# Bioconcentration of Dissolved Organic Compounds from Oil Sands Process-Affected Water by Medaka (*Oryzias latipes*): Importance of Partitioning to Phospholipids

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Ⓢ *Supporting Information*

**ABSTRACT:** The complex mixture of dissolved organics in oil sands process-affected water (OSPW) is acutely lethal to fish at environmentally relevant concentrations, but few bioconcentration factors (BCFs) have been measured for its many chemical species. Japanese medaka (*Oryzias latipes*) were exposed to 10% OSPW, and measured BCFs were evaluated against predicted BCFs from octanol–water distribution ratios ($D_{OW}$) and phospholipid membrane–water distribution ratios ($D_{MW}$). Two heteroatomic chemical classes detected in positive ion mode ($SO^+$, $NO^+$) and one in negative mode ($O_2^-$, also known as naphthenic acids) had the greatest $D_{MW}$



values, as high as 10 000. Estimates of $D_{MW}$ were similar to and correlated with $D_{OW}$ for $O^+$, $O_2^+$, $SO^+$, and $NO^+$ chemical species, but for $O_2^-$ and $SO_2^-$ species the $D_{MW}$ values were much greater than the corresponding $D_{OW}$, suggesting the importance of electrostatic interactions for these ionizable organic acids. Only $SO^+$, $NO^+$, and $O_2^-$ species were detectable in medaka exposed to OSPW, and BCFs for $SO^+$ and $NO^+$ species ranged from 0.6 to 28 L/kg, lower than predicted (i.e., $1.4-1.7 \times 10^3$ L/kg), possibly because of biotransformation of these hydrophobic substances. BCFs of $O_2^-$ species ranged from 0.7 to 53 L/kg, similar to predicted values and indicating that phospholipid partitioning was an important bioconcentration mechanism.
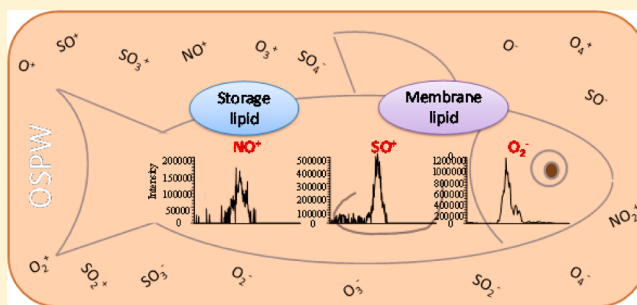
## ■ INTRODUCTION

The oil sands of northern Alberta, Canada, are among the largest proven petroleum reserves in the world, and in 2014 production of crude bitumen reached 1.9 million barrels per day.[1] For each barrel of bitumen produced, more than one barrel of acutely toxic[2] oil sands process-affected water (OSPW) is added to growing inventories in tailings ponds.[3] The seepage of OSPW from tailing ponds to the Athabasca River and its tributaries is of concern, and a detailed water chemistry study suggested that OSPW was already reaching the Athabasca River through groundwater flow.[4] Moreover, future end-pit lakes containing aged or treated OSPW will be hydraulically connected to the natural aquatic system.[5] Commissioned in late 2012, the first full-scale end-pit lake is located on the site of Syncrude Canada, Ltd., and is called Base-Mine Lake. This water is not currently discharged, but it is now

important to study the fate and behavior of chemicals dissolved in its OSPW.

The toxicity of OSPW is attributable to a complex mixture of dissolved organic compounds derived from bitumen. Applications of ultrahigh-resolution mass spectrometry characterization show the presence of thousands of acidic and nonacidic heteroatom-containing chemical species in OSPW. Each species is identifiable by its unique empirical chemical formula (derived from accurate mass) containing carbon, hydrogen, and various combinations of oxygen, sulfur, or nitrogen heteroatoms (e.g., $C_xH_xO_xS_xN_x$). Most of the chemical species are not pure compounds but are complex mixtures of structural isomers that

are exceedingly difficult to separate and identify.[6,7] For qualitative purposes, the thousands of species can also be binned into classes based on their empirical formula and described by numbers of oxygen, sulfur, or nitrogen, and whether they are detected in negative (−) or positive ionization (+) modes, that is, $O_X^{+/-}$, $NO_X^{+/-}$, $SO_X^{+/-}$, $S_2O_X^{+/-}$, $NO_XS^{+/-}$, $N^{+/-}$, and $S^{+/-}$.[8] For example, all simple carboxylic acids are detected in negative mode and are binned to the $O_2^-$ empirical formula class (commonly termed naphthenic acids), whereas polar neutral compounds, such as dihydroxyl, diketo, or keto-hydroxyl species are detected in positive mode and binned to the $O_2^+$ class.

As a first estimate of the bioconcentration potential of organic compounds in OSPW, in previous work polydimethylsiloxane (PDMS) coated stir bars were used to estimate the octanol−water distribution ratio ($D_{OW}$), or apparent $K_{OW}$, for 2114 organic species at native OSPW pH (pH 8.4). Using a limited training set of neutral and ionizable compounds, results of this study demonstrated the unexpected hydrophobic nature of certain chemical species in OSPW, in particular for $NO^+$ and $SO^+$ species whose $D_{OW}$ were as great as 203 000.[9] PDMS and octanol are used as surrogates for storage of neutral compounds in lipids of biota, thus partitioning from water to PDMS or octanol can be used to satisfactorily predict bioconcentration potential for neutral organic compounds, including ionizable compounds that are predominantly neutral in test solutions.[10]

However, for ionizable organic compounds that are predominantly charged at physiological pH, the extent of partitioning into biota would likely be underestimated by use of partitioning to octanol or PDMS.[11] This is because lipids in biota consist not only of neutral storage lipids, but also of membrane lipids with polar and ionizable head groups (e.g., phospholipids).[12] $D_{OW}$ cannot adequately describe the interaction of ionizable compounds with such membranes because it neither mimics the ordered structure and anisotropy of biological membranes, nor electrostatic interactions between ionizable compounds and zwitterionic head groups in bilayer membranes.[11−13] For example, it has been reported that at pH 7.4, affinities of 18 drugs for membranes were greater than affinities predicted based on $D_{OW}$.[14] Moreover, phospholipids make up the functional compartments of cells and organelles, thus measurements of membrane−water partitioning for chemical species in OSPW would not only allow more accurate prediction of bioconcentration, but also of acute toxicity prediction for chemicals acting through mechanisms involving membrane disruption.

The traditional method to assess partitioning of organic compounds from water to membranes is equilibrium dialysis with liposomal systems, which is accurate but time-consuming. Faster alternative methods have been developed, including retention factors on chromatographic stationary phases containing immobilized artificial membranes (IAM) consisting of phospholipids.[15] Nevertheless, electrostatic interactions with incompletely shielded charged surfaces of IAM columns can lead to biased results.[16] An emerging rapid method involves solid-supported lipid membranes, composed of silica beads coated with a realistic phospholipid bilayer. This system has been shown to be capable of high-throughput and accurate determination of phospholipid membrane−water distribution ratios.[14]

Most previous in vivo bioconcentration studies with OSPW have focused only on naphthenic acids, since these were previously assumed to be major contributors to toxicity of

OSPW.[17,18] An effects-directed analysis study recently confirmed that naphthenic acids are a major cause of the acute toxicity, but that other polar neutral substances containing nitrogen and oxygen ($NO^+$ empirical formula class) and sulfur and oxygen ($SO^+$ empirical formula class) also contributed.[19] Naphthenic acids have been detected in tissues of rainbow trout fingerlings exposed to OSPW, and also in tissues of wild fishes collected from rivers near oil sands deposits.[17,18] On the basis of muscle of rainbow trout (*Oncorhynchus mykiss*) that had been exposed to a commercial mixture of naphthenic acids, the BCF of one species of naphthenic acid with 13 carbons and 3 double bond equivalents (DBE) ($C_{13}H_{22}O_2$) was estimated to be approximately 2 at pH 8.2, and approximately 4 at pH 7.6.[18,20] However, the $D_{OW}$ for naphthenic acids increases with number of carbons and decreases with increasing DBE.[9] Thus, data for a single naphthenic acid species is insufficiently informative, and a more detailed in vivo study of bioconcentration is needed to assess the accumulation potential for the wide range of organic chemical species in authentic OSPW.

In this study, phospholipid membrane−water partitioning constants were measured for all detectable OSPW chemical species using solid-supported lipid membranes (TRANSIL), and BCFs were predicted based on a QSAR model that combined partitioning to storage lipids and phospholipids of fish. These predictions were then compared to empirical BCFs measured in juvenile Japanese medaka (*Oryzias latipes*) exposed to 10% OSPW.

## ■ MATERIALS AND METHODS

**Chemicals and Materials.** Acetic acid, formic acid, dichloromethane (DCM), methanol, acetonitrile (HPLC grade), and water (Optima grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Lauric-$d_{23}$ acid ($C_{11}D_{23}CO_2H$) internal standard was purchased from Sigma-Aldrich (St. Louis, MO, USA) and progesterone (2,3,4-$^{13}C_3C_{18}H_{30}O_2$, 99%) internal standard was from Cambridge Isotope Laboratories, Inc.(Tewksbury, MA, USA). TRANSIL$^{XL}$ membrane affinity kit was obtained from Sovicell GmbH (Leipzig, Germany).

**OSPW Sample.** The OSPW sample was collected July 15, 2014 on the site of Syncrude Canada, Ltd. (Fort McMurray, Alberta, Canada) from Base-Mine Lake. This body of water was originally an active tailings pond but is now the first end-pit lake in the Athabasca oil sands region. Constructed from OSPW overlying fluid fine tailings, Base Mine Lake was commissioned in December 2012, at which time the supply of fresh tailings and OSPW was turned off. Thus, OSPW in Base Mine Lake now represents a relatively "aged" sample. The OSPW sample was collected by pumping into 20 L high-density polyethylene pails from a floating barge. After collection, the OSPW was kept at 4 °C in a walk-in refrigerator until use. The fish exposures began September 17th 2014, and membrane partitioning experiments were done with the same water in October 2014.

**Stock Preparation for Phospholipid Membrane Distribution Testing.** A total of 1 L of OSPW was filtered with a 0.45 $\mu$m filter (Millipore, Billerica, MA) to remove suspended solids. The pH of the filtered water was reduced to 2 using 95% $H_2SO_4$ and then extracted with 2 × 200 mL of DCM. The extract was combined and evaporated to near dryness with a rotary evaporator (model R-210, Buchi, Toronto, Ontario, Canada). The remaining volume was transferred to a 20 mL glass vial and taken to full dryness under a gentle stream of nitrogen at room temperature (Turbovap LV, Biotage, Charlotte, NC). This was dissolved in 1 mL of dimethyl

sulfoxide (DMSO) to obtain a 1000× stock solution of dissolved organics in OSPW. This concentrated stock was then used to prepare 50× stock solutions for the phospholipid partitioning experiments.

**Determination of Phospholipd Membrane Distribution.** The phospholipid membrane partitioning for each organic species in OSPW was evaluated using the TRANSIL$^{XL}$ membrane affinity kit which is designed to measure the distribution coefficient of test compounds between phosphatidylcholine membranes and aqueous Dulbecco's phosphate buffered saline (DPBS) buffer at pH 7.4. Membranes are supplied as surface-modified silica beads noncovalently coated with a phospholipid bilayer (phosphatidylcholine) in aqueous DPBS buffer at pH 7.4. Each kit was composed of eight vials with varying contents of total membrane, which was used in accordance with the user guide. Briefly, two vials containing only buffer served as controls to measure background and any nonspecific adsorption. The other six vials contained silica beads with total membrane contents ranging from 0.14 to 4.40 $\mu$L lipid per mL of buffer. An aliquant of 10 $\mu$L of 50-fold stock solution was added to every vial. After vortex mixing for 15 s, mixtures were incubated overnight. To separate membrane and adsorbed content from the medium, vials were centrifuged for 10 min at 750 g and supernatant was collected for identification and semiquantification of species by HPLC-Orbitrap.

**Calculation of Phospholipid Membrane-Water Distribution Ratios ($D_{MW}$).** $D_{MW}$ is defined as the ratio between the total concentration of a chemical species in the membrane ($c_l$) to the total concentration of the same species in buffer ($c_b$) (eq 1).

$$D_{MW} = \frac{c_l}{c_b} \tag{1}$$

The terms $c_l$ and $c_b$ can be calculated for each vial by use of the mass balance (eq 2).

$$n_t = c_b \times V_b + c_l \times V_l \tag{2}$$

where $n_t$ is total mass of the species and $V_b$ and $V_l$ are the volumes of buffer and total lipid membrane in every vial, respectively. Equation 2 can be rearranged (eq 3) so that $D_{MW}$ can be determined from the slope of a line by plotting the ratio of $n_t$ divided by $c_b$ as a function of $V_l$ for each vial.

$$\frac{n_t}{c_b} = \frac{c_l}{c_b} \times V_l + V_b = D_{MW} \times V_l + V_b \tag{3}$$

The TRANSIL$^{XL}$ membrane affinity kit is generally recommended for measuring log $D_{MW}$ values >2 because compounds with lower log $D_{MW}$ typically yield supernatant concentrations in the assay that can deviate only marginally from control signals. However, in the current study, statistically significant log $D_{MW}$ values could be determined down to 1.2 with "good" Transil quality index scores (i.e., TQI > 7). Thus, in the current study, log $D_{MW}$ of 1.2 was an operationally defined limit of detection for significant membrane binding.

As previously noted, most chemical species in OSPW are mixtures of structural isomers, and the current $D_{MW}$ measurements, as well as previous $D_{OW}$ measurements, are weighted averages for all components sharing the same chemical formula.[9] Assuming that all isomers of a chemical species are not equivalent (i.e., have different true $D_{MW}$ or $D_{OW}$), it must be acknowledged that there is no single distribution coefficient that can link aqueous concentration of a mixture to the corresponding concentration of the same mixture in a

hydrophobic phase (e.g., storage lipid or phospholipid).[21] This is because it can depend on variable composition of the mixture, and to some extent on the ratio of water:hydrophobic phase. Nevertheless, for the current measurements of $D_{MW}$ the water/phospholipid ratio was varied and resulted in straight line relationships, thus the impact of the latter limitation is likely only minor here.

**Bioconcentration of Chemical Species by Fish.** Juvenile medaka were selected from a culture tank and randomly placed into 30-L glass tanks at 25 ± 1 °C. The experimental setup consisted of the following treatment tanks, with each tank containing 9 fish: 2 tanks of control (i.e., laboratory dilution water, pH 8.1) female medaka, 2 tanks of control male medaka, 3 tanks of female medaka exposed to a 10-fold dilution of OSPW (pH 8.24 ± 0.12) and 3 tanks of male medaka exposed to a 10-fold dilution of OSPW. To remove suspended solids, OSPW was filtered through a 0.45 $\mu$m glass filter paper (Millipore, Billerica, MA) before dilution. A semistatic experimental condition was used, with 50% of test water (15 L) renewed every 24 h in each tank. Exposure water pH (mean = 8.24 ± 0.12), which is consistent with the pH in the Athabasca River system (pH 8.0–8.5),[22] and dissolved oxygen concentration (>70%) were monitored daily in each tank. The photoperiod was 16:8 h light/dark, and fish were fed approximately 30 mg flake food per tank per day.

A single fish from each exposure tank and each control tank was collected at each predetermined time during the uptake phase of the experiment: 6, 24, 72, 144, and 288 h. Samples of water (1 L) were collected below the surface of each tank into polypropylene bottles at the same time that fish were sampled. At 288 h, the remaining fish were transferred to tanks containing clean fresh water, in which they were allowed to depurate. Extra care was taken to reduce contamination of water during the depuration phase by rinsing fish for 10 min in an intermediate 10 L tank containing clean water before being transferred to the tanks containing clean water where depuration would be followed. During the depuration phase, one fish from each tank and each control tank were sampled at each time: 6, 24, 72, and 144 h. Samples of water (1 L) were also collected at 6 and 72 h of the depuration phase. During the entire experiment, no fish died in any of the exposures.

Studies were performed in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan and medaka were from a culture maintained in the ATRF. All handling of fish and exposures were in accordance with protocols approved by the University of Saskatchewan Committee on Animal Care and Supply and Animal Research Ethics Board (UCACS-AREB; no. 20090108)

**Identification and Semi-quantification of Chemical Species in Fish and Water.** After spiking with recovery standards, whole fish were homogenized in 1.5 mL plastic (polypropylene copolymer) centrifuge tubes using a Tissue-Lyser II ball mill with stainless steel beads (Qiagen, Hilden, Germany). Homogenates were first extracted with 2 × 1 mL DCM and then extracted twice again with 1 mL DCM after adding 50 $\mu$L formic acid to adjust the pH. The extract was dried under gentle high-purity nitrogen gas, and taken up in 400 $\mu$L of 1:3 (v/v) water:acetonitrile. Solutions were centrifuged at 16089 g for 10 min to pellet the protein, and supernatants were transferred to hybrid SPE-precipitation cartridges (for phospholipid removal) with the zirconia coated silica as stationary phase (500 mg, 6 mL, Sigma-Aldrich, St. Louis, MO, USA) preconditioned with 6 mL 1% formic acid in
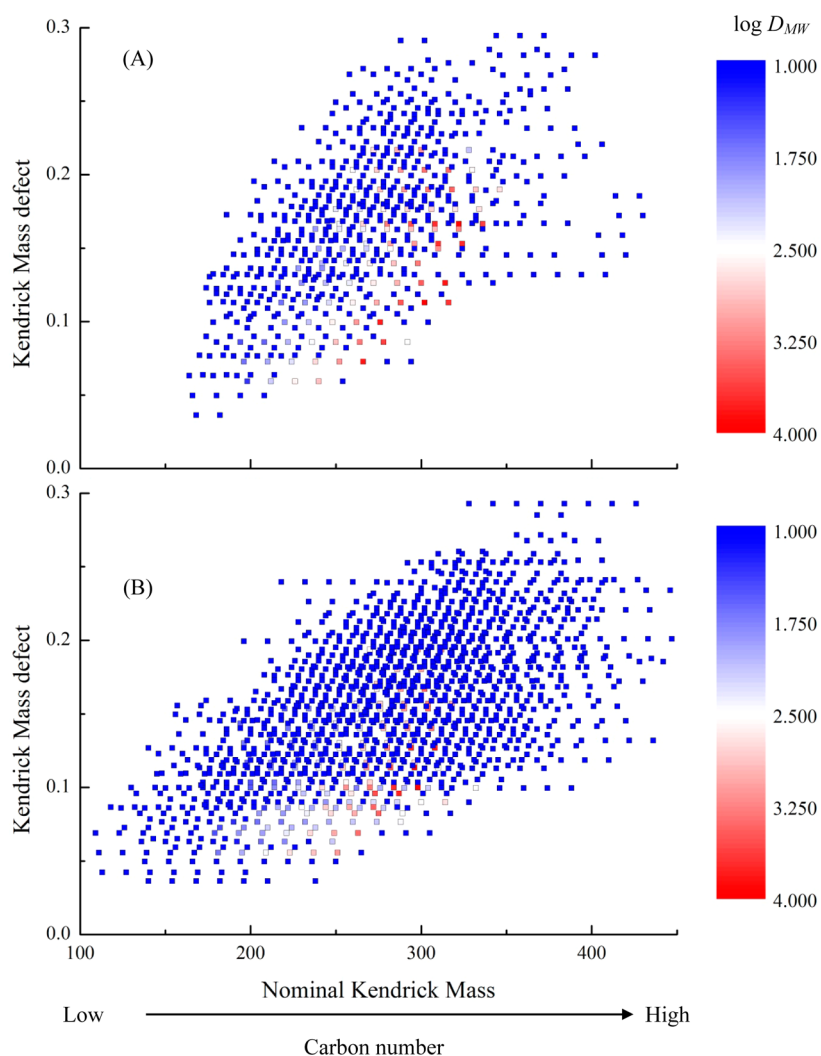
**Figure 1.** Heat maps of log $D_{MW}$ for organic compounds in OSPW observed by HPLC-Orbitrap in electrospray (A) negative mode, and (B) positive mode. Each colored square marker represents an individual chemical species with a distinct chemical formula (i.e., $C_xH_xO_xS_xN_x$), and horizontal lines generally represent a homologous series of species that differ only in carbon number.

acetonitrile. Cartridges were then eluted with 6 mL formic acid in acetonitrile, then 6 mL 1% ammonium formate in methanol. Extracts were dried under gentle nitrogen, taken up in 200 $\mu$L of acetonitrile and analyzed by use of HPLC-Orbitrap after filtration with 0.22 $\mu$m syringe filters (Millipore, Billerica, MA, USA). Water samples from the fish tanks were spiked with internal standards and analyzed directly by HPLC-Orbitrap after filtration with 0.22 $\mu$m syringe filters. All fish sampled were extracted and analyzed individually, and all analytes detected in samples were blank subtracted by comparison to control fish.

**Instrumental Analysis.** An Accela HPLC (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an Orbitrap Elite, hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for identification and quantification of species. Chromatographic separation was carried out on an XSELECT CSH C18 XP column (130 Å, 2.5 $\mu$m, 3 mm × 150 mm) (Waters, Milford, MA, USA) at 25 °C with a flow rate of 0.5 mL/min. An injection volume of 20 $\mu$L was used for supernatant sampled from TRANSIL$^{XL}$ membrane affinity kit, 10 $\mu$L for extracts of medaka and 50 $\mu$L was used for samples of water. Mobile phases consisted of (A) 0.1% acetic acid in water and (B) 100% methanol, and the gradient flow was 5% B for 1

min, followed by a linear gradient ramp to 90% B at 9 min, to 99% B over 5 min, and returning to 5% B in 1 min followed by a 4 min hold prior to the next injection.

The Orbitrap was operated with an electrospray (ESI) source operating in either positive or negative mode; separate injections of samples were made for each mode. Needle voltage was set at ±5 kV, while the sheath, aux, and sweep gas flows were set to 40 (arbitrary unit), 5 (arbitrary unit), and 2 (arbitrary unit), respectively. Capillary temperature was 300 °C. Acquisition was performed in scan mode from $m/z$ 100 to 500, with $m/z$ resolving power set to a nominal value of 240 000. Mass calibration and tuning was done externally by direct infusion of Pierce ESI negative ion calibration solution, or Pierce LTQ Velos ESI positive ion calibration solution (Thermo Fisher Scientific, Rockford, USA). The negative ion mode calibration solution covered a mass range from $m/z$ 265 to $m/z$ 1880 and the positive ion mode calibration solution covered a mass range from $m/z$ 74 to $m/z$ 1822, and both confirmed good $m/z$ accuracy (<2 ppm) between $m/z$ 50 and 2000. All data acquisition and analysis was performed with Thermo Xcalibur software.
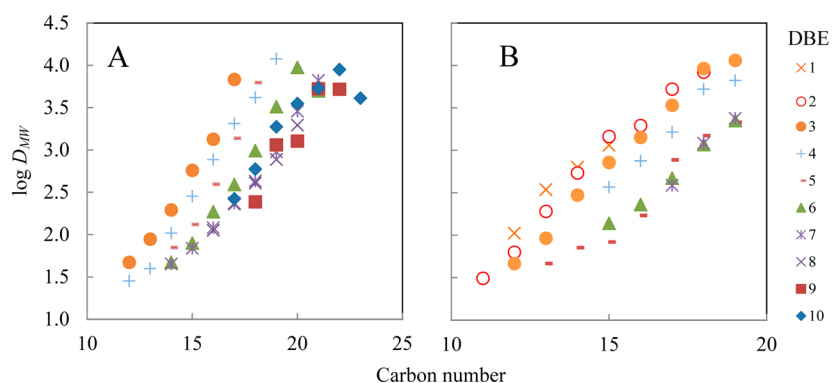
**Figure 2.** Effect of number of carbons and double-bond equivalents (DBE) on log $D_{MW}$ of example organic species in OSPW: (A) $O_2$ species observed in ESI negative mode and (B) SO species observed in ESI positive mode.
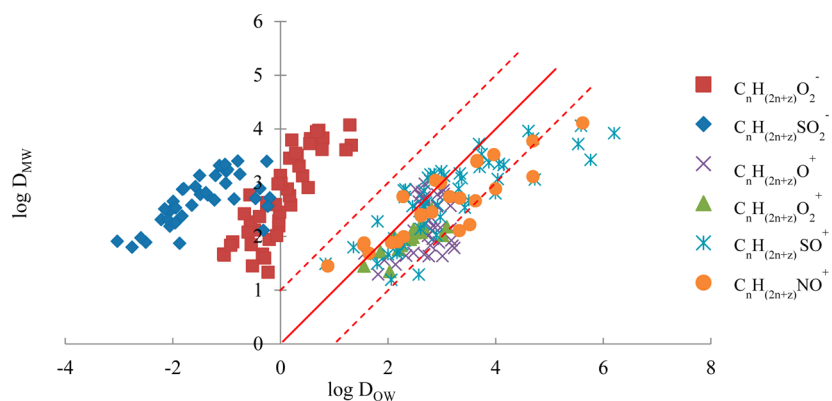


**Figure 3.** Comparison between log $D_{MW}$ and log $D_{OW}$ for 6 selected empirical formula classes in OSPW. The solid dashed line represents 1:1 agreement, and the dashed orange lines represent the range of ±1 log unit. Acidic compounds ($O_2^-$ and $SO_2^-$) had greater affinities for phospholipids than for octanol (i.e., PDMS).

## ■ RESULTS AND DISCUSSION

**Phospholipid Membrane–Water Distribution Ratios.** Among all organic species detected in OSPW in positive ionization mode (1442 species), most showed no detectable decrease in the buffer with increasing concentration of phospholipid, indicating very low log $D_{MW}$ for most species (i.e., log $D_{MW} \ll 1$). However, 9.1% of OSPW chemical species observed in positive ion mode (131 of 1442 species) had log $D_{MW}$ greater than 1.0, indicating a subset of species having a preference for the membrane compared to buffer. These 131 species belonged to one of only four empirical formula classes: $O^+$ (38 species), $O_2^+$ (16 species), $SO^+$ (52 species), and $NO^+$ (25 species). The maximum log $D_{MW}$ value of $O^+$ species was 3.0, for $O_2^+$, it was 2.5, for $SO^+$ it was 4.1 and for $NO^+$ it was 4.1.

Among all organic species detected in negative ionization mode, 14% (94 of 679 species) detected had log $D_{MW}$ greater than 1.0. These species all belonged to two heteroatomic classes of compounds: $O_2^-$ (naphthenic acids, 56 species) and $SO_2^-$ (38 species). The maximum log $D_{MW}$ for $O_2^-$ was 4.1, and for $SO_2^-$, it was 3.4. Relative to $O_2^+$ species with the same empirical formula, log $D_{MW}$ values of $O_2^-$ species were greater. While naphthenic acids should be exclusively ionized at the pH of these tests, the $O_2^+$ species are neutral substances containing one or more hydroxyl- or keto- functionalities.[23] Thus, the presence of a negative charge in $O_2^-$ species likely allows electrostatic interaction with the positive charge of the choline

functional group in phosphatidylcholine, and a higher measured $D_{MW}$.[13,24]

The $D_{MW}$ for each individual organic species can be displayed by heat maps of Kendrick mass defect versus nominal Kendrick mass (Figure 1). Kendrick plots are commonly used for visualizing data from petroleum-derived samples, where each dot in these plots corresponds to an organic species with a distinct empirical formula (i.e., $C_aH_bO_cN_dS_e^{+/-}$). A series of data points in the horizontal direction corresponds to a family of compounds belonging to the same heteroatom class with the same number of double bond equivalents, and an increasing number of carbon atoms from left to right. The color gradient, from blue to white to red, indicates log $D_{MW}$ increasing from <1 to 4. From these plots it is evident that chemicals with the greatest log $D_{MW}$ occur as homologous series of compounds (horizontal lines) within the same general composition.

For those empirical formula classes where log $D_{MW}$ was greatest, there were relationships between phospholipid membrane-water distribution ratios and chemical structure. For example, for $O_2^-$ and $SO^+$ species log $D_{MW}$ increased with the number of carbon atoms, but generally decreased with increasing double-bond equivalents (Figure 2). These general trends were also evident for $O^+$, $O_2^+$, $SO^+$, $NO^+$, $O_2^-$, and $SO_2^-$ species.

Comparison of current log $D_{MW}$ measurement with log $D_{OW}$ values, estimated by equilibration of OSPW with polydimethylsiloxane (PDMS) coated stir bars,[9] for these five classes of compounds (i.e., $O^+$, $O_2^+$, $SO^+$, $NO^+$, $O_2^-$, and $SO_2^-$) are shown in Figure 3. For those species detected in positive ion

mode (i.e., $O^+$, $O_2^+$, $SO^+$, and $NO^+$), log $D_{MW}$ was reasonably well correlated with log $D_{OW}$ (ANCOVA, $R^2 = 0.61$, $p < 0.001$) with most values agreeing to within $\pm 1$ log unit. Such correlation is expected because hydrophobic interactions are likely the major drivers for both parameters of these polar neutral substances. Nevertheless, for the most hydrophobic substances the linear relationship with log $D_{MW}$ tended to flatten out at higher values of log $D_{OW}$ (i.e., log $D_{OW} > 4.5$) for some $NO^+$ and $SO^+$ species.

Similar phenomena were observed in previous studies. For example, when log liposome-water partition coefficient was compared to the octanol–water partition coefficient (log $K_{OW}$) for 181 compounds, approximately 1:1 agreement was observed with differences being 0.4 on average and mostly within 0.8 log units, but considerably larger scatter was evident for compounds with log $K_{OW}$ greater than 6.[25] Furthermore, the correlation between log partition coefficient for distributions of compounds between water and phosphatidylcholine dimyristoyl membrane (log $K_{MW}$) and log $K_{OW}$ of compounds with log $K_{OW}$ ranging from $-2$ to 9 has been investigated.[20] In that study, compounds with log $K_{OW}$ between 1 and 5.5 had log $K_{MW}$ that were approximately equal to the log $K_{OW}$, whereas for those with log $K_{OW}$ greater than 5.5, the linear relationship broke down when log $K_{OW}$ values were greater than $K_{MW}$.[26] The breakdown of the relationship between log $K_{OW}$ and log $K_{MW}$ was attributed to the increase of molar volume ($>230$ cm$^3$ mol$^{-1}$) of the compounds, requiring more energy to form a cavity in the structured membrane phase to accommodate the compounds.[26] The apparently lower cutoff region in the current study (i.e., log $D_{OW} > 4.5$) might be due to the different membranes used here, the presence of sulfur and nitrogen heteroatoms, or different functional groups in the current compounds.

For species detected in negative mode (i.e., species in the $O_2^-$ and $SO_2^-$ chemical classes) there was also a linear correlation between log $D_{MW}$ and log $D_{OW}$ (Figure 3) ($p < 0.001$), however log $D_{MW}$ values were much greater than corresponding log $D_{OW}$ values. For $O_2^-$ species, $D_{MW}$ values were $36-3.8 \times 10^3$ times greater than the corresponding $D_{OW}$ ($8.1 \times 10^2$ on average). For $SO_2^-$ species, the differences between $D_{MW}$ and $D_{OW}$ were even greater, with log $D_{MW}$ being $2.7 \times 10^2 - 8.7 \times 10^4$ times greater ($2.3 \times 10^4$ on average). For both $O_2^-$ and $SO_2^-$ classes of compounds, the higher $y$-intercepts (Figure 3) indicate a much stronger affinity for phospholipid, compared to storage lipid. Organic compounds detectable in negative ionization mode are generally dissociated organic acids, and these results are suggestive of strong electrostatic interactions between negatively charged organic acids and the positively charged choline moiety of the phospholipid membrane as described in previous studies with organic acids.[14] Naphthenic acids are carboxylic acids (e.g., p$K_a$ = $5-6$)[27] that should be predominantly ionized at pH 7.4. The structure and physical properties of $SO_2^-$ compounds has not been reported, but could be similar to naphthenic acids with the exception that a single sulfur atom might be incorporated into the carbon backbone. We speculate that the linear relationship between log $D_{MW}$ and log $D_{OW}$ values for species in both $O_2^-$ and $SO_2^-$ classes indicates that $D_{MW}$ is also influenced by hydrophobicity (e.g., by increasing molecular size or carbon number), but the absolute difference in $D_{MW}$ and $D_{OW}$ values indicates that electrostatic interactions were the dominant mechanism of phospholipid membrane partitioning. It is nevertheless important to note a limitation of this comparison,

that log $D_{OW}$ was assessed at the environmentally relevant pH of 8.4, whereas log $D_{MW}$ was assessed at a physiological pH of 7.4 to maintain the stability of phospholipid bilayer.

Each OSPW organic species is composed of a complex mixture of structural isomers which are only partly separated by the current chromatographic method. As illustrated by Figure S1, log $D_{OW}$ and log $D_{MW}$ will be different for different isomers. For example, for isomers of $C_{15}H_{24}OS^+$ shown in the right panel, isomers eluting in the middle of the chromatographic hump showed relatively greater bioconcentration potential than the isomers eluting before and after. Thus, the log $D_{OW}$ and log $D_{MW}$ values reported here are weighted mean values for the current sample, and some variability could be expected for samples from different sources having different isomer compositions. The structure for the vast majority of these isomers is unknown, and isomer-specific data are not currently possible to measure due to limitations of chromatography.

**Prediction of BCFs for OSPW Organic Species.** Ignoring the effect of any biotransformation, an upper-limit estimate of steady-state fish bioconcentration factors (BCFs) was estimated using a simplified version of the ionogenic organic chemical bioconcentration model.[11] Briefly, the steady-state BCF (L/kg) for each species (sum of neutral and ionized) can be estimated from log $D_{OW}$, log $D_{MW}$ and the volume fractions of neutral storage lipids ($f_{SL}$), polar phospholipids ($f_{PL}$), and water ($f_w$) in fishes (eq 4).

$$\text{BCF} = f_{SL} \times D_{OW} + f_{PL} \times D_{MW} + f_w \tag{4}$$

On the basis of the methods suggested by Hendriks et al.,[28] $f_{SL}$ was assumed to be 4%, $f_{PL}$ was assumed to be 1%, and water was 80%. The structure and chemical functionality for most chemical species in OSPW are not known, but given that some species are organic acids and bases, $D_{OW}$ and $D_{MW}$ measurements under laboratory conditions were assumed to be environmentally relevant weighted averages of partitioning for both the neutral and ionized forms of each detected chemical species. Partitioning to nonlipid organic matter (NLOM) was omitted from the model because this parameter was not directly measured, and in fish this term is most often negligible relative to lipids.[11]

For all organic species with log $D_{OW}$ or log $D_{MW}$ greater than 1.0, estimated log BCFs are shown in Figure S2. The estimated log BCF ranged from $-0.1$ to 2.1 L/kg for $O^+$, $-0.1$ to 4.8 L/kg for $SO^+$, $-0.1$ to 4.2 L/kg for $NO^+$, $-0.1$ to 3.1 L/kg for $O_2^+$, $-0.1$ to 2.1 L/kg for $O_2^-$, and $-0.1$ to 1.4 L/kg for $SO_2^-$. Thus, $SO^+$ and $NO^+$ species were anticipated to have the greatest BCFs, followed by naphthenic acids ($O_2^-$) and $O^+$ species

**Extraction and Analysis of Fish Tissue.** Prior to this study, there were no published methods for extraction and analysis of the wide range of OSPW organics in fish tissue. Methods do not yet exist because developing an optimal method for such a complex mixture of organic acids and neutrals, whose structures are also largely unknown, is a difficult undertaking. Six medaka homogenates were separately spiked with OSPW stock solution and internal standards were used to estimate recoveries of chemical species by liquid–liquid extraction after homogenized and solid phase extraction cleanup. Recoveries of $O^+$ species ranged from 20% $\pm$ 4% to 66% $\pm$ 3% (mean 46% $\pm$ 13%), for $O_2^+$ species from 27% $\pm$ 4% to 82% $\pm$ 2% (mean 50% $\pm$ 14%), for $SO^+$ from 13% $\pm$ 2% to 76% $\pm$ 6% (mean 45% $\pm$ 15%), for $NO^+$ from 35% $\pm$ 5% to 117% $\pm$ 5% (mean 65% $\pm$ 17%), for $O_2^-$ from 30% $\pm$ 4% to 91% $\pm$ 14% (mean 61% $\pm$ 15%), and for $SO_2^-$ from 2% $\pm$ 1%
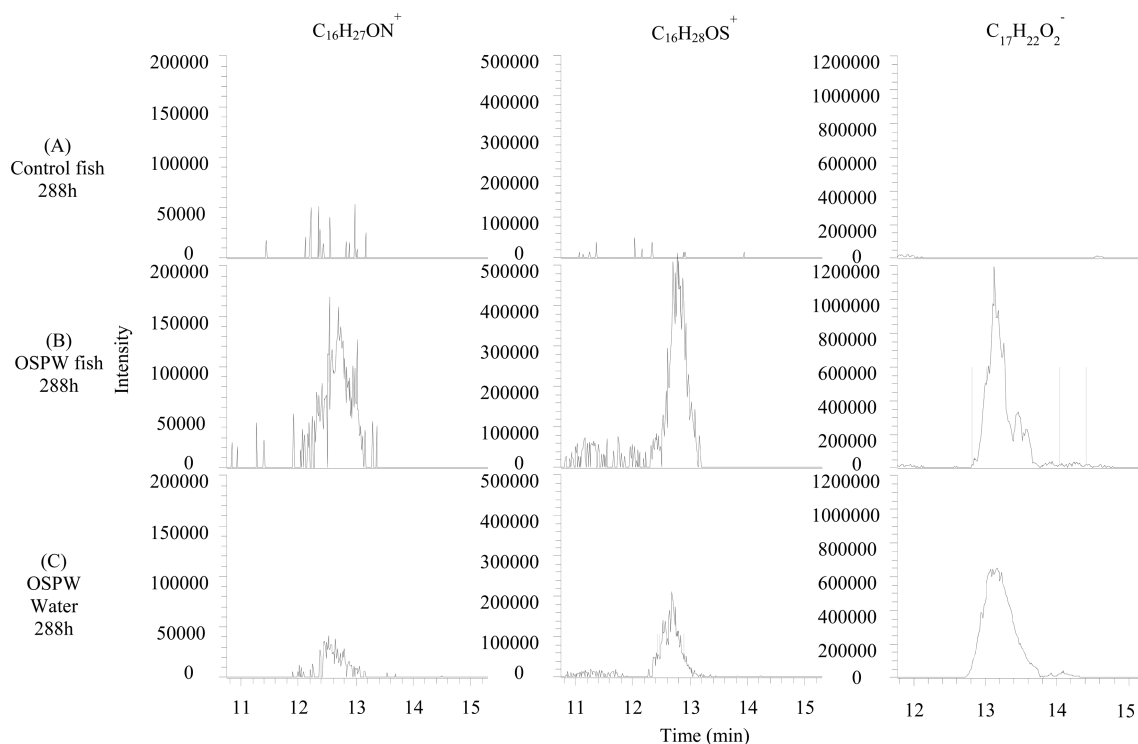
**Figure 4.** Example extracted ion chromatograms from HPLC−Orbitrap analysis showing the species $C_{16}H_{27}O\,N$ and $C_{16}H_{28}O\,S$ in positive mode, and $C_{17}H_{22}O_2$ in negative mode in (A) tissue extract of control fish (C1) at 288 h, (B) tissue extract of OSPW-exposed fish (E1) at 288h, and (C) exposure water (10% dilution of OSPW).

to 45% ± 7% (mean 16% ± 9%) (Figure S3). Absolute recoveries of $O^+$, $O_2^+$, $SO^+$, $NO^+$, and $O_2^-$ varied from 12% up to 117% but with rather good precision in all cases (e.g., typically <20% rsd) while most recoveries of $SO_2^-$ were less than 20%. Further attempts at optimization of the method improved the recovery of some species at the expense of others, thus the current method was deemed acceptable for the current study. BCFs for $SO_2^-$ were not reported due to their very low recovery. For certain naphthenic acids with carbon number greater than 17 and with DBE from 3 to 5, there were interferences, presumably from endogenous fatty acids, that were also in control fish which prevented recoveries from being calculated accurately. Thus, BCFs for these particular naphthenic acids were also not reported.

**Steady State BCFs in Medaka.** During the uptake period, the relative concentration of organic species in OSPW exposure water were monitored over time by use of total peak areas per unit volume of water analyzed. Relative standard deviations of concentrations of organic compounds in water were less than 30%, which suggested that the exposure concentration remained relatively stable throughout the uptake phase due to the small mass of fish in a large volume of water in each tank (0.16 g of fish (wet mass) per liter of water per day). Therefore, the mean water concentration for each chemical species was used as a constant estimate of $C_W$ for determination of steady-state BCF. During depuration, no contamination of OSPW chemical species was detected in any water samples from any tanks.

Only three classes of compounds were observed in fish exposed to diluted OSPW, corresponding to those same three chemical classes that were predicted to be most accumulative by the ionogenic bioconcentrtion model discussed above: $SO^+$, $NO^+$ and $O_2^-$ classes. Typical chromatograms were shown for

example chemical species in control fish, exposed fish and OSPW exposure water (Figure 4). For an example $NO^+$ species ($C_{16}H_{27}ON^+$) (Figure 4, left panels) a chromatographic hump eluted between 12 and 13.2 min in the exposure water and exposed fish tissue, but not in control fish. For an example $SO^+$ species ($C_{16}H_{28}OS^+$) (Figure 4, middle panels) a chromatographic hump eluted between 12.2 and 13.2 min in the exposure water and exposed fish tissue, but not in the control fish. For example $O_2^-$ species ($C_{17}H_{22}O_2^-$) (Figure 4, right panels) a chromatographic hump eluted between 12.6 and 14 min in exposure water and tissue of fish exposed to OSPW, but not in tissue of control fish. As discussed, each chromatographic hump is actually composed of a complex mixture of structural isomers whose retention times are all very similar, but not identical. It was therefore interesting that the shape of the chromatographic hump in fish tissue was sometimes different than that associated with the same class of species in exposure water. This indicates that some isomers were more accumulative than others. For example, in Figure 4B the latest eluting isomers in fish tissue (presumably the most hydrophobic of all isomers) appear as a more pronounced shoulder peak, which suggests that these are somewhat more accumulative than those isomers in the main hump.

Uptake and depuration of organic species in OSPW by fish can furthermore be demonstrated for example chemical species: $C_{16}H_{27}ON^+$, $C_{16}H_{28}OS^+$, and $C_{17}H_{22}O_2^+$ (Figure S4). Uptake reached a rapid equilibrium within 72 h. After transfer to clean water, depuration of organic species detected in positive mode occurred very quickly, with the total signal decreasing to less than 10% within 6 h in most samples of fish and then becoming less than the limit of quantification. For naphthenic acids, the total signal remained at approximately 20% after 6 h, and for some $O_2^-$ species (such as $C_{19}H_{24}O_2^-$) could still be detected

in fish tissues after 144 h of depuration (Figure S4). After an initial rapid elimination within the first 6 h, the rate of depuration became slower, suggesting biphasic elimination for some naphthenic acids. This could be related to the fact that these species accumulate in both neutral lipid and phospholipid compartments, with one compartment clearing much more slowly than the other.

For comparative purposes, elimination data were fit to a simple (one compartment) first-order decay model. Rate constants for depuration ($k_d$) were estimated as the slope of the linear regression between the ln (concentrations in fish) and depuration time, and the half-lives ($t_{50}$) were calculated[23] (eq 5).

$$t_{50} = \ln(2)/k_d \qquad (5)$$

For all SO$^+$ and NO$^+$ species, elimination was too rapid to calculate $k_d$, but on the basis of an upper limit of their limits of detection, their $k_d$ values must be >0.21 h$^{-1}$, corresponding to half-lives <3.3 h. Over the first 24 h of depuration, values of $k_d$ ranged from 0.10 to 0.22 h$^{-1}$ for naphthenic acids, representing biological half-lives in the range of 3.2–6.8 h. Moreover, there was a positive linear relationship between half-life and carbon number among the naphthenic acids (Figure S6). The time to reach 95% steady state ($t_{95}$) is determined solely by the rate of depuration (eq 6), which is derived from the general kinetic equation describing uptake and depuration (first-order kinetics).[29]

$$t_{95} = 3/k_d \qquad (6)$$

Thus, taking these depuration half-lives a steady-state should theoretically have been reached within 30 h, which is consistent with the observed results.

After 72 h, BCFs were calculated as the average of the ratio between the wet-mass based intensity of each chemical species in fish tissue relative to the intensity per volume in exposure water. BCFs, based on wet mass, ranged from 0.6 to 28 L/kg for SO$^+$ and NO$^+$ species, and from 0.7 to 53 L/kg for naphthenic acids. The BCF of one particular naphthenic acid species ($C_{13}H_{22}O_2$) was 4.4 L/kg, which is comparable to the BCF reported in previous studies (approximately 2, at pH 8.2 and approximately 4, at pH 7.6).[18,20] There was a positive relationship between the number of carbon atoms and the log BCF (Figure S7). The relative standard deviations for log BCF among the 56 chemical species detected in fish were all lower than 20%, except for 4 chemical species. The log BCFs of males and females were compared with an independent samples $t$ test, and there was no significant difference between male and females ($p > 0.05$) except for 6 chemical species, of which log BCF in females was slightly higher than in males. Thus, male and female BCF data were combined. For naphthenic acids and SO$^+$, DBE also showed an effect on the log BCF (ANCOVA, $p < 0.05$) (Figure S8). For NO$^+$, the effect of DBE was not observed since there were so few NO$^+$ species detected.

When values for log BCF determined in vivo were plotted against predicted log BCF (Figure 5) a good linear correlation between measured and predicted BCFs among positive mode species ($p < 0.001$, $R^2 = 0.835$, slope = 0.511, intercept = −0.065) and negative mode species ($p < 0.001$, $R^2 = 0.74$, slope = 0.752, intercept = −0.057) were observed. This supports the validity of predicting bioconcentration for a wide range of ionogenic and non ionogenic species by use of a combination of $D_{OW}$ and $D_{MW}$. The log BCF of naphthenic acids were reasonably well predicted by use of log $D_{OW}$ and log $D_{MW}$, with



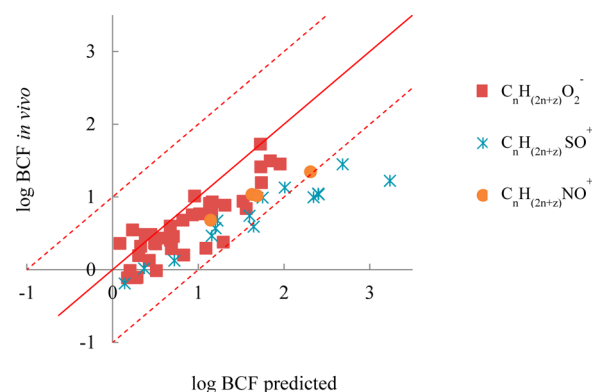**Figure 5.** Relationship between wet-mass measured log BCF in medaka and calculated log BCF based on log $D_{OW}$ and log $D_{MW}$ for organic species in OSPW. The solid line represents the 1:1 relationship and the dotted lines delimit the 1 order of magnitude deviation interval.

all data falling within the range of ±1 log unit. However, larger differences between measured and predicted BCF was evident for SO$^+$ and NO$^+$ species, whereby measured values were 2 to 100-fold lower than predicted. This may suggest that biotransformation processes are important for elimination of organic compounds in OSPW, particularly for the SO$^+$ and NO$^+$ species. Future research should confirm metabolic transformations and the associated pathways to ensure that the transformed products are indeed cleared and not accumulated.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01354.

Selected ion chromatograms showing different distribution behavior of different isomers, range of wet mass normalized log BCF for organic compound species in OSPW, range of recoveries for organic compound species, intensities of example organic species in OSPW, relationship between log concentration factor of example species and time, relationships between half-lives and number of carbons in naphthenic acids in OSPW, and effect of number of carbons on log BCF and number of carbons and DBE on log BCF (PDF)

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**Notes**

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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# Bioconcentration of Dissolved Organic Compounds from Oil Sands Process-Affected Water by Medaka (*Oryzias latipes*): Importance of Partitioning to Phospholipids

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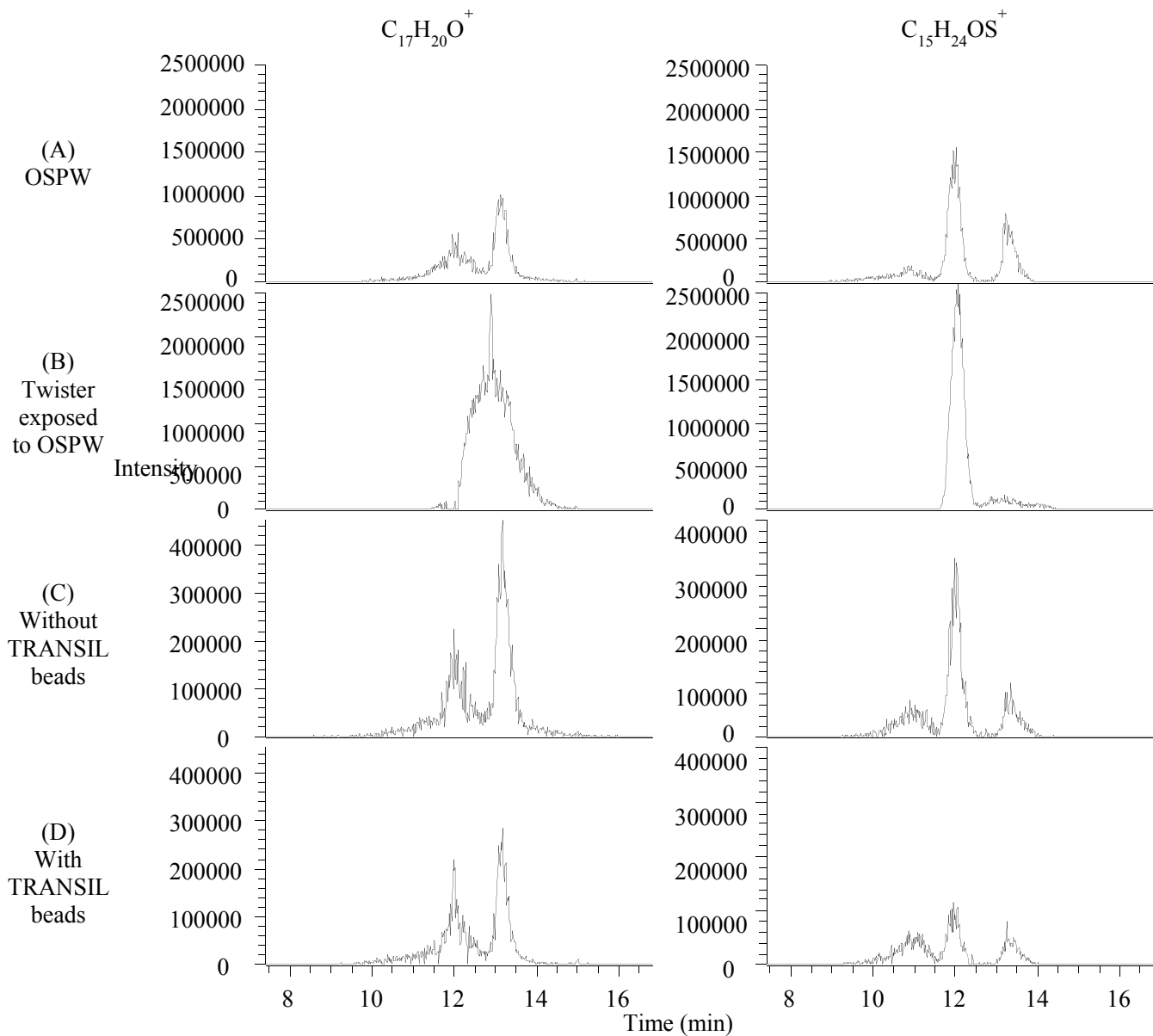
**Figure S1.** Example selected ion chromatograms from HPLC–Orbitrap analysis showing the species $C_{17}H_{20}O^+$ and $C_{15}H_{24}OS^+$ in (A) OSPW water, (B) Twister exposed to OSPW, (C) Supernatant of the vial without beads coated with phospholipid bilayer in TRANSIL kit, and (C) Supernatant of the vial with beads coated with phospholipid bilayer in TRANSIL kit.

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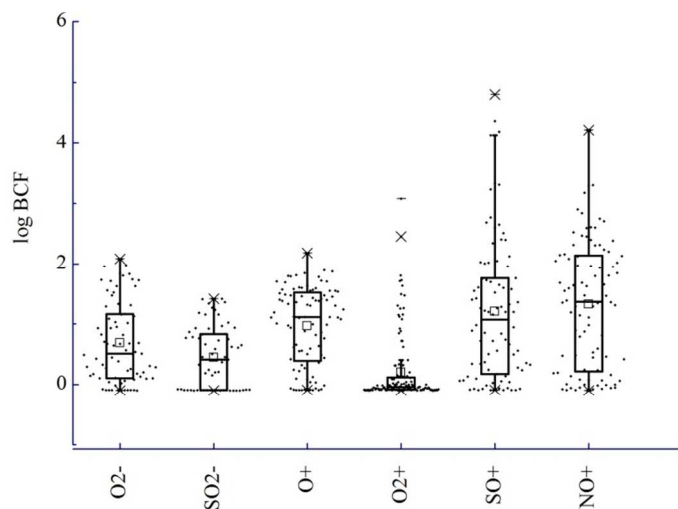
**Figure S2.** Range of wet mass normalized log BCF calculated based on log $D_{OW}$ and log $D_{MW}$ for organic
compound species in OSPW. Each dot within the box plots represents an individual organic species. Horizontal
lines represent medians, the $25^{th}$ and $75^{th}$ centiles define the boxes, while the whiskers represent the $10^{th}$ and
$90^{th}$ centiles. Small squares ($\square$) represent arithmetic means, crosses ($\times$) represent 1st and $99^{th}$ centiles, and
minima/maxima are shown as dashes (–).
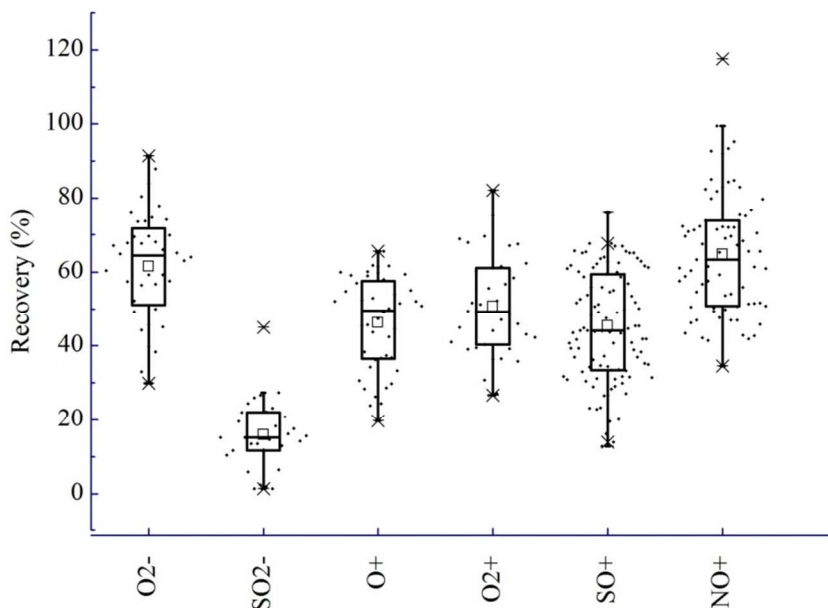
33 **Figure S3.** Range of recoveries for organic compound species in OSPW. Each dot within the box plots

34 represents an individual organic species. Horizontal lines represent medians, the $25^{th}$ and $75^{th}$ centiles define

35 the boxes, while the whiskers represent the $10^{th}$ and 90th centiles. Small squares (□) represent arithmetic

36 means, crosses (×) represent 1st and $99^{th}$ centiles, and minima/maxima are shown as dashes (–).
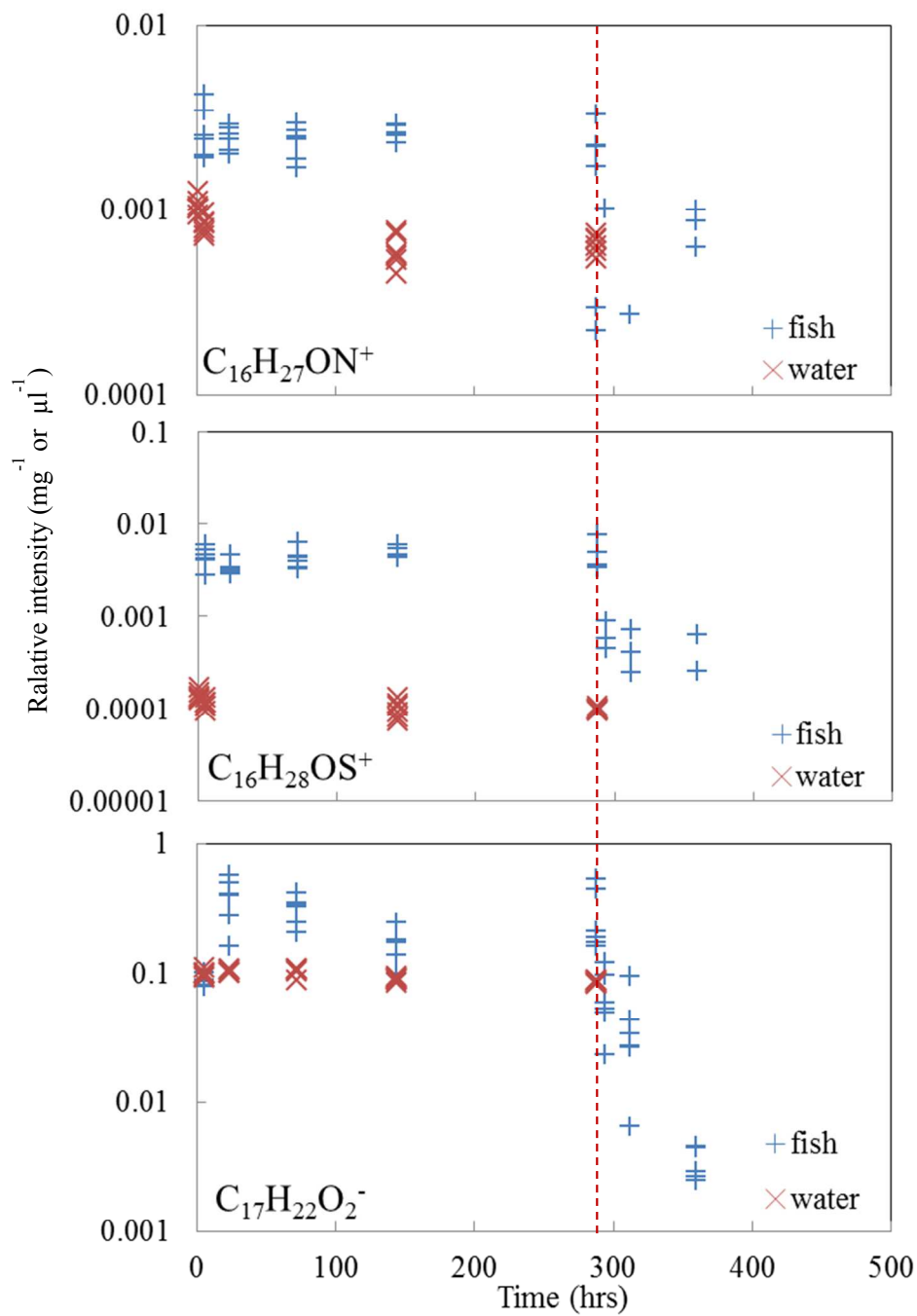
**Figure S4.** Intensities of example organic species in OSPW during uptake and depuration phases. Exposure period was from 0 to 288 h and the depuration period was from 288 to 412 h.
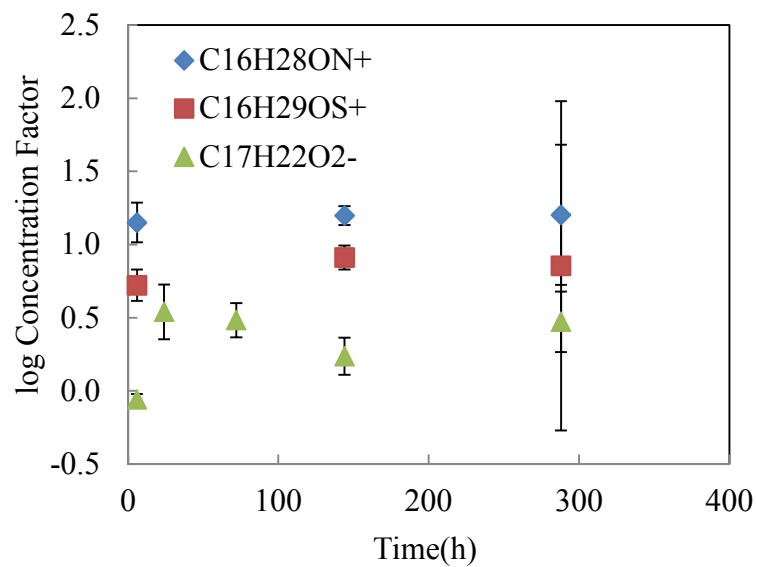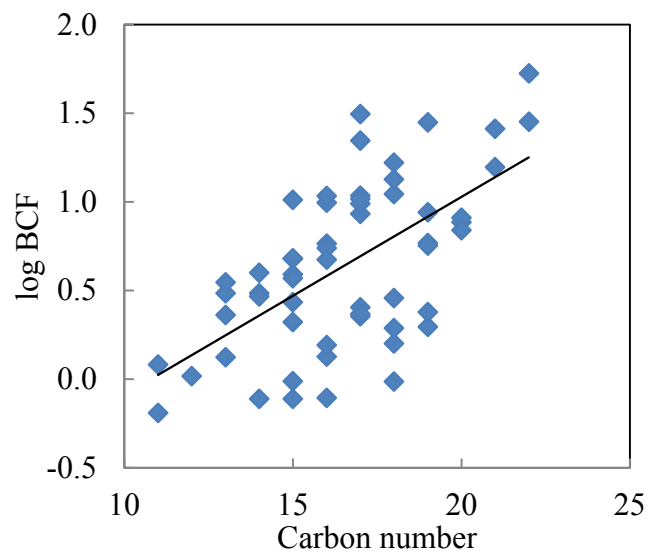
41     **Figure S5.** Log concentration factor of example species ($C_{16}H_{27}ON^+$ and $C_{16}H_{28}OS^+$, and $C_{17}H_{22}O_2^-$) in

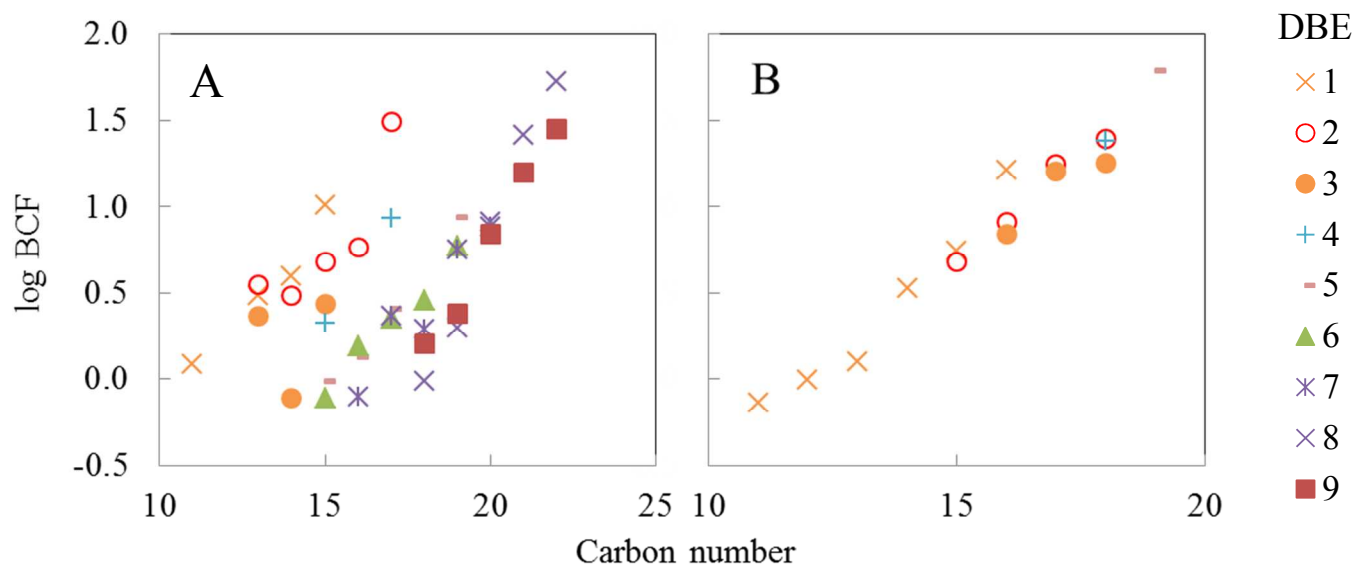42     OSPW. Exposure period was from 0 to 288 h.

43
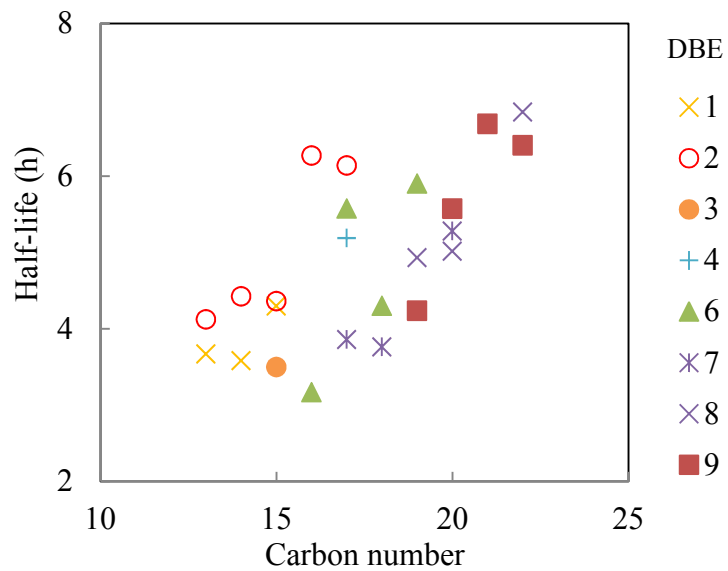
44  **Figure S6.** Relationships between half-lives and number of carbons in naphthenic acids in OSPW.

45

46    **Figure S7.** Effect of number of carbons on log BCF based on the ratio between the wet mass normalized
47    concentration in fish and the concentration in water of organic species in OSPW.

48

**Figure S8.** Effect of number of carbons and double-bond equivalents (DBE) on log BCF based on the ratio between the wet weight normalized concentration in fish and the concentration in water of example organic species in OSPW (A) O2 species observed in ESI negative mode; (B) SO species observed in ESI positive mode.