



Denatonium – A so far unrecognized but ubiquitous water contaminant?



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ABSTRACT

Denatonium is one of the bitterest substances known to man and therefore applied in numerous consumer products to prevent an accidental or intentional consumption. So far no information was available on the occurrence of this compound in the environment. A sensitive targeted method was developed and applied to water samples taken in the federal state of Baden-Württemberg, Germany. Denatonium was detected in 100% of the investigated 22 wastewater treatment plant (WWTP) effluents with a maximum concentration of 341 ng L⁻¹. Additionally, water samples were taken from the Ammer river over a period of one week and all wastewater impacted samples showed denatonium at concentrations up to 195 ng L⁻¹. Retrospective analysis of high-resolution mass spectrometric measurements of WWTP effluents from Italy and Switzerland confirmed and therefore point to an international occurrence of denatonium as anthropogenic contaminant.

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1. Introduction

Denatonium benzoate (trade name Bitrex[®]) is one of the bitterest substances known to man with recognition thresholds in the range of 3–23 µg L⁻¹ (Schiffman et al., 1994) and an unpleasant bitterness at elevated concentrations of 10 mg L⁻¹ (Cosmetic Ingredient Review Expert Panel, 2008). Volunteer studies from the 1980s and 1990s indicated its usefulness for poisoning prevention, because a reduced ingestion rate of denatonium benzoate-containing formulations was observed for children and non-target animals (Berning et al., 1982; Kleinkauf et al., 1999; Sibert and Frude, 1991). However, no effects on the frequency or severity of pediatric fluid consumption of antifreeze and windshield washer were identified, when poisoning data were compared before and after bittering was required in selected states of the USA (Mullins and Zane Horowitz, 2004; White et al., 2009). Nevertheless, Bitrex[®] is added nowadays to a wide range of products (e.g. finger paints, household products, and pesticides) and it is used for the denaturation of alcohol. Depending on its application,

concentrations between 200 and 300 mg L⁻¹¹ or 20–100 mg L⁻¹ are often used in animal repellants or to prevent accidental ingestion by humans (Cosmetic Ingredient Review Expert Panel, 2008; Kleinkauf et al., 1999), while the common denaturing procedure for alcohol in the European Union (EU) requires 10 mg L⁻¹ denatonium benzoate (European Commission, 2013).

This aversive agent is generally considered as a very safe compound, because animal studies indicate that it is neither carcinogenic, genotoxic (Cosmetic Ingredient Review Expert Panel, 2008), nor mutagenic (Consumer Product Safety Commission United States of America (1992)). It shows a moderate acute oral toxicity for different mammalian species with median lethal doses in the range of 593–865 mg kg⁻¹ (Consumer Product Safety Commission United States of America (1992)). Due to its unpleasant bitterness it is however very unlikely that humans ingest it in appreciable amounts. Available information about the acute inhalation toxicity and chronic toxicity are not conclusive. While no inhalation hazard was observed for rats exposed to a 0.1% w/v (i.e. 1 g L⁻¹)

¹ Denatonium concentrations are often reported in [ppm]. For consistent units, a constant density of 1 kg L⁻¹ was assumed for aqueous solutions of denatonium and [ppm] were directly converted to [mg L⁻¹].

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denatonium benzoate solution (Klein-Schwartz, 1991), the European Food Safety Authority (EFSA) reported a median lethal inhalation concentration of 0.2 mg L^{-1} (European Food Safety Authority, 2012). Monkeys showed an increased mortality when orally dosed with 8 or 16 mg denatonium benzoate $\text{kg}^{-1} \text{ day}^{-1}$ over one year, but no compound related effects were observed during a two year study with rats (Cosmetic Ingredient Review Expert Panel, 2008). A single case of Bitrex[®] related asthma and allergic skin reactions was reported in the 1980s, where adverse effects, i.e. erythema, were still observed at concentrations as low as 2 ng L^{-1} (Björkner, 1980). In contrast, neither skin sensitizing nor irritating properties were observed during several studies with overall more than 1100 humans, which were exposed to denatured alcohol containing denatonium benzoate (Cosmetic Ingredient Review Expert Panel, 2008).

Animal studies with aquatic species point towards a moderate acute toxicity with median lethal concentrations higher than 1000 mg L^{-1} for rainbow trout and $\geq 400 \text{ mg L}^{-1}$ for selected invertebrates (European Food Safety Authority, 2012). However, lower concentrations in the range of $5\text{--}10 \text{ mg L}^{-1}$ influenced the reproduction of *Daphnia magna* and reduced the biomass of the freshwater algae *Nitzschia palea* (European Food Safety Authority, 2012). Generally, the ecotoxicological risk for non-target animals was ranked as low by the EFSA, because only a negligible release of denatonium benzoate into the environment is expected when it is applied as repellent in forestry. However, they concluded that “if other uses resulting in higher exposure were intended in the future, a considerable amount of data would be needed to finalise the environmental exposure assessment” (European Food Safety Authority, 2012) and more studies are required for a comprehensive ecotoxicological risk assessment. Information about the use pattern and annual global production of this aversive agent is however not available, but approximately 10–100 tons are manufactured and/or imported each year in the European Economic Area (ECHA, 2016).

Denatonium was not considered so far as a suspect compound for the investigation of surface waters and wastewater, although other chemicals present in industrial products were already extensively studied, e.g. surfactants (Cowan-Ellsberry et al., 2014). Furthermore, non-target screening approaches are not suited for the identification of this compound, because protonation of the analyte is typically expected during electrospray ionization in the positive mode (Velpandian et al., 2012). Wrong molecular formulas are therefore assigned to permanently charged analytes, which was demonstrated recently for triphenylphosphonium compounds (Schlüsener et al., 2015).

We tentatively identified denatonium based on a commercially available, high-resolution spectral library and the main goal of this study was to provide first data on the occurrence of denatonium in the environment and to assess wastewater as a possible source. Therefore, a sensitive multi-analyte targeted method was developed and applied to samples from rivers and wastewater treatment plant (WWTP) effluents in the federal state of Baden-Württemberg, Germany. In the EU there are usually no regulatory thresholds for denatonium in industrial products, except for denatured alcohol. Therefore, 22 consumer products were bought from local supermarkets and analyzed to evaluate the range of denatonium concentrations in personal care products (PCPs), detergents, and antifreeze.

2. Material and methods

2.1. Chemicals

Optima[™] LC/MS grade acetonitrile (AcN), methanol (MeOH),

isopropanol (IPA), formic acid (FA), acetic acid (HAc), ammonium acetate (NH_4Ac) and water were purchased from Fisher Chemical (Belgium). The targeted analysis method was generally developed for 72 analytes, but results are presented in this study only for the following six compounds. Benzotriazole ($\geq 98\%$), denatonium benzoate ($\geq 98\%$), diatrizoic acid ($\geq 98\%$), paracetamol ($\geq 99\%$) and sulfamethoxazole ($\geq 98\%$) were obtained from Sigma-Aldrich (Germany), while lamotrigine ($\geq 98\%$) was purchased from TCI (Belgium). Analytes other than denatonium were used for quality control purposes and to compare the observations for the bittering agent to already known water pollutants. Individual stock solutions with analyte concentrations of 1 g L^{-1} were usually prepared with AcN, except for lamotrigine and diatrizoic acid which were dissolved in MeOH. A working solution containing all analytes was prepared in AcN with final concentrations of 10 mg L^{-1} and subsequent dilutions in tap water were used for external calibration. Stock and working solutions were stored in the freezer at $-18 \text{ }^\circ\text{C}$, while dilutions for the calibration were freshly prepared on a daily basis.

2.2. Sampling and sample preparation

In 2015, 22 consumer products (15 detergents, 6 personal care products (PCPs) and 1 antifreeze) were bought from local supermarkets and analyzed for their denatonium content. In order to assess the sources and the distribution of this bittering agent and other organic contaminants in the environment, two sampling campaigns were performed in the federal state of Baden-Württemberg, Germany, in summer 2014 and spring 2015. Surface water and WWTP effluent samples were collected from 13 different locations along six tributaries of the river Neckar and from 22 treatment plants, respectively. Time trends of pollutant concentrations were investigated for one of the surface waters, the river Ammer, by collecting 2 h composite samples over seven consecutive days. All other samples from these two campaigns were grab samples. Additionally, a total of five WWTP effluent samples from two plants in Switzerland and one from Italy were analyzed to investigate the occurrence of denatonium as water contaminant outside of Germany.

Environmental samples were transported to the laboratory on ice. Particles were removed either by centrifugation or filtration and the samples were stored in the dark at $4 \text{ }^\circ\text{C}$ for a maximum of 48 h until analysis. Consumer products were diluted prior to analysis by a factor of 10^5 without any further treatment and the dilutions were immediately analyzed after preparation. Further details about the sampling locations, consumer products, and sample processing are provided in the supporting information (SI section 1.1).

2.3. LC-ESI-QqQ analysis

Target analysis was performed using a 1260 Infinity HPLC system (Agilent Technologies, Germany), consisting of a degasser, a binary pump, an autosampler, a thermostat, and a column oven. Samples were kept in the autosampler at $10 \text{ }^\circ\text{C}$ and the injection volume was either $10 \mu\text{l}$ for the diluted consumer products or $100 \mu\text{l}$ for surface water and WWTP samples, respectively. Analytes were separated on a Poroshell EC-C18 column ($2.1 \times 100 \text{ mm}$, $2.7 \mu\text{m}$, Agilent Technologies, Germany) coupled to a Poroshell EC-C18 guard column ($2.1 \times 5 \text{ mm}$, $2.7 \mu\text{m}$, Agilent Technologies, Germany). In case of environmental samples, the mobile phases were (A) water + 0.1% HAc + 0.1 mM NH_4Ac and (B) AcN + 0.1% HAc. The following gradient program was used for separation: at 0 min 98% A, at 17 min 20% A, at 17.1 min 0% A, at 23 min 0% A, at 23.1 min 98% A, and at 32 min 98% A. Diluted consumer products were analyzed

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