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Monitoring Toxic Ionic Liquids in Zebrafish (*Danio rerio*) with Desorption Electrospray Ionization Mass Spectrometry Imaging (DESI-MSI)

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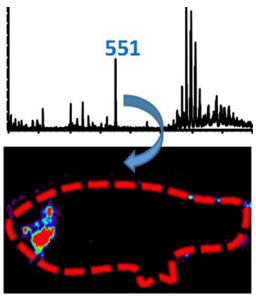

Abstract. Ambient mass spectrometry imaging has become an increasingly powerful technique for the direct analysis of biological tissues in the open environment with minimal sample preparation and fast analysis times. In this study, we introduce desorption electrospray ionization mass spectrometry imaging (DESI-MSI) as a novel, rapid, and sensitive approach to localize the accumulation of a mildly toxic ionic liquid (IL), AMMOENG 130 in zebrafish (*Danio rerio*). The work demonstrates that DESI-MSI has the potential to rapidly monitor the accumulation of IL pollutants in aquatic organisms. AMMOENG 130 is a quaternary ammonium-based IL reported to be broadly used as a surfactant in commercialized detergents. It is known to exhibit acute toxicity to zebrafish causing extensive damage to gill secondary lamellae and increasing membrane permeability. Zebrafish were exposed to the IL in a static 96-h exposure study in concentrations near the LC_{50} of 1.25, 2.5, and 5.0 mg/L. DESI-MS analysis of zebrafish gills demonstrated the appearance of a dealkylated AMMOENG 130 metabolite in the lowest concentration of exposure identified by a high resolution hybrid LTQ-Orbitrap mass spectrometer as the trimethylstearylammonium ion, $[C_{21}H_{46}N]^+$. With DESI-MSI, the accumulation of AMMOENG 130 and its dealkylated metabolite in zebrafish tissue was found in the nervous and respiratory systems. AMMOENG 130 and the metabolite were capable of penetrating the blood brain barrier of the fish with significant accumulation in the brain. Hence, we report for the first time the simultaneous characterization, distribution, and metabolism of a toxic IL in whole body zebrafish analyzed by DESI-MSI. This ambient mass spectrometry imaging technique shows great promise for the direct analysis of biological tissues to qualitatively monitor foreign, toxic, and persistent compounds in aquatic organisms from the environment.

Keywords: Ionic liquids, Toxicology, Environmental analysis, Imaging, Desorption electrospray ionization

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Introduction

Ionic liquids

Ionic liquids (ILs) are organic or semi-organic salts composed of a cation and an anion moiety, which are liquids at or below room temperature with low melting points. ILs have desirable physiochemical properties for the development of greener solvent alternatives to conventional organic solvents, including high thermal and chemical stability, nonflammability and nonvolatility [1]. ILs have negligible vapor pressures and cannot generate volatile organic compounds (VOCs) in the atmosphere causing unwanted air pollution; for this reason ILs have attracted significant attention as media for green synthesis. ILs have been implemented as lubricants, performance additives, biocatalysts, solvents for organic synthesis, as well as stationary and mobile phases for chromatographic separation [2]. Despite their wide applicability, commonly used

Dedicated to Dr. Cooks with admiration and affection.

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ILs have been found to exhibit higher toxicities than traditional organic solvents. The viability of these compounds as green solvents has been under scrutiny for quite some time because of their toxic effects on aquatic organisms from diverse trophic levels. Consequently, the release of toxic ILs into aquatic ecosystems poses a high threat to the environment and human health, such that toxic effects elicited may cause acute or chronic toxicity, sedimentation, or bioaccumulation [3].

The AMMOENG series (AMMOENG 100, 101, 102, 110, 111, 112, 120, and 130) are ILs structurally composed of tetrasubstituted-ammonium cations with varying saturated alkyl or oligo(ethyleneglycol) chains and small anion moieties (Cl^-, $MeOSO_3^-$, $H_2PO_4^-$, and $MeCO_2^-$). These ILs are available in large quantities and, hence, represent an interesting alternative to expensive and not readily available imidazolium-based ILs. AMMOENG type ILs have gained attention because of their surfactant properties with routine use in commercial products such as detergents and softeners [4]. Acute toxicity tests have been performed for many IL subclasses, including AMMOENG type ILs, such as marine luminescent bacteria (*Vibrio fischeri*) [5–7], limnic green algae (*Scenedesmus vascuolatus*) [8], fresh water plant (*Lemna minor*) [7], crustacean (*Daphnia magna*) [8–10], and zebrafish (*Danio rerio*) [8, 11, 12]. Pretti and coworkers tested 15 common ILs to zebrafish; among these, AMMOENG 130 was found to be more toxic than 13 other ILs with an LC_{50} of 5.2 mg/L. Histopathological examination of the fish gill tissue revealed marked damage, enlargement, and disorganization in secondary gill lamellae with disepithelialization in treated fish. Damage to the gill membranes was predicted to be a cause of altered lipid bilayers and enhanced membrane permeability to external ions [8, 11]. The majority of the ecotoxicologic data focuses on the influence of the cation head groups, the alkyl side chain length, and the anion toxicity [13]. One of the toxic contributions arises from the "alkyl side chain effect" where long hydrophobic alkyl chains promote increased toxicity [7, 14]. Independent of the organism and experimental method, ILs highly hydrophobic and lipophilic significantly increases toxic effects. There is growing evidence that the toxic mechanism of action for lipophilic ILs is to penetrate, dissolve, and disintegrate biological membranes affecting biological functions in cells. Therefore, ILs with long alkyl chains incorporate into phospholipid bilayers and alter the structural integrity of biological membranes [11, 15].

In organisms, lipophilic ILs are metabolized and eliminated only to a small degree, if at all, and hence have a greater potential to bioaccumulate [16]. Acute toxicity in combination with chronic toxicity, sedimentation, and bioaccumulation are crucial endpoints that need to be determined for environmental risk assessment to meet regulatory standards and guidelines, and most importantly to encourage greener practices [3, 17]. IL toxicity has been studied quite extensively, yet IL environmental fate, the metabolic fate within an organism, and the toxic mechanisms of action are still not well understood [15]. In spite of great efforts and advancements, there is continued uncertainty regarding the tendency, extent, and effects of bioaccumulation and/or bioconcentration for ionic species and a lack of the necessary chemical and analytical techniques to evaluate environmental accumulation upon exposure [17]. Traditional mass spectrometry methods such as gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography mass spectrometry (HPLC-MS) can provide compound elucidation, separation, and concentration within tissue [18], but unfortunately these methods can be laborious, time-consuming, and cannot provide spatial distribution of a compound's specific accumulation within aquatic organisms as can be achieved by ambient mass spectrometry (MS) imaging techniques.

Ambient MS Imaging

Ambient MS imaging has become an important tool for surface analysis of biological tissues in their native state, with minimal sample preparation. Ambient ionization techniques enable the interrogation of a wide range of samples by mass spectrometry with high throughput capabilities, fast analysis times, and high sensitivities [19]. Desorption electrospray ionization mass spectrometry has been an important technique in this field [20]. The DESI source consists of a pneumatically assisted charged spray solvent that impacts the sample surface, creating a thin liquid film by which primary droplets dissolve the analytes of interest forming secondary droplets that are then desorbed from the surface and directed into the mass spectrometer inlet for chemical analysis [19, 21].

DESI-MS imaging has become an attractive tool for the spatially resolved identification of chemical compounds in biological tissues. A software-controlled 2D moving stage is coupled to the DESI ion source that rasters across the sample surface in the x and y direction to scan the tissue for simultaneous MS analysis [22–25]. When DESI-MS is used in imaging modality, developments and applications are multifaceted and have been explored in the field of natural products [26–30], lipidomics [22, 23, 31], metabolomics [28, 32, 33], drug discovery [32], cancer typification [22, 34], and forensics [35, 36]. It has endless applications and capabilities for accurate compound identification, mapping chemical specific distributions, and aiding in the discovery of compounds' biological significance [37]. Endogenous biomarker and drug-induced accumulation are emerging areas of research evaluated by DESI-MS imaging for the specific distribution of drugs and metabolites in animal organs and whole body tissues [33, 38]. Likewise, exogenous compounds have also been mapped in whole body mice and locusts by DESI-MS imaging [39, 40]. Recently, this technique was successful in mapping endogenous fatty acids and multiple subclasses of phospholipids ranging from phosphatidylserines, phosphatidylinositols, and sulfatides in whole body zebrafish. Phospholipids were distributed in the brain and spinal cord tissue; as for the bile salt, 5α-cyprinol 27-sulfate, it was confined to the gastrointestinal system of the fish [41]. In this study, the zebrafish was chosen as the model organism since it has become an emerging research model because of its degree of homology with the human genome to

study human diseases, and in developmental biology, genetics, and environmental toxicology for in vivo and in vitro studies [42, 43].

It is the aim of this work to introduce DESI-MS imaging as an accumulation screening tool for toxic ILs in zebrafish. DESI-MS can provide efficient desorption and high sensitivities [44] since ionic species, to some extent, are partially dissociated and therefore charged before MS ionization processes enhancing chemical specificity [45]. This technique can provide simultaneous IL and metabolite identification, and distribution, in whole body tissues due to DESI-MS imaging capabilities of generating ion images of IL intensities versus tissue spatial coordinates. Hence, we intend to report for the first time a DESI-MS imaging approach to rapidly monitor the accumulation of a toxic IL in zebrafish.

Experimental

Materials and Reagents

A >95% AMMOENG 130 (dimethyldistearylammonium chloride) standard was purchased from IoLiTec Inc. (Tuscaloosa, AL, USA). Carboxymethyl cellulose sodium (CMC), tricaine methanesulfonate (MS-222), LC-MS grade water, and HPLC grade methanol were purchased from Sigma Aldrich (Oakville, ON, Canada). Flexible moulds were obtained from Polysciences Inc. (Warrington, PA, USA); Tissue-tek O.C.T. compound from Cederlane (Burlington, ON, CA). Professor Chun Peng from the Department of Biology at York University provided zebrafish from the Vivaria in four 2-L tanks containing four fish per tank for a total of 16 fish.

Static IL Exposure Study to the Zebrafish

A static 96-h IL exposure study was performed in accordance to the regulations for the care and management of fish set by the Canadian Committee on Animal Care (CCAC) and York University's Animal Care Committee (YACC) and the Economic Cooperation and Development (OECD): Fish Acute Toxicity Test No. 203. Four zebrafish were placed in each polycarbonate 2 L aquaria and were exposed to 1.25, 2.50, and 5.0 mg/L of AMMOENG 130 by administering the IL into the tank water, while a control group was monitored simultaneously. The water temperature was maintained at 22 ± 1 °C and fish were kept under laboratory illumination in a 12-hour daily photoperiod. No food was supplied during the study and fish were monitored periodically in time periods 1, 3, 6, 12, 24, 48, 72, and 96 h.

Zebrafish Sample Preparation and Cryosectioning

After the exposure period, surviving zebrafish were euthanized with 300 mg/L of MS-222 solution. Zebrafish were thoroughly washed with distilled water once removed from the euthanizing solution. Deceased fish were placed into flexible, peel away moulds for cryosectioning. A 5% CMC solution was prepared and poured into individual moulds containing a whole body zebrafish. Zebrafish carboxymethyl cellulose (CMC) embedded blocks were used to prepare 50 μm tissue sections on a Shandon E cryotome from Thermo Scientific (Nepean, ON, Canada). Tissue sections were placed on microscopic glass slides, kept in a –80 °C and later in a –18 °C freezer for immediate use. The tissue sections were air-dried for 30 min prior to DESI-MS imaging analysis.

Electrospray Ionization Mass Spectrometry and Tandem Mass Spectrometry of AMMOENG 130

A standard solution of 10 ng/mL of AMMOENG 130 was prepared in methanol for HRMS analysis using ESI-MS and ESI-MS/MS in a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap Elite; Thermo Scientific, USA). The spray voltage was set to 4 kV, the capillary temperature to 250 °C, the S-lens rf level to 68%. A nitrogen gas pressure of 100 psi and a flow rate of 5 μL/min of pure methanol were used. Full scan ESI-MS spectra of the IL were obtained in the mass range of *m/z* 200–1000. MS/MS was conducted on IL peaks using collision energies between 50% and 51% (manufacturer's unit).

DESI-MS and DESI-MS Imaging Acquisition Parameters

DESI-MS profiles of zebrafish gills on sections of whole body zebrafish tissue were acquired using a Thermo Scientific LTQ linear ion trap mass spectrometer (San Jose, CA, USA) equipped with a lab-built DESI source and a 2D moving stage for imaging [46]. DESI ion source parameters such as the capillary tip to surface distance, the mass spectrometer inlet to capillary tip distance, and the incident angle were optimized for zebrafish tissue as follows: an incident angle of 52°, 1 mm capillary tip to surface distance, and a 3–5 mm distance from the mass inlet to the solvent capillary tip was used. A nitrogen gas pressure of 100 psi and a flow rate of 2 μL/min of pure methanol was chosen for optimal desorption and ionization of AMMOENG 130 from 50 μm zebrafish tissue sections.

For DESI-MS imaging, the DESI ion source was mounted onto a 2D moving stage capable of moving in the x and y direction for image acquisition. A total of n = 4 fish per concentration for a total of n = 16 fish were analyzed for IL distribution. DESI-MS imaging of whole body zebrafish was performed in positive ion mode with pure methanol at a flow rate of 2 μL/min, the ion injection time was set to 150 ms, and 3 microscans were summed. Tissue imaging experiments were performed with a 2D moving stage by collecting mass spectral data by continuously scanning in horizontal rows in the x direction with a velocity of 200–350 μm/s every 200 μm over a mass range of *m/z* 200–1000. The zebrafish tissue imaging dimensions ranged from 3 to 4 cm in width versus 1 to 1.5 cm in length dependent upon the size of the tissue section under MS analysis. Imaging acquisition times varied between 1.5 and 2.5 h with a spatial resolution of 200 μm for all images.

Data Processing

The mass spectra were processed by Qual Browser Xcalibur 2.0 and ImageCreator ver. 3.0 software was used to convert the Xcalibur 2.0 mass spectra files (.raw) into a format compatible with BioMap (freeware, http://www.maldi-msi.org/). BioMap processed the mass spectral data to acquire 2D ion images of tissue surface coordinates versus ion intensity.

IL Degradation Study

A degradation study was conducted to assess the stability and degradation of AMMOENG 130 in water during 96 h. AMMOENG 130 was placed in 250 mL capped bottles in concentrations of 1.25, 2.5, and 5.0 mg/L. A control with distilled water, but without the IL, was run in parallel with total volumes of 200 mL; 500 µL of the IL solution was directly infused in an ESI source coupled to the LTQ mass spectrometer. The IL was sampled from each concentration in 12 h intervals for 96 h for the detection of IL degradation products. The ESI-MS parameters were kept constant as previously described for the characterization of AMMOENG 130 with the exception of the mass range. The mass spectral data were collected between *m/z* 200 and 700.

Metabolite Extraction and Identification with HRMS

A portion of zebrafish gill tissue was manually dissected from the fish body, ground by a mortar and pestle, placed into a 1.5 mL Eppendorf tube, macerated, and extracted with methanol. The extract was centrifuged at 7000 rpm for 30 min. The supernatant was separated, collected, and transferred into an Eppendorf tube. The supernatant was dried down in a concentrator for 2 h until complete evaporation and resuspended in 400 µL of methanol.

The gill extract prepared was analyzed by ESI-MS and ESI-MS/MS via LTQ-Orbitrap Elite for HRMS analysis. The spray voltage was set to 4 kV, the capillary temperature to 250 °C, the S-lens rf level to 68%, and the flow rate to 5 µL/min. Full scan mass spectra of the gill extract were acquired in the mass range *m/z* 200–1000. MS/MS experiments were conducted by isolating the metabolite ion within ±0.01 Da and fragmenting with a collision energy of 50%–51% (manufacturer's unit).

Results and Discussion

AMMOENG type ILs have not been fully investigated in the literature in terms of characterization, metabolism, or toxic mechanisms of action; therefore, AMMOENG 130 was characterized by ESI-MS and ESI-MS/MS. The ionic liquid's chemical formula $C_{38}H_{80}NCl$ with a molecular weight of 586.5 g/mol AMMOENG 130 was detected in positive ion mode as the cation of *m/z* 551, $[C_{38}H_{80}N]^+$; other ionic species of *m/z* 523, *m/z* 495, and a low intensity ion of *m/z* 312 were detected in the ESI-MS spectrum and identified by collision-induced dissociation (CID) as $[C_{36}H_{76}N]^+$, $[C_{34}H_{72}N]^+$, and $[C_{21}H_{36}N]^+$, respectively (Figure 1). The monoisotopic AMMOENG 130 ion of *m/z* 550.6 was isolated and fragmented by CID, producing MS/MS fragments of *m/z* 298.4 and 296.4 (Figure 1b). The most intense fragment of *m/z* 298.4 arises from the loss of the hydrophobic alkyl chain ($C_{18}H_{37}$). The MS/MS fragment of *m/z* 296.4 corresponds to the loss of the alkyl chain ($C_{18}H_{37}$) with subsequent loss of H_2 to form a carbon-nitrogen double bond for increased stabilization.

Subsequently, tandem mass spectrometry (ESI-MS/MS) was conducted on all other ionic species in the full scan spectrum. The ion of *m/z* 523 was identified as hexadecylstearyldimethylammonium ion $[C_{36}H_{76}N]^+$, the ion of *m/z* 495 as the tetradecylstearyldimethylammonium ion/dihexadecyldimethylammonium ion $[C_{34}H_{72}N]^+$, and the ion of *m/z* 312 as trimethylstearylammonium ion $[C_{21}H_{46}N]^+$; chemical structures and their corresponding fragments are reported in Figure 2 and Table 1, respectively. The ESI-MS/MS spectra of all ionic species can be found in the Supporting Information (Supplementary Figures S1–S3).

IL Exposure to the Zebrafish and Mortality

The fish were exposed to AMMOENG 130 for 96 h to induce accumulation. AMMOENG 130 was administered into the tank water containing four fish at each concentration of 1.25, 2.5, and 5.0 mg/L. Zebrafish were monitored for visible physical and behavioral changes compared with control during the exposure period (Supplementary Table S1). During the first 12 h, fish in all concentrations demonstrated normal behavior like the control. Zebrafish exposed to 5.0 mg/L and 2.5 mg/L were found deceased after 24 h. Deceased fish displayed signs of epidermal discoloration and inflammation of the buccal cavity and gills. Some fish exposed to these concentrations exhibited severe abdominal distension and inflammation. The fish abdomen was visibly distended beyond its normal girth (Supplementary Figure S4). Inflammation and growth dilution are characteristic signs of acute toxicity and chemical excretion. Growth dilution is a pseudo-elimination process by which substances are not physically eliminated; however, the concentration is diluted inside the organism by an increase in the fish tissue volume by fluid retention [17]. During 48–96 h, fish in 1.25 mg/L displayed a general decrease in activity, staying motionless for large periods of time with spurs of rapid and erratic swimming compared with control [11]. Fish in the control group and those exposed to 1.25 mg/L survived the 96 h study and were euthanized prior to DESI-MS analysis.

DESI-MS Analysis of Zebrafish Gills

Gills are the first point of contact and entry of water-borne contaminants into the fish from respiratory exchange; therefore, the presence of the IL was investigated there. DESI-MS profiles were acquired for control zebrafish gills and a plethora

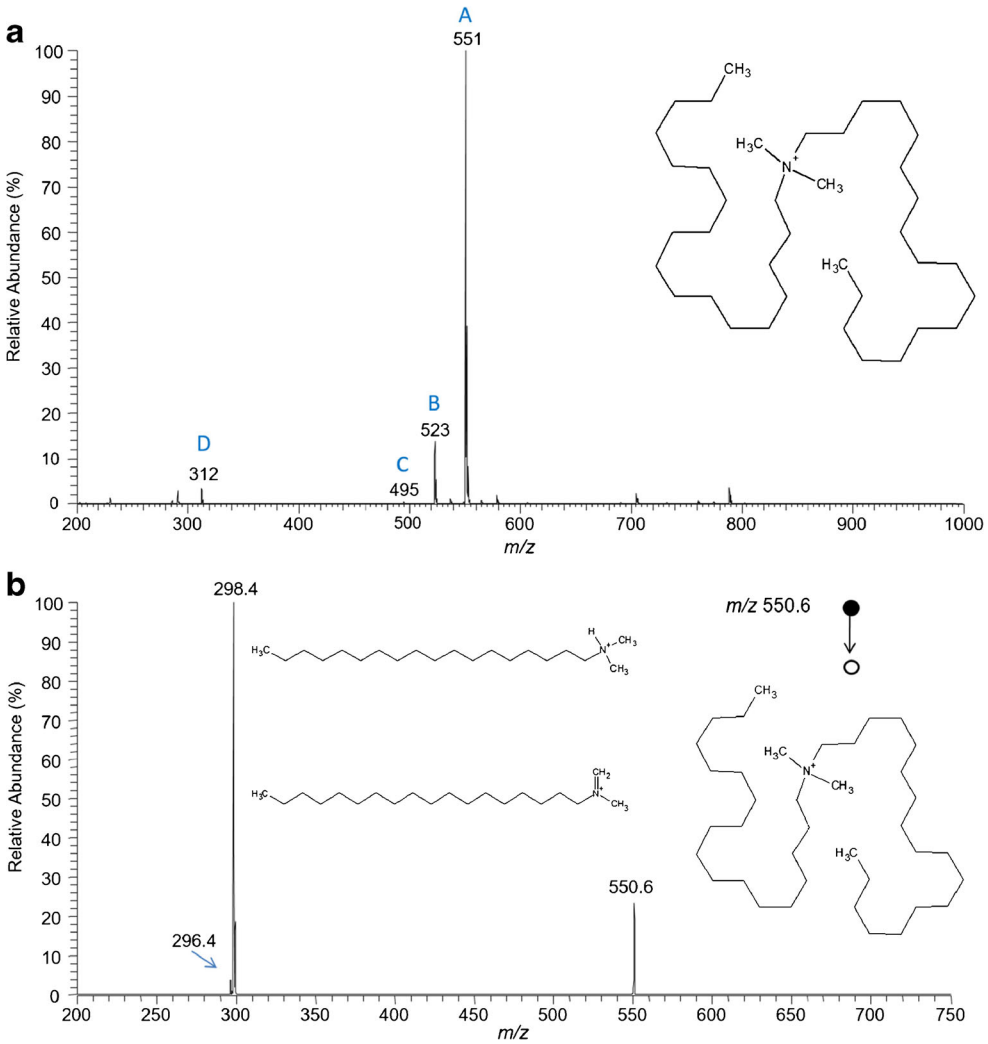

Figure 1. Characterization of AMMOENG 130 by ESI-MS and ESI-MS/MS. (**a**) ESI-MS spectrum of AMMOENG 130. IL ions of *m/z* 551, 523, 495, and 312 corresponding to AMMOENG 130 ion $[C_{38}H_{80}N]^+$, hexadecylstearylammonium ion $[C_{36}H_{76}N]^+$, tetradecylstearyldimethylammonium/dihexadecyldimethylammonium ion $[C_{34}H_{72}N]^+$, and stearyltrimethylammonium ion $[C_{21}H_{46}N]^+$, respectively. (**b**) ESI-MS/MS spectrum of AMMOENG 130 fragmented with a collision energy of 25% (manufacturer's unit) resulting in two fragments of *m/z* 298.4 and *m/z* 296.4

of phospholipids; mainly phosphatidylcholines (PC) in the range of *m/z* 750–1000 were found (Figure 3a). The characterization of the most intense phospholipids in zebrafish has been previously described by DESI-MS/MS in negative and positive ion mode [41, 45]. PCs dominate in freshwater fish gills playing important roles in structural membrane integrity and fluidity, membrane-mediated cell signaling, and cell apoptosis [47]. In seawater fish gills, mono-

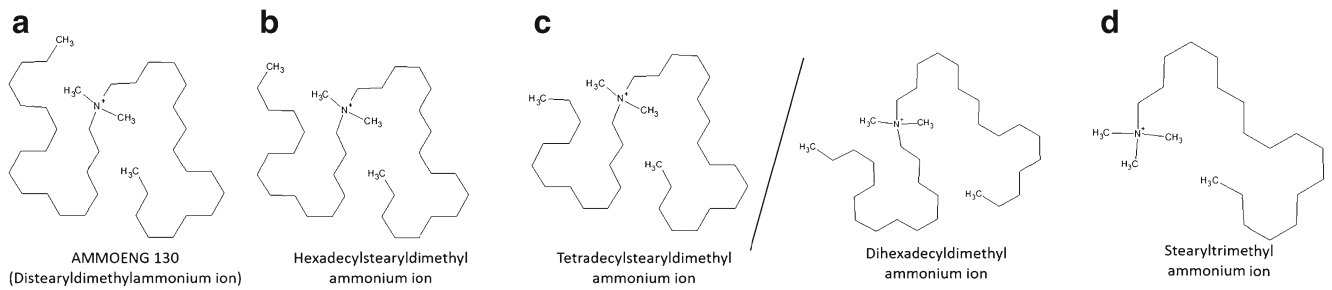

Figure 2. Chemical structure of AMMOENG 130 and other IL species. (**a**) AMMOENG 130, distearyldimethylammonium ion, $[C_{38}H_{80}N]^+$ of *m/z* 551. (**b**) Hexadecylstearyldimethylammonium ion, $[C_{36}H_{76}N]^+$ of *m/z* 523. (**c**) A mixture of Tetradecylstearyldimethylammonium ion/Dihexadecyldimethylammonium ion, $[C_{34}H_{72}N]^+$ of *m/z* 495. (**d**) Stearyltrimethylammonium ion, $[C_{21}H_{46}N]^+$ of *m/z* 312

Table 1. Electrospray Ionization Mass Spectrometry and MS/MS Characterization of AMMOENG 130

Ion	Molecular ion	Monoisotopic mass (Da)	Observed *m/z*	MS/MS fragments	Collision energy (%)
A	$[C_{38}H_{80}N]^+$	550.6290	551	298.4, 296.4	25
B	$[C_{36}H_{76}N]^+$	522.5977	523	298.4, 270.4	25
C	$[C_{34}H_{72}N]^+$	494.5664	495	270.4, 298.4, 242.4, 268.4	30
D	$[C_{21}H_{46}N]^+$	312.3630	312	296.4	35

unsaturated phosphatidylethanolamines (PEs) are predominant. PEs in saltwater fish have been found to act as regulators because of the salinity of the water environment to assist in osmoregulation. The absence of PEs in

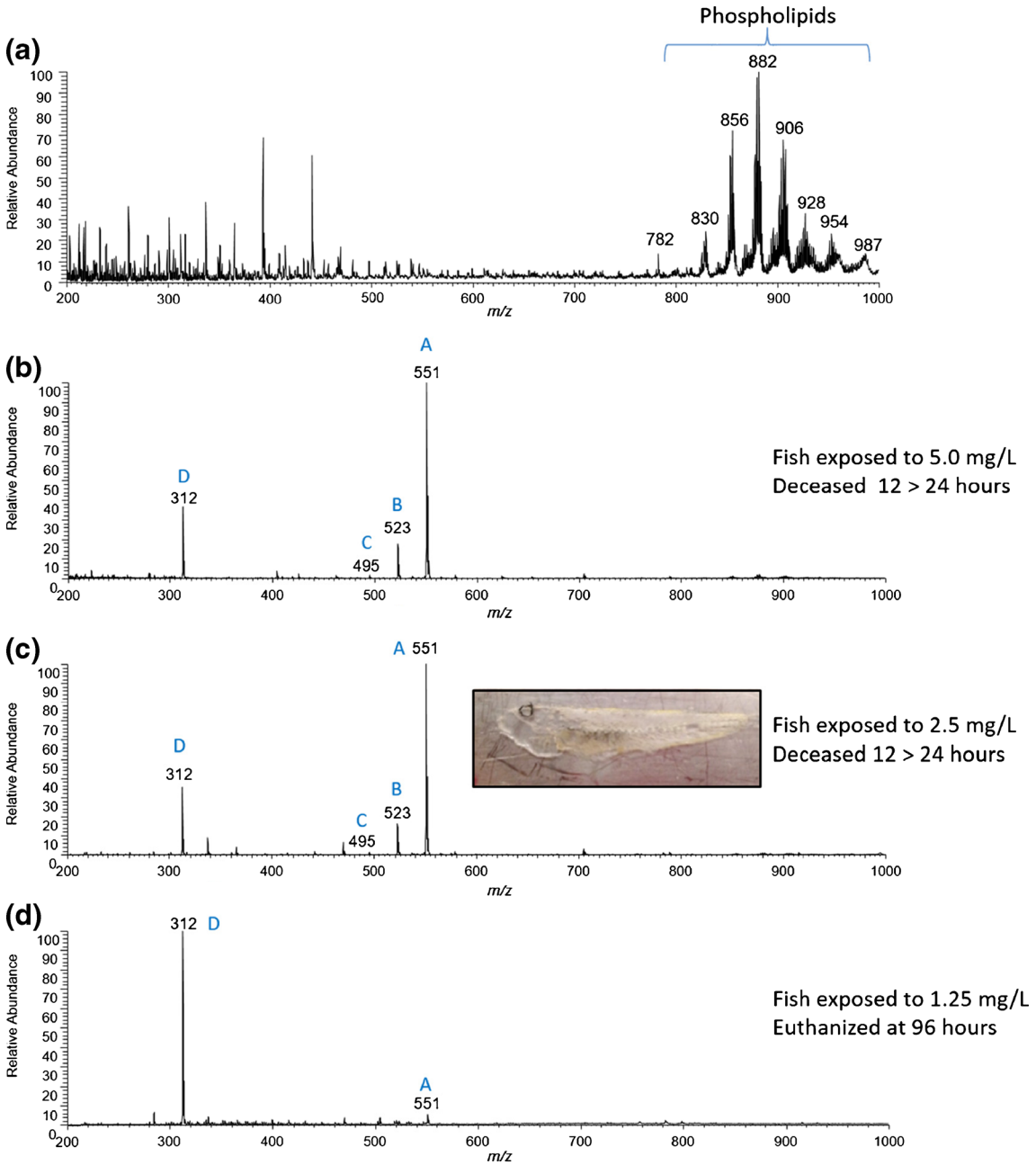

Figure 3. DESI-MS analysis in positive ion mode directly from zebrafish gills after AMMOENG 130 exposure. (**a**) Control fish gills displaying phospholipids between *m/z* 750 to 1000. (**b**) Fish exposed to 5.0 mg/L. (**c**) Fish exposed to 2.5 mg/L. The inset image shows the zebrafish tissue on a microscopic glass slide for DESI-MS analysis. (**d**) Fish exposed to 1.25 mg/L

freshwater fish shows that fish do not appear to undergo such an osmotic challenge [48].

Fish gill profiles exposed to varying concentrations of AMMOENG 130 were collected by DESI-MS analysis (Figure 3). The workflow involved from the exposure of the IL to the zebrafish up to the DESI-MS analysis is shown in Supplementary Figure S5.

The high abundance of the IL in the gills leads to ion suppression of the naturally occurring phospholipids between *m/z* 750 and 1000 (Figure 3b–d). Zebrafish gill profiles in concentrations from 1.25 to 5 mg/L demonstrated marked changes in IL abundance after death. Interestingly, the ion of *m/z* 312 increased significantly to ~40% abundance at exposure concentrations of 2.5 and 5.0 mg/L (Figure 3b, c). The absence of hexadecylstearyldimethyl ammonium (*m/z* 523) and tetradecylstearyldiethylammonium (*m/z* 495) was observed in fish gills from the lowest exposure concentration. The simultaneous decrease in the abundance of AMMOENG 130 and the increase of the ion of *m/z* 312 as the major contributor in the gills led us to speculate that either AMMOENG 130 degradation was taking place in water during the study, or the metabolic conversion of AMMOENG 130 into a metabolite was at play (Figure 3d).

Metabolite Identification

The characterization of the metabolite was conducted by ESI-MS and ESI-MS/MS through HRMS analysis from zebrafish gill extract. The metabolite was identified by HRMS analysis as stearyltrimethylammonium ion, $[C_{21}H_{46}N]^+$. The accurate mass detected was 312.3619 Da and the exact mass as 312.3630 Da with a mass error of 3.5 ppm (Figure 4a). AMMOENG 130 was found in the zebrafish gill extract (*m/z* 550.6267) with a mass error of 4.5 ppm. The major fragments upon CID of the metabolite was an ion of *m/z* 296.3303 (Figure 4b). The major fragment of *m/z* 296.3303 is a common fragment in AMMOENG 130 (Figure 1b). The exact mass of the fragment was calculated to be 296.3317 Da, corresponding to a mass error of 1.4 ppm.

We suspect that zebrafish actively metabolized AMMOENG 130 during the course of exposure into a more polar compound for elimination and to reduce the IL's persistence and toxicity. An IL degradation study was carried out to exclude the ion of *m/z* 312 as an AMMOENG 130 degradation product in water throughout the course of exposure.

AMMOENG 130 Degradation and Metabolism

IL chemical degradation and biodegradation pathways by microbial breakdown have been investigated with modest success. Thus far, the greatest chemical degradation efficiency for imidazolium ILs was achieved in combination of UV light and oxidative catalysts of hydrogen peroxide and titanium dioxide in which 99% was degraded after 3 d. In contrast, microbial IL degradation seems far more feasible by incorporating biodegradable ester and amide side chains, enabling these functional groups to be microbially/enzymatically attacked to improve biodegradability [9, 15, 49]. Considering the stringent conditions and specific polar functionalities to promote IL degradation, degradation was not expected under ambient conditions over a short degradation time. Nevertheless, an IL degradation study was performed to monitor the production of AMMOENG 130 degradation products [49]. AMMOENG 130 was sampled by ESI-MS by dissolving the IL in water in concentrations of 1.25, 2.5, and 5.0 mg/L to mimic IL exposure to the zebrafish. The IL was monitored for 96 h, yet only data from time periods of 0 and 96 h are reported (Supplementary Figure S6).

Degradation products were not encountered in our experiments regardless of the IL concentration and exposure time. The intensity of all ions, most importantly of *m/z* 312, remained constant, fluctuating slightly within 1%–2% in abundance, which can be explained by the changes encountered upon sampling different regions of the water and slight instrument variability. AMMOENG 130 degradation was predicted to be highly unlikely, since structurally, AMMOENG 130 consists of a dimethylated quaternary ammonium head group with two unfunctionalized, saturated $C_{18}H_{37}$ alkyl chains. The sole presence of water was not sufficient to cause oxidative cleavage to produce degradation products. Degradation mechanisms require the presence of enzymes or highly nucleophilic species to breakdown lipophilic ILs [15]. Quaternary ammonium ILs prone to degradation are composed of functionalized cation head groups requiring high temperatures and long periods of time for degradation to occur [50].

Animal metabolism of xenobiotics occurs as means of detoxification and chemical elimination with the primary function of converting the parent compound into more hydrophilic compounds for excretion. Phase I metabolism (N-dealkylation, N-demethylation, N-oxidation) and phase II metabolism (sulfation and glucuronidation) are common enzymatic metabolic processes that are controlled by a range of cytochrome P450 (CYP) families and subfamilies in zebrafish [51]. In this case, the metabolic conversion of AMMOENG 130 into the metabolite may originate from dealkylation of the loss of $C_{17}H_{34}$. Metabolic reactions involving dealkylations are a major pathway in all organisms. In fish and mammals, trifluralin accumulates extensively and a large fraction is converted into metabolites. The major metabolic route of trifluralin consists of the oxidation of one of the N-propyl side groups followed by dealkylation. Among trifluralin metabolites, a broad range of biotransformations occurs such as N-oxidations, N-dealkylations, nitro reductions, and conjugations with amino acids [52, 53].

IL Accumulation in Whole Body Zebrafish

DESI-MS imaging was used to localize the accumulation of AMMOENG 130 in zebrafish tissue in all concentrations. Tissues were collected and analyzed from varying depths of the fish body, yet sections closer to the center of the fish body containing most biological systems such as brain, gill, and organs were mapped for AMMOENG 130.

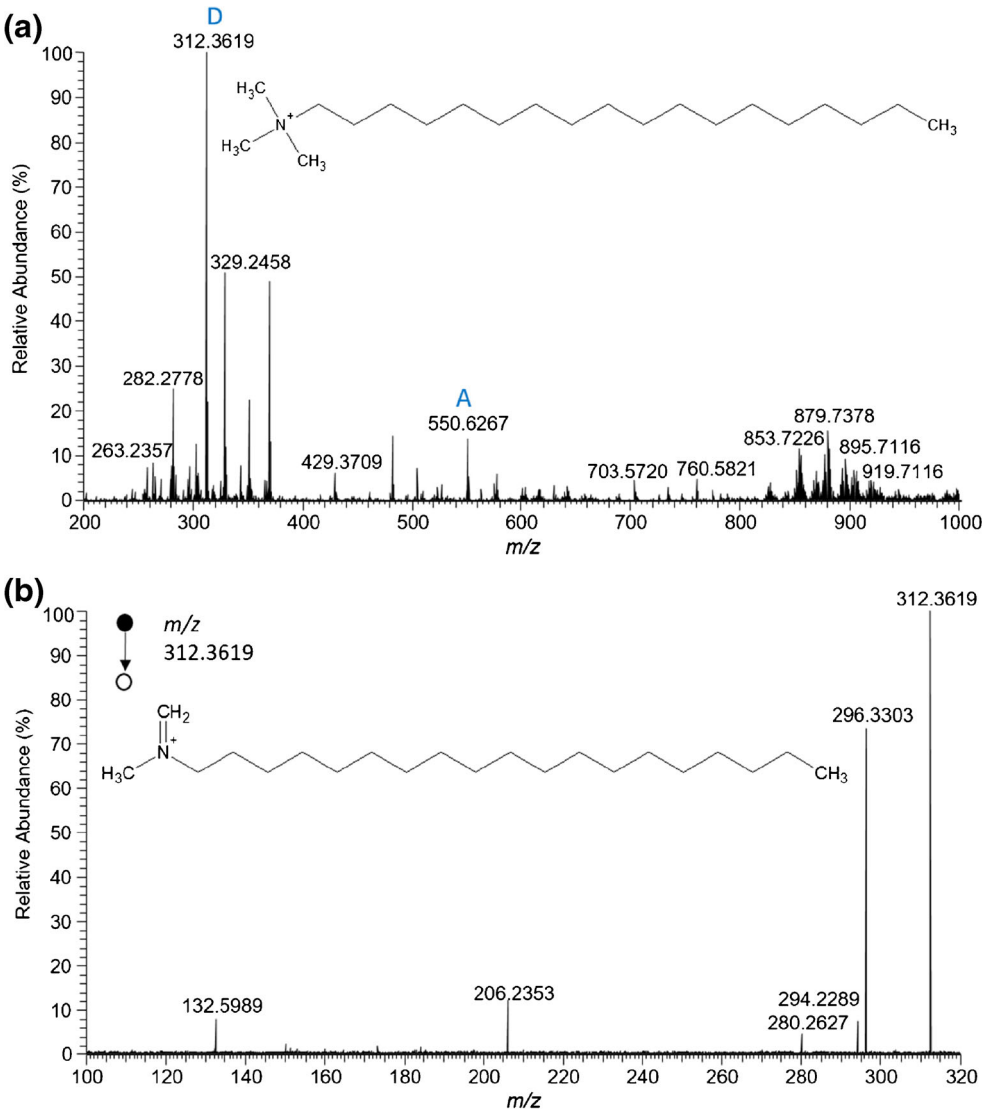

Figure 4. Metabolite characterization from zebrafish gill extract by HRMS analysis prepared from fish exposed to 1.25 mg/L of AMMOENG 130. (**a**) Full scan ESI-MS spectrum of the gill extract. AMMOENG 130 of *m/z* 550.6267 and the metabolite of *m/z* 312.3619. (**b**) ESI-MS/MS of the metabolite by CID using a collision energy between 50-51% (manufacturer's unit) displaying the major fragment of *m/z* 296.3303, and minor fragments of *m/z* 294.2289, 280.2627, 206.2353, and 132.5983

In fish exposed to IL concentrations of 5.0 mg/L, AMMOENG 130, hexadecylstearyldimethylammonium, and the metabolite accumulated in the gills (Figure 5). Zebrafish gills are responsible for a variety of physiological functions, including respiration, excretion of nitrogenous waste, osmoregulation, ionoregulation and acid-base regulation [54, 55]. Upon physiological damage of gill lamellae, the gill surface area for respiration is decreased, the fish gas-exchange process is disrupted, and is no longer functional [11]. The gills are a major site of damage induced by environmental pollutants, and it has been suggested as a crucial site of metabolism and the excretion of toxins [54]. Accumulation of AMMOENG 130 in the gills was found at a concentration of 2.5 mg/L (Figure 6). The accumulation in the outermost part of the tissue near the gills is most likely accumulation in fish epithelia. The complex and multifunctional epidermal barrier is capable of defending against the introduction of harmful xenobiotics; however, lipophilic ILs upon insertion can cause havoc on the structural integrity of membranes, and thus xenobiotics accumulate. The highly toxic exposure concentrations near the LC_{50} induced mortality quite quickly and did not allow the IL to accumulate in other organs during a short time of exposure of merely 24 h.

Zebrafish was mapped for AMMOENG 130 exposed to 1.25 mg/L; however, only AMMOENG 130 (*m/z* 551) and the metabolite (*m/z* 312) were detected (Figure 7). The regions of accumulation in this lower concentration differed from concentrations of 5.0 and 2.5 mg/L because of a longer exposure time of 96 h.

The IL was primarily localized in the respiratory and nervous system of the zebrafish. AMMOENG 130 was distributed in the brain, gills, and other unidentified regions (Figure 7c),

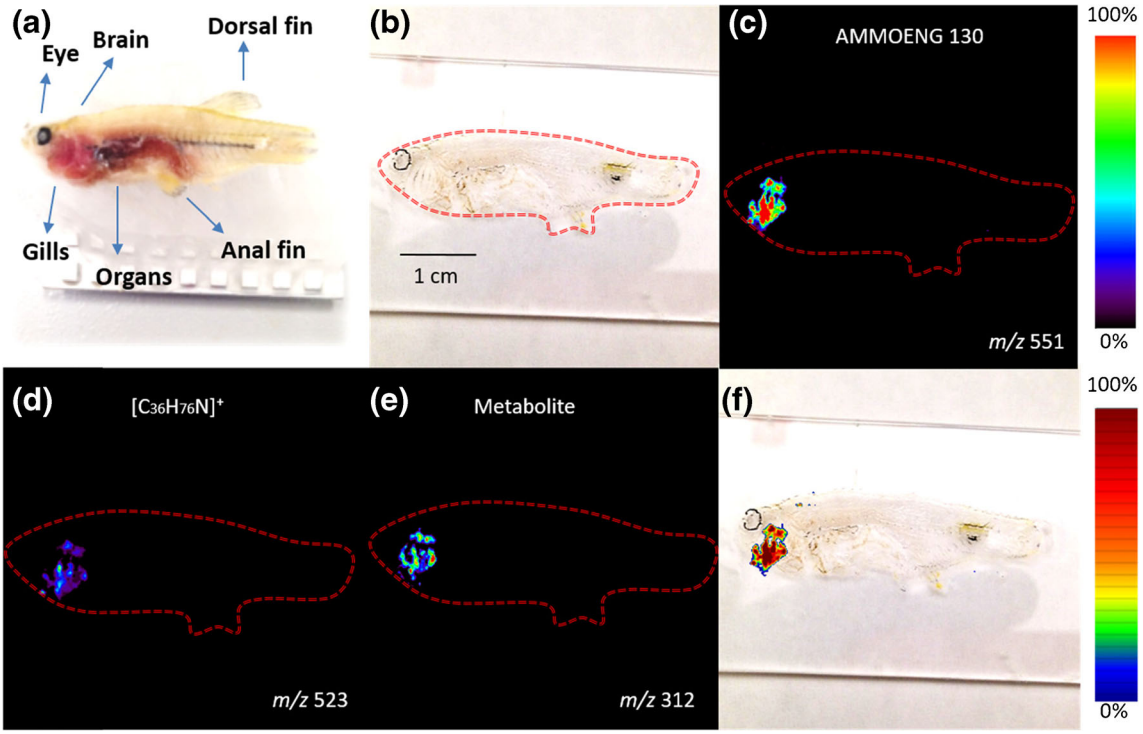

Figure 5. Accumulation of AMMOENG 130 in whole body zebrafish exposed to concentrations of 5.0 mg/L analyzed by DESI-MS imaging. (**a**) Zebrafish image outlining organ systems. (**b**) Optical image of the zebrafish tissue section under analysis. (**c**) Accumulation of AMMOENG 130 (*m/z* 551). (**d**) Accumulation of hexadecylstearyldimethylammonium (*m/z* 523). (**e**) Metabolite accumulation (*m/z* 312). (**f**) Overlay of the optical image with the DESI-MS image of AMMOENG 130. All images and overlays produced in both scale bars were normalized with Biomap

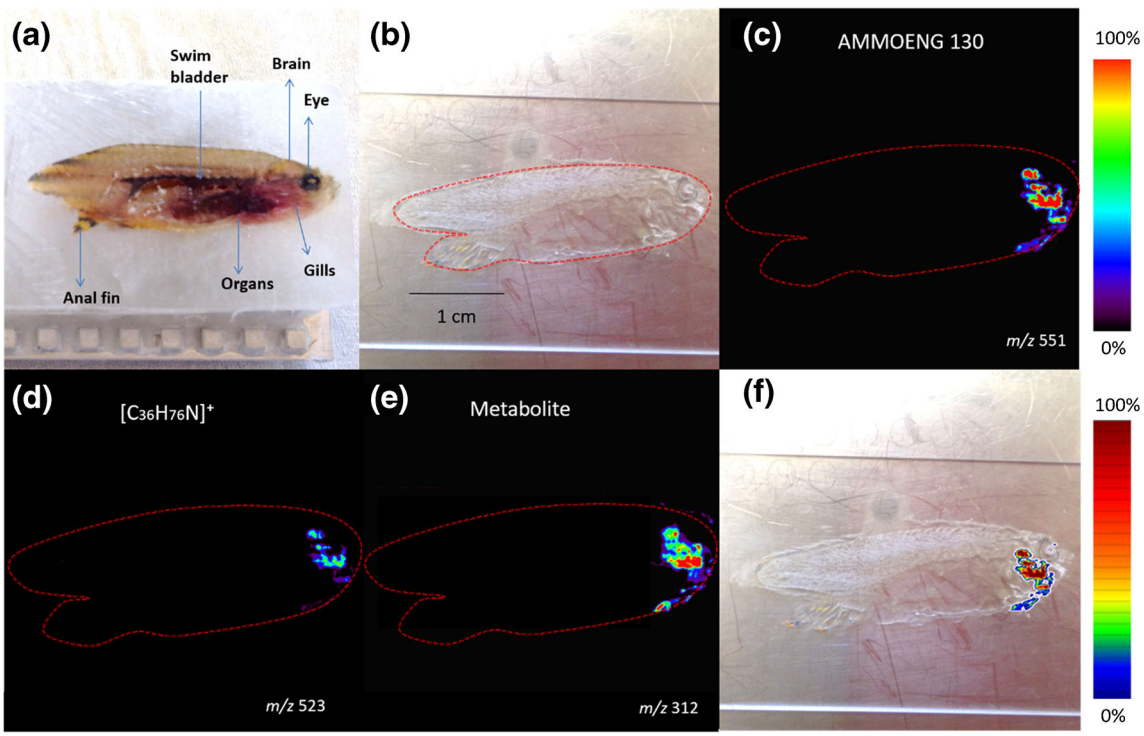

Figure 6. Accumulation of AMMOENG 130 in whole body zebrafish exposed to concentrations of 2.5 mg/L by analyzed DESI-MS imaging. (**a**) Zebrafish image of organ systems. (**b**) Optical image of the zebrafish tissue section under analysis. (**c**) Accumulation of AMMOENG 130 (*m/z* 551). (**d**) Accumulation of hexadecylstearyldimethylammonium (*m/z* 523). (**e**) Metabolite accumulation (*m/z* 312). (**f**) Overlay of the tissue optical image with the DESI-MS image of AMMOENG 130. All images and overlays produced in both scale bars were normalized with Biomap

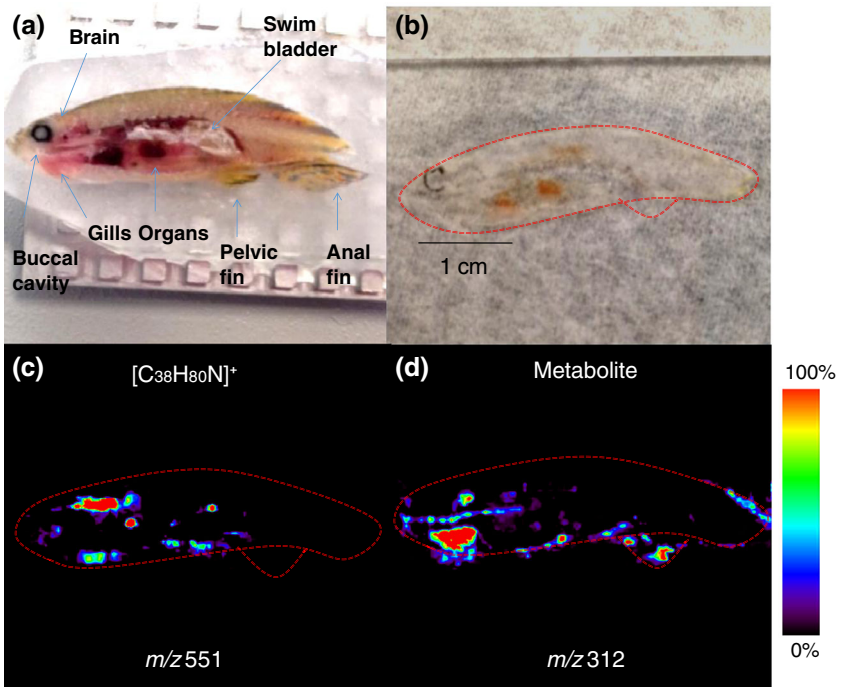

Figure 7. Accumulation of AMMOENG 130 analyzed by DESI-MS imaging in whole body zebrafish exposed to a concentration of 1.25 mg/L. (**a**) Optical image of CMC embedded fish during cryosectioning outlining organ systems. (**b**) Optical image of the zebrafish tissue section under analysis overlapped with the DESI-MS image of the metabolite. Images were normalized in both scale bars with Biomap. (**c**) Accumulation of AMMOENG 130 (*m/z* 551). (**d**) Metabolite accumulation (*m/z* 312)

whereas the metabolite was mainly present in high abundance in the gills and in lower abundance in the brain (Figure 7d). Both the IL and the metabolite showed an accumulation pattern in membrane-rich areas. AMMOENG 130 was found in highest abundance in the brain, suggesting that the IL was capable of penetrating the blood brain barrier (BBB), the brain's main defense system against toxic substances, with subsequent accumulation in the nervous system. Lipophilic ILs, like AMMOENG 130, resemble endogenous lipids in membranes; the amphiphilic nature of these species containing a positively charged hydrophilic head group and long hydrophobic alkyl chains allows for membrane insertion. Yet, to ultimately breach the brain via the BBB highly depends on the size of the xenobiotics in question. The zebrafish BBB is a highly selective size-dependent barrier that allows the passive diffusion of lipid-soluble molecules across the membrane with greater ease than hydrophilic molecules [56]. Therefore, AMMOENG 130 most likely has greater ease than the metabolite of diffusing through the BBB as can be seen with the higher accumulation of AMMOENG 130 in the brain than the metabolite in Figure 7c. With respect to metabolism, we can speculate that the biotransformation occurred in fish gills and the metabolite either partly diffused from gills into the brain and/or AMMOENG 130 was metabolically transformed locally in the brain to give rise to the metabolite. In either case, both plausible metabolic routes indicate the metabolism of AMMOENG 130 in two major organ systems: the respiratory system and the nervous system. However, the high abundance of metabolite in the gill tissue leads us to suggest that gills play a major role in the metabolic biotransformation of AMMOENG 130.

Accumulation of AMMOENG type ILs in the nervous system is of concern in regards to respiratory toxicity and neurotoxicity. The metabolite consists of a trimethylated ammonium head group with a $C_{18}H_{37}$ hydrophobic alkyl chain; the cation moiety structurally resembles biological neurotransmitters choline and acetylcholine in the brain. Choline transport through the blood brain barrier is carrier-mediated, saturable, and the uptake of nitrogen-methylated choline analogs can block choline binding active sites, causing inhibition. Quaternary ammonium binding recognition is a major transport restraint in the BBB. A pyrene derivative, 2-[-4-(1-pyreny l0 butyryloxy-ethyl]-trimethylammonium ion has been shown to be a choline competitive inhibitor with a 20-fold increased in affinity compared with choline in rat synaptosomes. The increased affinity was a result of the hydrophobicity of the compound interacting with the membrane core or adjacent hydrophobic binding regions [57]. Subsequently, AMMOENG 130 and the metabolite may act as an inhibitor, effectively entering the BBB, accumulating in the brain, and potentially causing neurotoxic effects. These findings are rather concerning since AMMOENG type ILs may possibly be neurotoxic for zebrafish. Nevertheless, AMMOENG 130 and its metabolite's neurotoxicity must be investigated further to arrive at conclusive results. The results were highly reproducible; in all fish, we did not observe accumulation in other regions. Several tissue sections were analyzed per fish to ensure that the results were consistent. Approximately 5–10 tissue slides were

analyzed per fish at the center of the fish body where most organs are located. Many other tissue slides were also used for the analysis from varying depths of the fish body to ensure that accumulation did not take place elsewhere.

The accumulation of AMMOENG 130 was not detected in the liver, pancreas, or gastrointestinal system by MS analysis; the conversion of AMMOENG 130 to the metabolite may originate as means of extrahepatic metabolism. Zebrafish cytochrome P450 2J (CYP2J) subfamilies have been identified as catalysts for arachidonic acid metabolism in extrahepatic tissues of many species, including the zebrafish, with a high degree of similarity to the activity of CYP2J enzyme in humans [51]. Spot fish (*Leiostoma xanthrus*) exposed to radioactive tributyltin (^{14}C-TBT) in the water metabolized ^{14}C-TBT into dibutyltin, monobutyltin, and other conjugates in extrahepatic tissues. TBT concentrations monitored after 2 and 4 d remained considerably higher in gill tissue compared with other tissues [58]. We have presented here the accumulation and metabolism of AMMOENG 130 into a dealkylated metabolite by DESI-MS imaging as a proof of principle study in which this ambient MS imaging technique has potential to rapidly monitor environmental accumulation of persistent ILs in small aquatic organisms.

DESI-MS as a Potential Tool for Monitoring Environmental Pollutants

The viability of DESI-MS as a potential monitoring technique was explored to assess if environmentally relevant ILs exposed aquatic organisms could be evaluated by this technique. To study the limit of detection (LOD) of AMMOENG 130, PTFE coated microscopic glass slides were used as the substrate, and AMMOENG 130 solutions in methanol were spotted in a wide range of concentrations. The DESI-MS spectrum was collected for 2 min for 1 μL spots of 1, 10, 100 ng/L, and 1 μg/L, yet none of these concentrations were detected by DESI-MS. The AMMOENG 130 peak of *m/z* 551 was not distinguishable from the average noise level. Higher concentrations were spotted from 10, 100 μg/L, 1, 10 mg/L to 100 mg/L corresponding to a mass of 10, 100 pg, 1, 10, and 100 ng in the 1 μL spotted area. The AMMOENG 130 peak was detected in all concentrations; however, at 10 μg/L, AMMOENG 130 was six times greater than the signal to noise (S/N) ratio. Then, AMMOENG 130 concentrations of 8, 9, 10, 11, and 12 μg/L were spotted onto the PTFE substrate. The IL peak was not detected from the noise level at concentrations of 8 and 9 μg/L. However, the IL peak was found in concentrations of 10–12 μg/L ranging from 3 to 6 times greater than the S/N. The LOD for several pharmaceutical compounds have been previously investigated with DESI-MS on PTFE substrates [60]. Compounds such as propanolol, dobutamine, and verapamil were analyzed in (+)DESI-MS where LODs ranged between 10 and 10,000 μg/L, whereas LODs for compounds such as chloramphenicol and ibuprofen detected in (–)DESI-MS ranged between 10 and 100,000 μg/L [59]. Therefore, the LOD of AMMOENG 130 was either comparable or substantially better than several pharmaceutical compounds.

Analytical parameters such as the LOD, limit of quantification (LOQ), linearity, dynamic range, inter-day, and intra-day precision could not be assessed because of the lack of an isotopically labeled standard or analog. When assessing analytical parameters in biological tissue (i.e., zebrafish), biological matrix effects must be considered specifically for the tissue type for IL recovery from that particular matrix and for potential matrix ion suppression effects that may increase analytical parameters. Nevertheless, DESI-MS and DESI-MSI show promise to evaluate environmentally relevant IL concentrations in aquatic organisms. Quantification with DESI-MS is still an emerging research area in ambient mass spectrometry. Analytical improvements need to be made in terms of reproducibility and uniform deposition of the internal standard onto the sample surface for validation of the quantification method by DESI-MS. As of now, DESI-MS and DESI-MSI quantitation require validation with external and more routinely used quantitative methods. DESI-MS quantitation has not been investigated thoroughly enough to be established as a quantitative technique; therefore, further investigations in this area are still necessary for future applications in ambient mass spectrometry [25, 60].

Conclusions

It was the intent of this study to introduce DESI-MSI as a novel, rapid, and sensitive technique to explore the accumulation of toxic IL in a popular aquatic vertebrate model. DESI-MSI has provided simultaneous characterization, accumulation, and metabolite detection of AMMOENG 130 with minimal sample preparation and short analysis time. DESI-MSI provided new insights into the accumulation pattern of AMMOENG 130 and a dealkylated metabolite in fish respiratory and nervous system. The accumulation of the metabolite in the brain leads us to question whether this compound could illicit neurotoxic effects and potentially act as a neurotoxin to zebrafish. Innovative techniques and methodologies are required to investigate the bioaccumulation of toxic compounds from the environment for environmental hazard and health risk assessments. IL persistence within aquatic organisms can lead to indirect toxic effects by secondary poisoning in ecosystems and negatively impact wildlife. The application of this technique may be extended to bioaccumulation studies involving IL exposure of environmentally relevant concentrations in zebrafish and in other small aquatic organisms. In the future, biomonitoring of ILs can be investigated in a quantitative study with DESI-MSI as a means of accurately determining IL concentrations within zebrafish tissue after environmental exposure. Hence, this research opens new avenues in the study of ecotoxicology of small aquatic organisms from environmental exposure of not only ILs but also other chemical pollutants, pesticides, and drugs to biomonitor ubiquitous, persistent, and toxic compounds.

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