili et al., 2012). Gammarids are expected to be optimal model organisms for body burden monitoring. They have already been used as model organisms for assessing both adverse effects (Cold and Forbes, 2004; Rasmussen et al., 2012) and uptake of organic micropollutants under laboratory conditions (Ashauer et al., 2012, 2006; Gross-Sorokin et al., 2003; Miller et al., 2015).

Although numerous multi-target screening tools based on GC-MS and LC-MS are available for water and sediment samples (Hernández et al., 2011; Hug et al., 2014; Krauss et al., 2010) only few methods are available for screening in macroinvertebrates (Huerta et al., 2015; Miller et al., 2015; Tlili et al., 2012; Berlioz-Barbier et al., 2014; Tixier et al., 2003). A major challenge is sample preparation with sufficient recovery of a broad range of chemicals. Sample preparation is required not just to extract the desired substance from the tissue but also to remove the complex mixture of biological matrix compounds that might interfere with the analysis of the targeted pollutants in order to improve the sensitivity and accuracy of the analysis (Pan et al., 2014; Ribeiro et al., 2014).

For biological environmental samples previous studies have utilized pressurized liquid extraction, soxhlet extraction and microwave-assisted extraction often followed by additional steps to remove matrix interferences prior to instrumental analysis (Pan et al., 2014). The vast majority of the analytical methods are based on time and/or solvent consuming procedures and they only targeted selected compounds or compound groups. It is imperative to develop more versatile methodological procedures, easily modifiable and able to overcome the shortcomings of the traditional methods. A new approach may be the use of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) which presents several advantages such as higher recoveries for a wide polarity and volatility range of analytes, reduces the amount of sample used and may significantly save solvents, waste and time required for the analysis (Chiaia-Hernandez et al., 2013). QuEChERS has been successfully applied for preparation of a wide variety of samples, including food, plants, vegetables, fruits, soils and water samples (Lehotay et al., 2010; Norli et al., 2011; Wilkowska and Biziuk, 2011). This method is based on a salting-out extraction with an organic solvent followed by dispersive solid phase extraction (dSPE) clean-up step. Since the development of this method, subsequent studies have adjusted and optimised the procedure according to the substance classes targeted and the complexity or characteristics of the matrices (Jia et al., 2012; Johnson, 2012; Lehotay et al., 2010; Norli et al., 2011; Plassmann et al., 2015). In the present study QuEChERS is adapted to and validated for invertebrate samples for a broad range of compounds.

Evaluation of novel analytical procedures under real world conditions, characterised by complex mixtures at often low internal concentrations, is a key to propose them for monitoring purposes. The Danube River appears to be an optimal case to test tools for multi- and non-target screening of invertebrates since this river receives chemicals from a large range of pollution sources. Overall, concentrations may be seen as typical for large rivers instead of reflecting hot spots of contamination. In the Joint Danube Survey 3 (JDS3) and under the frame of the project SOLUTIONS (Brack et al., 2015), macroinvertebrates have been collected from several sites from the upper course of the Danube River in Austria down to the delta in Romania for analysing body burdens as one parameter that might explain changes in invertebrate communities. This allowed for testing the new analytical method for applicability under routine monitoring conditions.

The objectives of this study were (i) to develop a method which allows to extract and quantify organic micropollutants of diverse chemical classes and physicochemical properties, (ii) to compare extraction and clean-up procedures for a subsequent analysis in liquid chromatography-tandem mass spectrometry (LC-MS/MS), (iii) to provide a suspect and non-target screening tool based on high-resolution (HR) MS full scan analysis for invertebrate analysis, and (iv) to apply the method on environmental samples from the Joint Danube Survey 3 to detect and quantify organic micropollutants in benthic macroinvertebrates.

2. Materials and methods

2.1. Reagents, chemicals and consumables

A list of 74 analytes with a wide range of properties (log *D* at pH 7 from −2.89 to 5.36) was selected for method development based on their occurrence in water samples and sediments (see Table S1, Supplementary materials). Representatives belonged to different pollutant families such as pesticides, pharmaceuticals and other wastewater-derived pollutants and some of their main metabolites. Details about these target compounds and other chemicals used are given in the Supplementary materials, S1.1.

2.2. Sample collection

Due to logistical reasons method development and validation were performed with the species *Gammarus pulex*, which was obtained in frozen state from FiMö Aquaristik GmbH (Bünde, Germany) and stored at −20 °C. Method application in the Danube River used *Dikerogammarus* spp, an invasive species replacing *Gammarus* species in this river ecosystem (Dick and Platvoet, 2000). Both species are closely related and coexist in a similar niche in freshwater ecosystems (Truhlar and Aldridge, 2015). Thus, no influence of the consideration of different species on body burden analysis is expected.

Gammarids of the species *Dikerogammarus* spp. for method application and evaluation were collected in 18 sampling spots from the Danube River along its watercourse as part of JDS3 in 2013 (Liška et al., 2015). *Dikerogammarus* spp. is an ubiquitous benthic macroinvertebrate in the Ponto-Caspian region of eastern Europe/