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ORIGINAL ARTICLE

Biodegradation and Ecotoxicity of Branched Alcohol Ethoxylates: Application of the Target Lipid Model and Implications for Environmental Classification

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Abstract With the advent of global regulations for safer detergent and an emphasis on a shift toward more environmentally friendly formulations, the environmental profile of surfactant chemistries have moved to the forefront of product formulation and design. The two cornerstones of surfactant environmental profiles are the ability to biodegrade in the natural environment and the ecological hazard profile. The objectives of this article are to describe biodegradation and aquatic toxicity data for a series of branched oxo-alcohol ethoxylate (AEO) surfactants; to apply the target lipid model (TLM) for deriving model-based threshold hazard concentrations (HC5) of AEO; and, finally, to accurately determine aquatic classifications for AEO surfactants for use in regulatory classification frameworks. Biodegradation results indicate a high level of biodegradability of branched AEO, with C8–C13-rich oxo-alcohols with 1–20 mol of ethoxylate meeting the readily biodegradable criteria. Results from acute and chronic toxicity tests indicated comparable or lesser aquatic toxicity *versus* linear AEO structures previously reported in the literature. The TLM model, applied *a priori*, resulted in good agreement with acute toxicity data (RMSE = 0.49) and is comparable to the root mean square errors (RMSE) previously determined for other narcotic chemicals (RMSE = 0.46–0.57). Model errors for invertebrates and fish were smaller than those for algae, with the TLM systematically overpredicting acute and chronic classification of two of seven branched AEO. Furthermore, TLM-predicted HC5 values were determined to be sufficiently conservative, with 100% of observed chronic data (N = 79) falling above the HC5 threshold values, providing a useful tool for the risk assessment of AEO.

Keywords Alcohol ethoxylates · Surfactant · Aquatic toxicity · Biodegradation · Target lipid · TLM

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Introduction

Alcohol ethoxylates (AEO) are neutral surfactant molecules, widely used in both industrial and consumer product applications (Environment Canada, 2013). They are the result of the chemical reaction of fatty alcohols of various origins and ethylene oxide. The result is a surfactant molecule with hydrophobic (fatty alkyl alcohol hydrophobe) and hydrophilic (ethylene oxide chains) portions. Often, commercial products are complex mixtures with a range of alkyl and ethoxylate chain lengths (P&G, 2015; Sasol, 2018; Shell, 2018). Generally, AEO have the formula R- $(OCC)_n$-OH, where R represents the fatty alcohol alkyl chain and n represents the degree of ethoxylation of the molecule. While both the chain length, R, and degree of ethoxylation, n, vary considerably among commercial products, common ranges for AEO are

Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

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R = 10–17 and n = 0–18 (Belanger et al., 2006; Environment Canada, 2013). The unique surfactant properties of these AEO along with their large molecular weight and variability in composition often result in large uncertainties around estimating their physical chemical properties (i.e., K_{OW}) and, consequently, their ultimate fate and potential hazard in the aquatic environment.

Since the widespread adoption of the first chemical surfactants in the mid-20th century, the environmental fate and effects of these substances have been a key performance property. In recent years, the environmental properties of nonionic surfactants have been of increasing interest to regulators and downstream users (Brown, 1995; Lacasse and Baumann, 2012; Lassen et al., 2013; Wenzel et al., 2004). Consequently, there has been increased emphasis on the importance of biodegradability of surfactants as a basic performance dimension. This has resulted in a vast proliferation of voluntary and regulatory industry efforts to minimize the use and release of substances that may persist in the environment (Lacasse and Baumann, 2012, Lassen et al., 2013, Wenzel et al., 2004). The European Union (EU) Safer Detergents Regulation (EC, 2005), which mandated standardized testing for primary (biotransformation) and ultimate (mineralization) biodegradability and the application of stringent requirements for consumer and industrial applications, was critical in establishing harmonized performance characteristics of surfactants that have impacted the global surfactants industry. In addition, the widespread adoption of the Globally Harmonized System (UN, 2017) of classification and labelling has increased the demand for surfactants with favorable safety profiles.

Biodegradation

Understanding the biodegradability of industrial chemicals is a key component required by many regulatory agencies globally (e.g., European Chemicals Agency [ECHA], Environment Canada, US Environmental Protection Agency [USEPA], etc.). The use of the Organization for Economic Cooperation and Development (OECD) 301 and 310 series of ready biodegradability tests are considered the standard for screening purposes (OECD, 1992b, OECD, 2014). These tests are generally considered to be conservative in nature. As such, a positive result on one of these tests is considered to be "indicative of rapid and ultimate biodegradation in most environments." These screening tests were designed in a manner such that positive results would be unequivocal, and in such cases, further investigation of the biodegradability of the chemical or environmental effects of any transformation products would normally not be required (ECHA, 2017).

Prior reports on highly branched AEO have consistently characterized an inverse relationship between degree of branching and biodegradation rate (Dorn et al., 1993; Kravetz et al., 1991; Marcomini et al., 2000a; Marcomini et al., 2000b; Mausner et al., 1969). It is critical to appreciate that biodegradation screening tests (OECD 301 series) are considered stringent and their interpretation conservative. Ready biodegradability tests are sufficiently stringent that the rapid and complete biodegradation of the compounds in aquatic environments is assumed (OECD, 1992b). It is well known that ready biodegradability tests can be variable, resulting in false negatives. This variability can be attributed to several factors; perhaps most influential is the variability in inoculum source, population, and the mass ratio of inoculum (Gartiser et al., 2017). Thus, "positive" results that meet the criteria are considered sufficient evidence of biodegradability and should generally supersede negative results (OECD, 1992b).

The degree of branching of multiple surfactant classes has been associated with slower or poor biodegradability relative to linear analogs in various ultimate biodegradability test methods. Conversely, published reports have demonstrated a favorable effect of branching on environmental toxicity (Dorn et al., 1993; Kaluza and Taeger, 1996; Kravetz et al., 1991).

Risk Assessment of AEO

The ecological fate and aquatic hazard of surfactants has been well-studied over the past several decades. Early efforts focused predominantly on ionic surfactant classes (i.e., linear alkyl-benzene sulfonate [LAS]) (Feijtel et al., 1995; Rapaport and Eckhoff, 1990; Waters and Feijtel, 1995). Subsequent work has focused on AEO and their relative risk profiles, compared to other classes of surfactants, due to high production volumes and direct-release use scenarios for consumer applications (Environment Canada, 2000; EPA, 2005; Goyer et al., 1981; Knepper et al., 2003; Little, 1977; Routledge and Sumpter, 1996; Servos, 1999; Talmage, 1994; van de Plassche et al., 1999). A more recent risk assessment by Belanger et al. derived species-sensitivity distributions (SSD) and, ultimately, HC5 (hazardous concentration protective of 95% of species) threshold values based on chronic toxicity data for AEO mixtures and homologues using 17 species and 60 ecotoxicity tests (Belanger et al., 2006). These HC5 values were compared to typical AEO concentrations in the environment in North America and Europe, concluding that low levels of risk were present for aquatic environments based on these derived criteria. While the risk assessment of Belanger et al. was comprehensive, branched AEO were not included, and thus, there remains some uncertainty regarding potential risk to the aquatic environment.

A significant barrier to deriving HC5 criteria from experimental chronic data (as used in the 2006 Belanger et al. risk assessment) for new substances or mixtures is that it requires a significant number of species to develop a

sensitivity distribution to allow for extrapolation down to the 5% (HC5) level of species protection (Aldenberg and Jaworska, 2000; Posthuma et al., 2001). Alternatively, HC5 criteria can be derived (computationally) for chemicals that exhibit nonspecific "narcotic" toxic modes of action using the target lipid model (TLM) developed by Di Toro et al. (Di Toro and McGrath, 2000; Di Toro et al., 2000).

Target Lipid Model

The TLM is a quantitative structure–activity relationship (QSAR) model, which relates observed toxicity (i.e., median lethal concentration – LC50) to a substance's octanol–water partition coefficient. There are several key assumptions upon which the TLM model framework is constructed: (1) the lipid fraction of the organism is the relevant site of action for narcotic chemicals, (2) the critical concentration of a chemical required to exhibit a toxic effect (the critical body burden) expressed in [μmol g^{-1} lipid] is organism-specific and independent of the chemical structure, and (3) the partitioning behavior of narcotic chemicals between lipid and water is described by the octanol–water partitioning behavior through a universal "narcosis slope" and is independent of the organism (Di Toro and McGrath, 2000; Di Toro et al., 2000). The TLM was originally derived for a set of known narcotic chemicals (n = 140) and test organisms (sp = 33) (Di Toro and McGrath, 2000) but has since been expanded and revised to include additional species (McGrath et al., 2004), additional classes of petroleum products (i.e., gasoline constituents, mono- and polycyclic aromatic hydrocarbons) (McGrath et al., 2005; McGrath and Di Toro, 2009), and additional environmental compartments (i.e., soil and sediment) (Redman et al. 2014b). The TLM forms the basis for the PETROTOX and PETRORISK tools, widely used in hazard and exposure assessments for petroleum hydrocarbon products and hydrocarbon solvents in North America and the European Union (EU) (Redman et al., 2012; Redman et al., 2014a; Redman et al., 2017).

Previous work has demonstrated that the mode of action of the ecotoxicological effects of AEO surfactants is considered to be narcotic (Escher et al., 2002; Escher and Hermens, 2002; Roberts, 1991; Roberts and Marshall, 1995), and Droge et al. (2008, 2009) have demonstrated that sediment toxicity can be predicted from aqueous toxicity based on equilibrium partitioning theory if the correct sorbate descriptors of AEO and sorbent properties of the sediment are taken into account. Consequently, it is possible to apply the framework of the TLM to AEO aquatic toxicity and to derive TLM-based HC5 criteria without the need for extensive chronic toxicity testing programs. To date, no attempt has been made to validate and apply the TLM to predict the aquatic toxicity of AEO. This may be due to the fact that there are significant limitations to the derivation of reliable experimental log (K_{ow}) data for surfactants (Hodges et al., 2019), leading to substantial uncertainties in the predicted aquatic toxicities of single homologue and commercial surfactant mixtures.

This article aims to address several specific objectives: first, it presents previously unpublished biodegradation and aquatic toxicity data (some of which have been previously reported in conferences and in company reports (Markarian et al., 1989, 1990) for a number of branched AEO, characterizing the environmental properties of surfactants derived from highly branched alcohol feedstocks. Second, this article compares aquatic toxicity data (including existing literature data for single AEO homologues, as well as commercial mixtures) to predicted levels using the TLM. Finally, this article evaluates aquatic toxicity effects levels and available biodegradation data for AEO within the context of hazard classification under the UN GHS (Globally Harmonized System of Classification and Labeling of Chemicals).

Experimental Procedures

Alcohol Ethoxylates

AEO surfactants were derived from branched C8-rich, C9-rich, C10-rich, C11-rich, and C13-rich oxo-alcohols. AEO were either commercial products or toll processed, with varying degrees of ethoxylation ranging from 1 to 20 mol of ethoxylation (EO). For comparative purposes, additional data are presented for AEO derived from a branched C12-rich oxo-alcohol and a semilinear C13/C15 mixture, which are both no longer commercially available. Compositional and chemical characteristics of these alcohols are presented in **Table** 1. Estimated physical–chemical properties for the associated ethoxylate substances are presented in Table S1..

Structural, physicochemical, and toxicological data for additional linear commercial AEO mixtures, as well as single-constituent homologues, were obtained from the literature. Specifically, data were considered from previous risk assessments (Belanger et al., 2006) in addition to data for substances used in the calibration and validation of previous QSAR models (Wind and Belanger, 2006; Wong et al., 1997). The literature data collected includes *ca.* 100 acute and chronic endpoints covering 14 species (three fish, three algal, and eight invertebrate sp.), with substances ranging from C8 to C18 alcohol chain length, with 2–13 mol EO (Belanger et al., 2006; Morrall et al., 2003; Wind and Belanger, 2006; Wong et al., 1997). For a complete list of chemicals, endpoints, and physical chemical data, see Table S2.

Table 1 Characteristics of alcohols and level of ethoxylation of test substances

Sample name; code	Carbon number										Avg. branches/molec.	Details major isomers/ feedstock	EO range
	6	7	8	9	10	11	12	13	14	15			
Branched C8-rich oxo-alcohol; C8br	<0.1	3	92	5							1.59	Methyl-1-heptanols, dimethyl-1-hexanols. *Feedstock*: Heptene (proplyene/butene dimer)	4–10
Branched C9-rich oxo-alcohol; C9br			3	77	19	1					1.88	Methyl-1-octanols, dimethyl-1-heptanols. *Feedstock*: Octene (Butene-rich olefin dimer)	1–20
Branched C10-rich oxo-alcohol; C10br				3	90	7					2.03	Dimethyl-1-octanols, trimethyl-1-heptanols. *Feedstock*: Nonene (propylene trimer)	3–9
Branched C11-rich oxo-alcohol; C11br				0	9	85	6				2.23	Dimethyl-1-nonanols, trimethyl-1-octanols. *Feedstock*: Decenes (propylene/butene trimer)	3–10
Branched C12-rich oxo alcohol; C12br					6	18	55	20	1		3.1	Trimethyl-1-nonanols, tetramethyl-1-octanols. *Feedstock*: Undecenes (propylene/butene trimer)	7–12
Branched C13-rich oxo alcohol; C13br						1	23	70	6		3.06	Trimethyl-1-decanols, tetramethyl-1-nonanols. *Feedstock*: Dodecenes (propylene tetramer)	3–12
Semilinear C13/15 oxo alcohol #1; C1315 (a) #2; C1315 (b)								67		33	0.33	Blend of linear C13 and C15 alcohols; *ca*. 33% methyl and ethyl branching at α-Carbon *Feedstock*: C12/14 linear a-olefins	7–12

Biodegradation Testing

Ultimate ready biodegradability tests followed the OECD 301F manometric respirometry test guideline (OECD 1992b). Ready biodegradability was determined for 21 branched AEO. All test systems were placed on a manometric respirometer, manufactured by Coordinated Environmental Service, Ltd. (Kent, UK), which automatically recorded the oxygen uptake. Biodegradation was based on oxygen consumption; the theoretical oxygen demand (ThOD) was calculated from the results of an elemental analysis of the test substance. Sodium benzoate was used as a positive control. The fresh activated sludge inoculum was obtained from a municipal wastewater treatment plant (Somerset-Raritan Valley Sewage Authority, Bridgewater, NJ, USA), selected because it deals predominantly with nonindustrial, municipal wastewater. Operating temperature at the plant varied seasonally, with ranges of 10–13 °C in winter, 14–17 °C in spring/fall, and 22–25 °C in summer. Fresh activated sludge was obtained 1 day before the start of the test and was homogenized in a blender for 2 min at low to medium speed. The homogenized sample was allowed to settle for 30 min to 1.5 h, after which the supernatant was decanted to avoid carryover of sludge solids. The microbial activity of the supernatant was determined, using an Easicult®-TTC dip slide, to be 10^5 to 10^6 CFU mL^{-1}. Microbial sludge supernatant was added at a 1% loading volume to the test medium. The test medium was prepared according to the guideline and consisted of glass-distilled water and mineral salts (phosphate buffer, ferric chloride, magnesium sulfate, calcium chloride). Test medium and activated sludge, which was aerated for 24 h with carbon dioxide-free air, were added to each respirometer flask. Test vessels were glass flasks, placed in a water bath and electronically monitored for oxygen consumption. Triplicate test systems were run for each test substance, along with positive control and blank samples. The test substance concentration ranged from 20 to 50 mg (in *ca*. 900 mL) to provide at least 50–100 mg ThOD/L (range of ThOD specified by the test guideline). One study was conducted using several concentrations of C13br-7EO (14, 28, 57, and 111 mg ThOD/L) and C13br-12EO (28, 55, and 110 mg ThOD/L) to evaluate the effects of concentration on biodegradation. Substances were weighed on glass fiber filters and then placed into each individual replicate flask to aid dispersion of the test substance in the medium and to increase the bioavailability to the test organisms. Test temperature was maintained at 22 °C ± 1 °C (with some minor deviation as noted in Table S3). All test vessels were stirred constantly using magnetic stir bars (standard for the 301F

test; note that this may also increase bioaccessibility from sorbed phases such as glass walls). Details on specific study information are provided in Table S3.

Bioaccumulation Test Methods

A single study was performed to evaluate the bioaccumulation potential of an AEO derived from branched C11-rich oxo-alcohol with 3 mol of EO (C11br-3EO) in rainbow trout, *Oncorhynchus mykiss*. The study was performed in 2005 but followed the test procedures later adopted in the OECD 305 test guide for dietary exposure bioaccumulation in fish (OECD, 2012). The study included 10-day uptake and 14-day depuration phases. During uptake, juvenile fish (117 days old) were fed *ca*. 3% of average body weight per day with either a treated (mean measured 527 mg kg^{-1} test substance) or untreated (control) diet (Finfish Starter, #1 crumble—Zeigler Bros., Inc., Gardners, PA, USA). Fish feed was spiked at a target concentration of 500 μg g^{-1} by adding 55 μL of the test substance to a total of 100 g of diet and shaking manually for 1 min followed by overnight mechanical tumbling. During the depuration period, all fish were fed the untreated diet. Test chambers were 40 L glass aquaria equipped with stainless steel standpipes; 31 L of dilution water was delivered to the test chambers at a rate of 102–118 mL min^{-1} using a peristaltic pump (five to six volume replacements day^{-1}; organism loading: 0.14 g of fish per liter of dilution water per day). Fish samples were collected on day 10 of the uptake phase and on days 1, 3, 7, and 14 of the depuration phase. Due to the potential for rapid biotransformation, gastrointestinal tracts were removed from fish samples prior to tissue analysis. This step was taken to ensure that tissue measurements reflected absorbed substance and not test material that was associated with residual spiked diet in the gut. Fish samples were stored by freezing at −140 °C pending extraction. Fish carcasses (0.6 and 3 g) were placed in *ca*. 20 mL glass vials, minced with 3 g of hydromatrix, and allowed to dry in a fume hood overnight and transferred to 11 mL accelerated solvent extraction (ASE) cells along with 2 mL of methanol. Fish lipid content was measured 2 days before (on the stock population) and at the end of the uptake phase.

Toxicity Testing

Aquatic toxicity studies for C12br-7EO, C12br-12EO, C13br-7EO, C13br-12EO, C1315-7EO, and C1315-12EO were conducted in 1988–1990 at the aquatic toxicity laboratory located in East Millstone, NJ. These studies followed standard USEPA test guidelines (EPA, 1987a, b, c; Horning and Weber, 1985). More recently (2018), acute aquatic toxicity tests for C10br-9EO, C13br-3EO, and C13br-5EO were conducted at the laboratory located in Annandale, NJ, following standard OECD test guidelines (OECD, 1992a; OECD, 2004, 2011) and OECD Good Laboratory Practice (GLP) compliance (OECD, 1998). Minor differences between study details can be attributed to the standard laboratory conditions at either site. Study-specific information (e.g., concentrations, water quality, observations) is detailed in Tables S4 (acute) S5 (chronic) and S6 (analytical results).

For all studies, a stock solution of the substance in diluent was prepared in an appropriately sized glass vessel and mixed on a magnetic stir plate with a Teflon®-coated stir bar until the test substance was fully dissolved, which usually took between 15 and 60 min. The stock was then diluted to prepare a geometric series (factor of 1.8–2.2) of five or six treatment solutions plus a dilution water control. All treatment solutions appeared clear without evidence of substance insolubility. Observations were performed daily, although many of the studies included additional observation periods (e.g., 3 h, 6 h).

Pseudokirchneriella subcapitata (formerly *Selenastrum capricornutum*) were cultured and tested using Algal Nutrient Media (Miller et al., 1978). All tests were started with algal cells (*ca*. 1×10^4 cells mL^{-1}) in the log phase of growth, from 4- to 5-day cultures, and were performed in a temperature-controlled environmental chamber. For tests conducted in 1988–1990, test chambers were disposable, sterile, polycarbonate 125 mL Erlenmeyer flasks containing 50 mL of the inoculated test solution with autoclaved foam stoppers; flasks were randomly placed on a rotating shaker table (100 rpm). Effect observations were obtained by Chlorophyll a fluorescence readings that were converted to cell numbers using a regression formula developed through hemacytometer cell counts. The test duration was 96 h under continuous cool-white fluorescent light at 4300 ± 10% lux at 24 °C ± 2 °C. In 2018, algal nutrient media was amended with 400 mg L^{-1} $NaHCO_3$, added as a carbon source in a no or minimal headspace environment. Test chambers were 50 mL glass Erlenmeyer flasks with polytetrafluoroethylene (PTFE)-lined screw caps, containing *ca*. 64 mL of test solution and a Teflon®-coated stir bar. Algal cell density was determined daily using a hemacytometer. Test chambers were randomly assigned daily on a multiposition magnetic stir plate. Tests were performed for 72 h under continuous cool-white fluorescent light at 4440–5920 lx at 23 °C ± 2 °C. Relative growth rate (EPA, 1987a) (log10 based) or average specific growth rate (OECD, 2011) (ln based) was calculated according to the respective test guidelines, and percentage inhibition of the control was computed.

For daphnid and fish studies conducted in 2018, diluent was "moderately hard" (80–100 mg L^{-1} as $CaCO_3$) or "hard" (>140 mg L^{-1} as $CaCO_3$) reconstituted water prepared with UV-sterilized, deionized (DI) well water and reagent-grade salts (APHA, 2017). In earlier studies, the dilution water was a laboratory blend of filtered well water

and reverse-osmosis water, meeting the same hardness specifications as the reconstituted water. Tests were performed in a temperature-controlled water bath or environmental chamber, with a 16:8 h light:dark photoperiod (*ca.* 400–1100 lx). Unless stated otherwise, test chambers were covered with a glass sheet to minimize evaporation or contamination.

Daphnia magna were cultured in-house. Neonates (<24 h old) were randomly assigned to test chambers that were randomly positioned in the test area. Tests were conducted over 48 h at 20 °C ± 2 °C. For tests conducted in 1988–1990, test chambers were autoclaved 400 mL glass beakers containing 300 mL of test solution. Two replicate chambers containing 10 daphnids each were prepared for each treatment and the control. For 2018 tests, test chambers were 130 mL glass bottles completely filled with solution (no headspace), closed with a screw top PTFE-lined cap. Each treatment and control consisted of four replicate chambers, each containing five daphnids.

Ceriodaphnia dubia were cultured in-house. Neonates (<24 h old, released within a 6 h period) were randomly assigned to test chambers that were randomly positioned in the test area. Survival and reproduction tests were conducted over 7 days at 25 °C ± 1 °C. The diluent was laboratory blend water, which had been aged for 7 days and spiked with YCT (yeast/cereal leaves/trout chow). Test chambers were either 20 mL glass scintillation vials or 30 mL polypropylene beakers containing 15 mL of test solution. Ten replicate chambers containing a single daphniid each were prepared for each treatment and the control. Following daily observation and enumeration of young, parent organisms were transferred to fresh solution and fed 2×10^5 cells mL^{-1} of *P. subcapitata* algae and 0.1 mL of YCT. There are two notable differences between the 1985 test guideline followed for these studies and the current version. First, the test was started with neonates collected from separate parent organisms, fully randomized, whereas in the current version, a block randomization procedure (so that offspring from a single female are distributed evenly among the treatments) would be followed. Second, the test was terminated within ±2 h of exactly 7 days, whereas the current test would end after at least 60% of the control organisms produce their third brood or at the end of 8 days, whichever occurs first. These updates to the test guide reduce the variability within and across treatment levels.

Fathead minnow (*Pimephales promelas*) juveniles (7–22 weeks old, depending on the study) for acute toxicity tests were either obtained from Aquatic Research Organisms, Hampton, New Hampshire (and acclimated to dilution water for at least 14 days and to test temperature at least 7 days) or cultured at the test facility. Acute toxicity tests were conducted over 96 h at either 22 °C or 23 °C ± 1 °C. For tests conducted in 1988–1990, test chambers consisted of 5 L glass aquaria containing 2.0–4.1 L of test solution, providing a 0.32–0.67 g L^{-1} organism loading. Two replicate chambers containing 10 organisms (randomly assigned) each were prepared for each treatment and the control. In 2018, the test chambers consisted of *ca.* 4 L glass jars containing 3.8 L solution, closed with a polycarbonate screw cap, providing a 0.08–0.09 g L^{-1} organism loading. Each treatment and control consisted of two replicate chambers, each containing seven organisms. In 2018, organisms were transferred to fresh solution daily (daily renewal), while older studies were static (no solution renewal).

For larval survival and growth tests (*P. promelas*), organisms were cultured at the test facility. Newly hatched larvae were either randomly or sequentially distributed to test chambers. Tests were conducted over 7 days at 25 °C ± 2 °C. Test chambers consisted of 400 mL polypropylene beakers containing 250 mL of test solution. Four replicate chambers containing five organisms each were prepared for each treatment and the control. Fish were fed twice daily with <24-hour-old brine shrimp nauplii. Following daily observation, test solutions were renewed by siphoning out approximately 90% of solution and replacing with fresh solution. At termination, fish were euthanized using a benzocaine solution, placed (by replicate) on preweighed foil dishes, dried in a 100 °C oven for at least 2 h, and then weighed.

Rainbow trout (*O. mykiss*) were 6–10-week-old juveniles obtained from Spring Creek Trout Hatchery, Lewistown, MT. Fish were acclimated to dilution water for at least 14 days and to test temperature for at least 7 days. Tests were conducted over 96 h at 12 °C ± 1 °C. Test chambers consisted of 8.5–19 L glass aquaria containing 6.0–7.5 L of test solution. Two replicate chambers containing 10 organisms (randomly assigned) each were prepared for each treatment and the control.

The protocol and procedures used were ethically reviewed and approved by the laboratory's animal use coordinator (2005–2018) or laboratory director (1988–1990). All fish were treated humanely in accordance with published guidance (AVMA, 2013; NRC, 1985, 2010). The study design and personnel training were sufficient to minimize animal pain within the confines of the study objectives. All fish were euthanized using a benzocaine solution (1988–1990) or a tricaine methane sulphonate (MS-222) solution of 500 mg L^{-1} (2018) or 2 g L^{-1} (bioaccumulation study) buffered with sodium bicarbonate at pH 7.0 as per laboratory standard operating procedure, prepared in laboratory dilution water.

A few studies were performed by an external laboratory, the results of which are included here. Acute toxicity to luminescent bacteria *Photobacterium phosphoreum* was determined for C12br-7EO and L1315-7EO. Acute toxicity to zebrafish *Danio rerio* (Directive 84/449 EEC, Cl, No. L 251/146 (19.9.84) and chronic toxicity to *D. magna* (ISO TC 147/SC5/GT2 No. 28) were determined for C12br-7EO; both conformed to OECD GLP.

Analytical Methods

Elemental analysis for carbon, hydrogen, nitrogen, and oxygen (C, H, N, O) content was performed by Intertek, Whitehouse NJ. Analysis was performed using a Perkin-Elmer 2400 CHN Elemental Analyzer equipped with an oxygen accessory kit. The measured C, H, N, O values were used to calculate the ThOD of the sample for biodegradation testing.

Acute aquatic toxicity tests included analysis of both fresh and old test solutions for total organic carbon (TOC). At a minimum, the low-, mid-, and high-level treatments were analyzed, while for many of the toxicity tests, TOC analysis was performed on samples from each treatment level. TOC analyses for studies of C10br-9EO, C13br-3EO, and C13br-5EO were obtained using the oxidation combustion infrared analysis method with a Shimadzu TOC-V Total Organic Carbon Analyzer. For all other studies, TOC analysis was performed on an O.I. Model 700 Total Carbon Analyzer. All TOC analyses were performed in duplicate.

For C12br-7EO, C13br-7EO, and C1315b-7EO studies, total nonionic surfactants as cobalt thiocyanate active substances (cobalt thiocyanate active substances) were quantified. Water samples from the low-, mid-, and high-level test treatments were extracted and complexed using the Wickbold method (Wickbold, 1972). The complexed extracts were analyzed by optical spectroscopy following the procedure outlined in Standard Methods for the Examination of Water and Wastewater, method 512C (Greenberg et al., 1985).

For the bioaccumulation study, triplicate aliquots of feed were extracted and analyzed at the start and end of the uptake period (Day 0 and 10). One-gram feed samples were extracted with 25 mL of 50% methanol/50% ethyl acetate. Samples were extracted for 1 min by manual shaking followed by 60 min of mechanical shaking. The contents of the vial were permitted to settle for 60 min, after which aliquots were placed in autosampler vials. Fish were extracted using a Dionex ASE 200 Accelerated Solvent Extractor with a mixture of 80% methanol/20% ethyl acetate at 125 °C and 1500 psi. The raw ASE extracts were reduced to a final volume of 2.0 mL under a gentle stream of nitrogen. The concentrated extracts were then centrifuged, and a portion of each concentrated extract was passed through a conditioned Bakerbond Octadecyl (J.T. Baker) solid-phase extraction disposable clean-up column and rinsed with a mixture of 50% methanol/50% water prior to being eluted with 90% methanol/10% water. The final volume collected was adjusted to 0.5 mL. Standards were prepared by fortifying control fish with the test substance and taking them through the same extraction and clean-up procedures as the sample fish.

Solvent extracts of spiked and control diet and fish carcasses were performed in the multiple reaction monitoring (MRM) mode using liquidchromatography–mass spectrometry (LC–MS) (liquid chromatography–mass spectrometry) operated in the selective ion recording mode using a Micromass Quattro LC using MassLynx software (version 3.5) with a Hewlett-Packard 1050 quaternary pump and autosampler. Fortified standards of the test substance in control diet and fish were analyzed for method standardization. The corresponding practical quantitation limit (PQL) was approximately 0.2 μg or 0.14 μg g^{-1} for a fish sample weighing 1.5 g. Fish lipid was extracted by ASE and measured gravimetrically. The method was based on that described in Dionex Application Note 334 (1999).

Data Analysis

Toxicological Endpoint Analyses

For acute toxicity tests, the statistical method used to calculate LC50s and associated 95% confidence intervals was based on the dose–response pattern observed. Methods utilized included the PROC PROBIT procedure in SAS (SAS, 2013), a probit procedure based on Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1949), a nonlinear regression model (Logistic 3P) (Ratkowsky, 1993), the trimmed Spearman-Karber Method (Hamilton et al., 1977), and the Binomial Method (Stephan, 1977). The algal EC10s and EC50s for growth rate were calculated using a probit regression calculation based on the methods of Finney (Finney, 1971).

For chronic toxicity tests, the No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values were determined for the survival and growth data of the fathead minnow and for the parent survival and reproduction data of *C. dubia*. Fisher's exact test was performed on *C. dubia* survival data. For all remaining data, Dunnett's procedure (parametric data) or Steel's many-one rank test (nonparametric data) was used. Shapiro-Wilk's test for normality and Bartlett's test for homogeneity of variance were used to determine the parametric status of the data. These statistical procedures were part of the TOXSTAT (version 3.0, 3.2) software (Gulley et al., 1989; Gulley et al., 1990).

Bioaccumulation Endpoint Analyses

The dietary, lipid-corrected BMF (biomagnification factor); dietary assimilation efficiency; and growth-corrected whole-body half-lives were calculated (OECD, 2012). The uptake phase of the dietary bioaccumulation test is described by:

$$C_{\text{fish}}(t) = \frac{\text{E I}}{K_{\text{T}}}[1 - e - \text{K}_{\text{T}}\, t]\, C_{\text{diet}} \quad (1)$$

where $C_{\text{fish}}(t)$ is the concentration of the chemical in fish at time t (μg g^{-1}), C_{diet} is the concentration of the chemical in diet [μg g^{-1}], E is the assimilation efficiency of chemical from the diet, I is the ingestion rate [g food/g wet fish/day], K_{T} is the first-order elimination rate [day^{-1}], and t is exposure time (days).

During the depuration phase, the concentration in fish is described by the linear equation:

$$\ln C_{\text{fish}}(t) = \ln C_{\text{fish}}(0) - K_{\text{T}} t \quad (2)$$

where ln C_{fish} (0) and (t) denote the natural logarithm of the concentration in fish tissue at the start and at time t of the depuration period, respectively.

These kinetic data can then be used to estimate the intercept [ln C_{fish} (0)] and slope [K_{T}] in Eq. (2) using linear regression. As the intercept also characterizes the fish tissue concentration at the end of the exposure period, this value along with the K_{T} estimate derived from the depuration experiment and the time provided for uptake can be substituted into Eq. (1) to estimate the assimilation efficiency of the diet.

The total depuration rate obtained using Eq. (2) reflects gill elimination, fecal egestion, biotransformation, and growth dilution. The contribution of growth dilution (using growth rates during the study period) allows the calculation of a growth-corrected half-life for the calculation of the BMF, which can then be expressed on a lipid-normalized basis (OECD, 2012).

TLM Analysis

The TLM for predicting aquatic toxicity of polar and nonpolar narcotic chemicals is expressed as follows:

$$\log\left(LC_{50,ij}\right) = \log\left(CTLBB_i\right) - 0.94\log\left(K_{ow,j}\right) + \Delta c_j \quad (3)$$

where $LC_{50,ij}$ is the concentration of chemical "j" in organism "i," corresponding to 50% mortality [mmol L^{-1}]; $CTLBB_i$ is the critical target lipid body burden of organism "i" [mmol/kg-lipid]; $K_{ow,j}$ is the octanol–water partition coefficient of chemical "j" [L –water/kg-octanol]; and Δc_j are chemical class-specific correction factors for chemical "j" (Di Toro and McGrath, 2000; Di Toro et al., 2000; Kipka and Di Toro, 2009). Values for the critical body burdens, universal narcosis slope, and the chemical class correction factors have been updated consistently since the model's inception as new data have been developed and new species and chemicals added (Bragin et al., 2016; Di Toro and McGrath, 2000; Di Toro et al., 2000; Kipka and Di Toro, 2009; Redman et al., 2014b; Redman et al., 2017). For this analysis, values for the critical body burdens, universal narcosis slope, and the chemical class correction factors were taken from the most recent reanalysis and update of the TLM in 2018 (McGrath et al., 2018).

Historically, the TLM has been calibrated and applied using estimated octanol–water partition coefficients (Di Toro et al., 2000). This is due in part to the absence or high variability in experimental values for the large number of chemicals used in deriving and validating the original model. Early iterations of the model used SPARC Performs utomated Reasoning in Chemistry (SPARC) (SPARC Performs Automated Reasoning in Chemistry) estimations of log (K_{ow}), while later updates to the TLM switched to EPI Suite (KOWWIN)-based estimates of log (K_{ow}). For surfactants, large discrepancies between observed and predicted log (K_{ow}) have been reported for the KOWWIN-predicted log (K_{ow}). In addition, several experimental methods, tested by Hodges et al. in their previous work, were shown to have significant variability. More recently, polyparameter approaches, such as those of Abraham et al. (Abraham et al., 2004; Endo and Goss, 2014; Platts et al., 2000), have been shown to demonstrate excellent predictive capabilities for wide ranges of chemical classes and functional moieties. Consequently, octanol–water partition coefficients for the AEO were computed using the Abraham polyparameter linear free-energy relationship (pp-LFER) developed by Goss (Goss, 2005), which is expressed as:

$$\log\left(K_{ow,j}\right) = e_{ow}E_j + s_{ow}S_j + a_{ow}A_j + b_{ow}B_j + v_{ow}V + c_{ow} \quad (4)$$

where the uppercase descriptors represent the excess molar refractivity (E), polarizability (S), hydrogen bond acidity (A), hydrogen bond basicity (B), and McGowan volume (V) of a chemical "j." Lowercase descriptors represent complementary solvent–system interactions, with c_{ow} representing nonspecific interactions and carrying the units of the partition coefficient (Abraham and Acree, 2010; Abraham et al., 2004; Abraham and Zhao, 2004; Endo and Goss, 2014; Goss, 2006; Goss and Schwarzenbach, 2001; Platts et al., 2000). Uppercase solute descriptors are estimated using the UFZ LSER database (Ulrich et al., 2017) *via* a fragment-based approach. Previous work has demonstrated excellent agreement between experimental and fragment-predicted Abraham descriptors for a wide range of chemistries (Japertas et al., 2014; Platts et al., 1999).

It should be noted that pp-LFER for membrane and storage lipid (as well as other biological phases) have been developed (Endo et al., 2011) and could, in theory, be directly substituted into Eq. (3), eliminating the need for an extrapolation from octanol to membrane–water, K_{mw}, partition coefficients. This would minimize the overall model uncertainty where the membrane–water partition coefficients are known experimentally (Müller et al., 1999). However, where K_{mw} must also be estimated (e.g., using a pp-LFER model), there will be an associated uncertainty. It is currently unclear if these uncertainties would be smaller or larger than those associated with the estimated log (K_{ow}) values for surfactants.

Previous work (McGrath et al., 2018) has demonstrated correlations between the estimated critical body burdens (CTLBB) and the universal narcotic slope (m). Consequently, to update the TLM using the KOWWIN log (K_{ow}), the CTLBB and slope had to be re-estimated as the model parameters are cross-correlated. Furthermore, in the calculation of

the HC5 value (Eq. (6)), the variance and the mean of the TLM variables are included in the extrapolation. To directly apply membrane–water partition coefficients within the TLM (Eq. (3)), a complete recalibration of the model is required. This work is ongoing but is out of scope for this analysis, which offers a *de novo* application and analysis of the TLM to AEO surfactants, demonstrating the existing model applicability and accuracy.

Consequently, the Goss pp-LFER was selected due to systematic underprediction of octanol–water partition coefficients for AEO by the KOWWIN (EPA, 2018) model typically used to estimate K_{OW} values for the TLM. In addition, several other fragment-based models and the membrane lipid–water pp-LFER model of Endo et al. were evaluated and compared (where possible) to experimental data (see Tables S7–S9). Finally, the KOWWIN and Goss pp-LFER models were compared to the K_{OW} values used to derive water quality guidelines by Environment Canada (2013) (their performance is summarized in Fig. S1.).

Chronic toxicity predictions using TLM are derived as follows from the acute TLM predictions (Eq. (3)):

$$CE_{i,j} = \frac{LC50_{i,j}}{ACR_i} \tag{5}$$

where $CE_{i,\ j}$ is the chronic effect predicted by the TLM (comparable to an NOEC or EC10) for organism "i" and chemical "j," $LC50_{i,\ j}$ is the acute 50% effect level predicted in Eq. (3) for chemical "j," and ACR_i is the acute to chronic ratio for organism "i." While individual acute to chronic ratios can vary considerably between studies, an average value for each organism (for predicting chronic effects) or an overall average value $E[ACR_i]$ (for extrapolating down to HC5-predicted effects levels) can be used (McGrath et al., 2018). It should be noted that predicted chronic effects levels (CE) do not correspond uniquely to a single chronic toxicological endpoint (i.e., NOEC, EC10) but are instead meant to be representative of either of these endpoints (McGrath et al., 2018).

The equation to extrapolate down to the hazard concentration threshold protective of 95% of species (HC5) from the acute TLM model (Eq. (3)) is derived by McGrath et al. (2004) and is as follows:

$$\begin{aligned}\log\left(HC5_j\right) = {} & E[m]\log\left(K_{ow,j}\right) + E[\log(CTLBB_i)] + \Delta c_j - \mathrm{E}[\log(ACR_i)] \\ & - k_z\sqrt{V[m]\log\left(K_{ow,j}\right)^2 + V[\log(CTLBB_i)] + V[\log(ACR_i)]}\end{aligned} \tag{6}$$

where E[] and V[] represent the mean and variance of the respective variables across all species contained in the TLM (defined previously), respectively, and k_z represents the 95% confidence extrapolation factor for a log-normal distribution (McGrath et al., 2018).

The performance of the TLM in estimating acute and chronic toxicity, as well as ultimately deriving model-based HC5 effects levels, were compared to experimental data by evaluating the root mean square error (RMSE) of the logarithmic toxic effects levels in [mg L^{-1}]. The RMS error for the acute and chronic TLM was computed as follows:

$$RMSE_k = \sqrt{\frac{\sum_{i=1}^{s}\sum_{j=1}^{n}\left(EC_{i,j,k} - EC_{i,j,obs}\right)^2}{N}} \tag{7}$$

where $RMSE_k$ is the root mean square error of the respective model "k," N is the total number of observed data points (for all species, "i," and all chemicals, "j"), and EC is a given effects concentration (LC50, NOEC, EC10, *etc*.) for the given chemical and species. As the RMS errors are computed on the log-transformed variables, an RMS error of 0.3 corresponds approximately to a factor of 2× the average predictive error in linear space, with an RMS error of 0.5 corresponding approximately to a factor of 3× the average predictive error. This range can generally be considered a practical limit of QSAR models for complex environmental systems (i.e., lipid–water, organic carbon–water) for which active site/structural heterogeneity introduces substantial uncertainty over more homogeneous systems (i.e., pure solvent–water) (Endo and Goss, 2014).

All model calculations, statistical analysis, processing, and data visualization were carried out in the R software package (R Development Core Team, 2018). Default parameters for the TLM were obtained from McGrath et al. (2018) and were used as is unless otherwise noted.

Results and Discussion

Biodegradation of Branched AEO

The European Commission Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) deemed it not necessary to utilize the 10-day window criteria (i.e., the pass level must be reached within 10 days after 10% degradation is reached in the test) for assessing the ultimate

biodegradability of surfactants in detergents (CSTEE, 1999). The basis for this recommendation is the kinetics associated with different homologues present in many commercial surfactants, differing intermediate metabolite kinetic rates, and potential interference of parent molecule degradation by metabolites. While not a requirement for drawing a conclusion of ready biodegradability, the 10-day window has been adopted as a more stringent requirement for some voluntary certification programs, and thus, those results are reported here.

Ultimate biodegradation results are presented in Table 2. AEO derived from branched C8-rich, C9-rich, C10-rich, and C11-rich oxo-alcohols with 1–20 ethoxylates all pass the readily biodegradable criteria within the 10-day window, with a single exception. C11br-3EO met the 28-day threshold for readily biodegradability but did not fulfill the 10-day window criteria. This is surprising as the C11-rich oxo-alcohol does pass the 10-day window, including when tested in conjunction with the C11br-3EO sample. AEO derived from branched C13-rich oxo-alcohols with eight or more moles of EO also meet the readily biodegradable criteria within the 10-day window. C13br-7EO passed the 28-day threshold but did not fulfill the 10-day window criteria. Studies performed at multiple substance loading rates indicate a dose–response effect where lower concentrations achieved a higher level of biodegradation over the 28 days. This is recognized as a potential issue with the Manometric Respirometry test, where inhibitory effects from relatively high concentrations of test substance may reduce the level of degradation achieved. Consequently, results in these test systems that reach the pass level demonstrate ready biodegradability with no restrictions (CSTEE, 1999).

To evaluate this further, acute toxicity to activated sludge was predicted using the TLM (Redman et al., 2007) and compared to the concentrations used in the biodegradation tests. The values are provided in Table S3 and demonstrate that, when tested well below their estimated EC50, ultimate biodegradation is easily achieved within the 10-day window criteria (usually 4–5 days), and overall biodegradation averages over 90%. More variability is observed when tested near (±30%) estimated EC50s, where most (70%) substances met the 10-day window requirement and all reach 60% biodegradation within 2 days of the 10-day window. Where test concentrations were well above (1.5–3×) the estimated EC50, the 10-day window was not achieved, and the time to pass the 60% threshold was slower (13–25 days). This trend indicates that the microbes may be affected and able to recover over the 28-day test duration but also that the TLM would be informative when selecting biodegradation test concentrations.

Prior studies have indicated that aerobic biodegradation of branched AEO depend on the structure and degree of branching of the alkyl chain. The Danish EPA summarized data on linear C9-18 AEO containing 5–14 EO units (Madsen et al., 2001), indicating that they are ultimately degraded under aerobic conditions, achieving between 64% and 86% degradation when using test methods most similar to this study (28-day closed bottle or CO_2 evolution test). Comparable levels of biodegradation (61–100%) were observed in this study for C8-13-rich branched alcohols containing 1–20 EO units. It is apparent, however, that lower biodegradation occurs in the most highly branched C13 with lower levels of EO than shorter alkyl chains with less branching (Fig. S2).

Table 2 Summary of OECD 301F ready biodegradation test results

Substance	Conc. (mg L^{-1})	Day 28% biodeg.	Result (10d window)
C8br-4EO	43	91.7	Readily (Y)
C8br-6EO	39	103[a]	Readily (Y)
	51	84.4	Readily (Y)
C8br-8EO	42	100	Readily (Y)
C8br-10EO	46	107[a]	Readily (Y)
C9br-1EO	41	81.7	Readily (Y)
C9br-3EO	42	90.6	Readily (Y)
C9br-5EO	41	82.9	Readily (Y)
	37	96.6	Readily (Y)
C9Sbr-7EO	40	102[a]	Readily (Y)
C9br-8EO	39	92.8	Readily (Y)
	37	98.8	Readily (Y)
C9br-20EO	37	95.4	Readily (Y)
C10br-3EO	36	83.8	Readily (Y)
	35	85.6	Readily
	50	79.5	Readily (Y)
C10br-7EO	39	87.5	Readily (Y)
	54	84.2	Readily (Y)
C10br-9EO	34	112[a]	Readily (Y)
C11br-3EO	34	77.2	Readily
	33	81.0	Readily
C11br-5EO	34	82.1	Readily (Y)
	37	80.7	Readily
C11br-7EO	36	106[a]	Readily (Y)
C11br-8EO	37	87.0	Readily (Y)
C11br-10EO	36	95.2	Readily (Y)
C13br-7EO	6.0, 12, 25, 48	85.7, 67.9, 66.6, 60.7[b]	Readily
	41	66.2	Readily
	26	68.9	Readily
C13br-8EO	58	67.8	Readily
	22	66.9	Readily (Y)
C13br-12EO	13, 25, 51	66.2, 68.2, 80.0[c]	Readily (Y, 1 conc)
	26	97.0	Readily (Y)

[a] Substance reached 60% within 6-9 days and ranged 76-95% at end of 10 day window (see Table S3).

[b] Tested at multiple concentrations simultaneously; potential inhibition of microbes at higher concentrations.

[c] Tested at multiple concentrations simultaneously; no apparent inhibition of microbes.

Bioaccumulation of Branched AEO

Juvenile rainbow trout were exposed to C11br-3EO *via* their diet. The mean measured exposure concentration in the diet was 527 μg g^{-1}. There was no significant loss of test substance in the diet over the uptake period. Fish lipid content at the end of the uptake phase was 2.24%. No difference in mortality or growth rate was observed between the treated diet and the control at the end of the study (24 days in total). The growth-corrected whole-body half-life was determined to be 0.36 days, with a corresponding lipid-corrected BMF value of 0.012. Previous aqueous, as well as dietary bioaccumulation, studies have been performed with C10-rich (C10br-0EO) and C13-rich (C13br-0EO) oxo-alcohols in juvenile rainbow trout

Table 3 Acute aquatic toxicity test results

Substance	Species	Endpoint	Effect concentration mg/L[a]
C10br-9EO	*P. subcapitata*	72h EC50	128 (117–141)
	D. magna	48h EC50	>100[b]
	P. promelas	96h LC50	39 (29–52)
C12br-7EO	*P. subcapitata*	72h EC50	36.9 (27.2–58.5)
	P. phosphoreum	15m EC50	6.4
	D. magna	48h EC50	6.84 (5.52–8.46)
	O. mykiss	96h LC50	6.7 (6.0–7.5)
	P. promelas	96h LC50	6.3
	D. rerio	96h LC50	13.5 (12.1–15.2)
C12br-12EO	*P. subcapitata*	96h EC50	210 (154–324)
	D. magna	48h EC50	30.2 (23.2–39.4)
	O. mykiss	96h LC50	31.5 (26.3–37.7)
	P. promelas	96h LC50	26.0 (23.3–29.0)
C13br-3EO	*P. subcapitata*	72h EC50	6.0 (5.6–6.5)
	D. magna	48h EC50	1.1 (0.98–1.3)
	P. promelas	96h LC50	>1.8[c]
C13br-5EO	*P. subcapitata*	72h EC50	8.4 (7.9–8.8)
	D. magna	48h EC50	4.5 (3.8–5.3)
	P. promelas	96h LC50	1.8 (1.6–2.1)
C13br-7EO	*P. subcapitata*	72h EC50	25.6 (19.7–46.9)
	D. magna	48h EC50	5.92 (5.02–6.99)
	O. mykiss	96h LC50	4.62 (3.40–6.28)
	P. promelas	96h LC50	4.41 (3.69–5.27)
C13br-12EO	*P. subcapitata*	96h EC50	172 (cnc)
	D. magna	48h EC50	37.0 (30.0–48.3)
	O. mykiss	96h LC50	13.9 (12.2–15.9)
	P. promelas	96h LC50	15.7 (13.2–18.8)
C1315a-7EO	*P. subcapitata*	72h EC50	0.61 (0.50–0.74)
	D. magna	48h EC50	0.74 (0.42–1.31)
	P. promelas	96h LC50	1.48 (1.25–1.75)
C1315b-7EO	*P. subcapitata*	72h EC50	0.74 (cnc)
	P. phosphoreum	15m EC50	1.71
	D. magna	48h EC50	0.61 (0.46–0.80)
	O. mykiss	96h LC50	1.2 (1.0–1.5)
	P. promelas	96h LC50	1.28 (1.12–1.47)
C1315a-12EO	*P. subcapitata*	72h EC50	5.7 (cnc)
	D. magna	48h EC50	3.20 (2.73–3.74)
	O. mykiss	96h LC50	3.78 (3.16–4.52)
	P. promelas	96h LC50	2.73 (2.44–3.04)

[a] 95% confidence interval in parentheses.

[b] 35% immobilization at high dose.

[c] 36% mortality at high dose.

(Camenzuli et al., 2019). In the aqueous tests, steady-state fish tissue concentrations were achieved within a 16-day (C10br-0EO) or 10-day (C13br-0EO) exposure period. The 5% lipid-normalized steady-state BCF_{ssl} values were 21 L kg^{-1} (C10) and 39 L kg^{-1} (C13) wet fish weight, and calculated dietary, lipid-corrected BMF values were ≤0.016 (at 500 and 2500 ppm treatment levels) for both alcohols, with growth-corrected whole-body half-lives of less than 0.6 days. These data are consistent with numerous studies demonstrating that alcohols and their ethoxylates are highly metabolizable (Belanger et al., 2009; Tolls et al., 2000).

Acute Toxicity of AEO

Analytical results demonstrated that measured concentrations correlated well with nominal concentrations (generally ±20%) and that the test substance was stable and present in solutions (little to no loss) for the study duration. For the C13br-3EO and C13br-5EO, TOC was not sensitive at concentrations below *ca.* 10 mg L^{-1}, so only the stock and highest treatment solutions were analyzed. Similar restrictions occurred with TOC analysis of initial tests of C1315a-7EO where nominal concentrations were below 5 mg L^{-1}, although later testing of C1315b-7EO at slightly higher concentrations included analytical confirmation confirming the presence and stability of the concentration. All the substances are readily soluble at the concentrations tested; therefore, all results are based on nominal concentrations.

Median lethal concentrations (LC50s) were greater than 10 mg L^{-1} for C10br-9EO, C12br-12EO, and C13br-12EO. Acute toxicity ranging from 1–10 mg L^{-1} was observed for C12br-7EO, C13br-3EO, C13br-5EO, C13br-7EO, and L1315-12EO. Only the semilinear L1315-7EO would be considered "very toxic to aquatic organisms" (LC50 0.1—1.0 mg L^{-1}). Individual study results are presented in Table 3. It should be noted that, for 96 h algal studies, when percentage inhibition of growth rate at 72 h was greater, those results are presented. These findings are consistent with evaluations of aquatic toxicity of AEO (Madsen et al., 2001), which have demonstrated an increase in toxicity with increasing hydrophobe chain length and with decreasing EO chain length (Fig. 1).

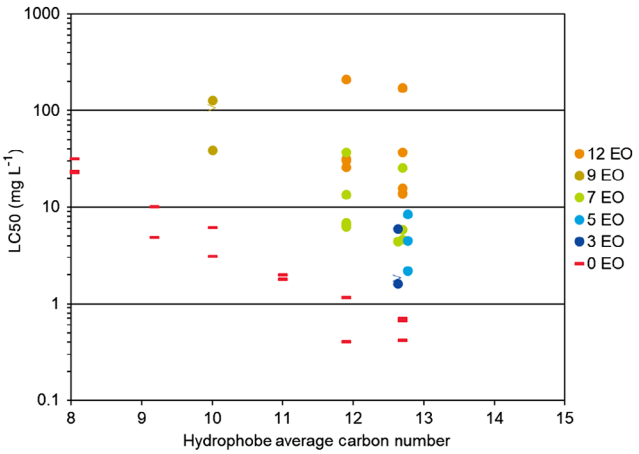

Fig. 1 Effect of hydrophobe (oxo-alcohol) and ethoxylate (EO) chain length on acute aquatic toxicity. Toxicity increases with increasing hydrophobe chain length and decreases with increasing EO. Greater than symbols indicate less than 50% effect at highest concentration tested. Horizontal solid black lines correspond to GHS classification thresholds. Toxicity of associated branched alcohols added where available (red squares)

Chronic Toxicity of AEO

Chronic aquatic toxicity data for three trophic levels were developed for 7 mol ethoxylates of a branched C12-rich and C13-rich oxo-alcohol and for a mixture of semilinear C13/C15 homologues. Chronic data for algae were obtained for a broader range of AEO (Table 4). It should be noted that the *C. dubia* study with C12br-7EO was terminated on day 7, while 70% of the controls had only two broods as per the previous test guidance. The current test guideline requires three broods in at least 60% of control organisms (within 8 days). There was a clear concentration response, and the result is consistent with the *D. magna* study with C12br-7EO (NOEC 2.5 and 5.5 mg L^{-1}).

A similar trend of increasing toxicity with increasing hydrophobe chain length and with decreasing EO chain length is apparent (Fig. S3), although data to evaluate the effect from EO length are available for algae only. Fish

Table 4 Summary of chronic aquatic toxicity test results

Substance	Species	Endpoint	Effect concentration (mg/L)
C10br-9EO	*P. subcapitata*	72h ErC10	54 (39–66)
C12br-7EO	*C. dubia*	7d NOEC/LOEC[a]	2.5/5.0
	D. magna	19d NOEC/LOEC[a]	5.5/8.0
	P. promelas	7d NOEC/LOEC[b]	0.625/1.25
	P. subcapitata	72h ErC10	15 (0–25)
C12br-12EO	*P. subcapitata*	96h ErC10	25 (0–89)
C13br-3EO	*P. subcapitata*	72h ErC10	1.9 (1.5–2.4)
C13br-5EO	*P. subcapitata*	72h ErC10	2.6 (2.3–2.8)
C13br-7EO	*C. dubia*	7d NOEC/LOEC[a]	1.25/2.5
	P. promelas	7d NOEC/LOEC[b]	0.75/1.5
	P. subcapitata	72h ErC10	9.6 (0–15)
C13br-12EO	*P. subcapitata*	96h ErC10	cnc
C1315a-7EO	*P. subcapitata*	72h ErC10	0.19 (0–0.32)
C1315b-7EO	*C. dubia*	7d NOEC/LOEC[a]	0.5/1.0
	P. promelas	7d NOEC/LOEC[b]	0.625/1.28
	P. subcapitata	72h EC10	0.38 (cnc)
C1315a-12EO	*P. subcapitata*	72h ErC10	3.5 (cnc)

[a] Reproduction, chronic effect.

[b] Growth, short-term chronic effect.

appeared to be the most sensitive species for the branched AEO C12br-7EO and C13br-7EO, whereas all three trophic levels of fish, invertebrates, and algae were comparably sensitive to the semilinear L1315-7EO.

Performance of TLM for Acute Toxicity of AEO

For the branched and semilinear AEO in this study (Tables 3 and S1.), as well as the compiled literature data (Table S2), the TLM was applied *a priori* in a blind prediction of acute toxicity effects levels for two algal species (*P. subcapitata* and *Scenedesmus subspicatus*), an invertebrate (*D. magna*), and several species of fish (*P. promelas*, *O. mykiss*, and *D. rerio*), as well as the microtox bioassay (*P. phosphoreum*). Critical body burdens for *P. subcapitata* were taken from Bragin et al. (2016), where they had re-estimated body burden values from the original TLM model. Critical body burdens for all other species were taken from the 2018 re-evaluated TLM model (McGrath et al., 2018), along with the universal narcotic slope and chemical class correction factors. For the AEO singular homologues, the exact structures were used to generate the molecular descriptors (Eq. (4)) for estimating log (K_{OW}) values for use in the TLM calculations (Eq. (3)). For both the linear and branched substances, initially, a representative structure was constructed using the average carbon and EO numbers, as well as information regarding the degree and location of branching. The results of the TLM prediction for acute toxicity of AEO are shown in Fig. S4.

The TLM for these representative structures provides generally good agreement (RMSE 0.051–0.879) across the seven test species, with the model often performing better for the branched AEO substances in the current study (Fig. S4). For *P. subcapitata*, despite the increased critical body burden, the TLM model systematically overpredicts toxicity for four of the seven branched AEO (Fig. S4d). In addition, linear AEO mixtures are systematically underpredicted for *P. promelas* and *D. magna* (Fig. S4a, b) compared to the branched substances in this study.

Previous work (Boeije et al., 2006) has highlighted the difficulty of using representative structures for predicting the ecotoxicity of AEO mixtures with broad ranges of carbon chain and ethoxylate numbers. As the toxicological response (i.e., LC50) does not vary linearly with carbon number, the use of an average structure for a broad distribution of carbon (i.e., a binary mixture) or ethoxylate number can result in substantial errors in predicted toxicity of the average compound *versus* the observed mixture. For mixtures of C8EO4 and C16EO8 (average structure C12EO6), EC50s for *D. magna* were overestimated by approximately a factor of 5×, where a mixture of C10EO8 and C14EO8 (average structure: C12EO8) was overestimated by a factor of 2.6×. This effect is particularly pronounced for binary mixtures with a broad range of alcohol chain length, level of EO, or both. This may explain the improved model performance of the more narrowly distributed branched AEO substances (this study), using a single representative structure, over that of the binary mixture linear AEO (Wong et al., 1997) for *D. magna* and *P. promelas*.

Consequently, the acute toxicity of the AEO substances were re-evaluated using a toxic unit (TU) addition approach, outlined by Loewe (1953) and previously applied by Di Toro and McGrath (2000) for describing the toxic effects of petroleum hydrocarbon mixtures. For this method, substances are subdivided into representative constituents, and the toxicities of the individual constituents are computed using the TLM model, with the effect concentration for the substance computed as follows:

$$EC_{\text{mix}} = \frac{1}{\sum TU_j} = \frac{1}{\sum f/_{EC_j}} \tag{8}$$

where EC_j is the TLM-predicted effect concentration (i.e., LC50) of constituent "j," f_j is the weight fraction of constituent "j" within the AEO mixture, TU_j is the computed toxic unit contribution of constituent "j," and EC_{mix} is the resultant effect concentration of the mixture by TU addition. The EC_{mix} computed from the TLM and relevant compositional information for the AEO mixtures were then compared to the experimental data and the predictions from the representative structure approach, described previously. The final acute TLM results using TU addition are shown in Fig. 2. RMSE errors (Eq. (7)) for the individual species are tabulated (Table S2) and are summarized by taxonomic group in Table 5.

TLM predictions for *D. magna* and *P. promelas* are significantly improved when individual constituent contributions are considered, as opposed to a single representative structure (and log (K_{OW})). Improvement in predicted acute effects are more pronounced for both the binary mixtures of AEO (Wong et al., 1997) and the semilinear AEO mixtures included in this study (see Table S2). This result is not unexpected as the underlying alkyl chain length distributions, essentially bimodal (*versus* a tight quasi-Gaussian distribution around the mean carbon chain length for the branched AEO (Table 1)), should yield considerably different results when average representative structures are used.

Little improvement was observed for branched AEO acute toxicity for *P. subcapitata*, which exhibited the weakest overall performance of the TLM (including the systematic overprediction of toxicity for six of the seven branched AEO). However, it should be noted that the TLM performs well for the semilinear (this study) and single structural homologues [52], with RMS errors comparable to those observed for the fish and invertebrate species (Table S2). Overall, the TU addition approach results in approximately 0.1 log-unit improvement in the

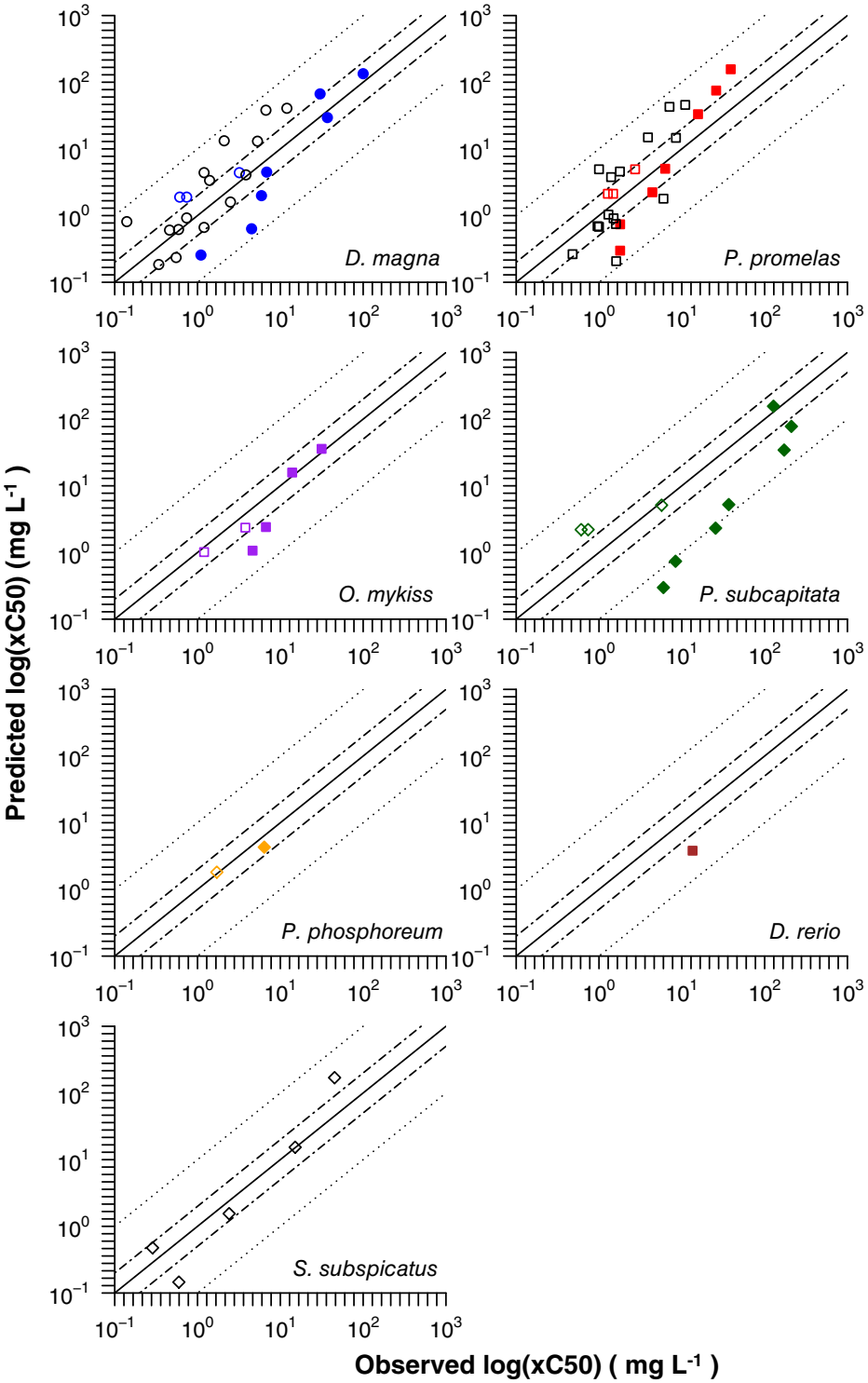

Fig. 2 Toxic unit addition (TU) TLM-predicted *versus* observed acute toxicities for seven aquatic organisms. Colored symbols indicate experimental data for branched AEO (filled) or semilinear AEO (open) from this study. Open black symbols indicate literature data. Solid lines represent 1:1 agreement, semidashed lines represent a factor of 2× predictive error, and dotted lines represent a factor of 10× predictive error. RMSE errors (Eq. (7)) for the individual species are tabulated and are summarized in Table S2

predictive accuracy of the TLM for the acute toxicity of the tested AEO species. The overall RMS error obtained for the AEO mixtures (RMSE = 0.491) is comparable to typical model errors for the much larger TLM datasets (RMSE = 0.460–0.566) reported previously (Kipka and Di Toro, 2009).

Table 5 RMS errors for TLM-predicted acute and chronic toxicity using representative AEO structures (AVG) and toxic unit addition (TU) methods

Group	Acute			Chronic	
	N	TLM (AVG)	TLM (TU)	N	TLM (TU)
Algae	15	0.663	0.665	27	1.037
Invertebrate	25	0.559	0.447	31	0.702
Fish	32	0.593	0.440	21	0.507
Total	74	0.589	0.491	79	0.793

Performance of TLM for Chronic Toxicity of AEO

Chronic toxicity for the nine AEO in this study and additional compiled literature data (Belanger et al., 2006) were evaluated, again using the TLM model, *a priori*. Several test species for which chronic data were available do not have established critical body burdens (CBB) within the TLM model framework. For these species, the mean value for the CBB TLM re-evaluation (McGrath et al., 2018) was used ($\bar{CBB} = 70.8\ \text{mmol kg}^{-1}$). For all species, the average ACR value ($\bar{ACR} = 5.22$) was used. This is consistent with the approach used for computing the HC5 from acute data (Eq. (4)). Due to the large number of species, organisms were grouped into three general taxonomic classes: algae, invertebrates, and fish for visualization. The results of the TLM prediction for chronic toxicity of AEO are shown in Fig. 3, with associated RMSE errors (Eq. (7)) for the individual species tabulated (Table S2) and summarized by taxonomic group in Table 5.

TLM prediction errors for chronic toxicity are slightly larger than those for acute toxicity of AEO. This is potentially due to the uncertainties discussed previously in test species' body burden, as well as the use of an average ACR value. In addition, it should be noted that, for chronic effects, multiple endpoints are considered (i.e., NOEC, EC10) and are included, both in this analysis and in the development and evaluation of the TLM model (McGrath et al., 2018). For broader dose–response curves or more variable experimental data, this can result in additional errors when multiple endpoint types are included in the same analysis.

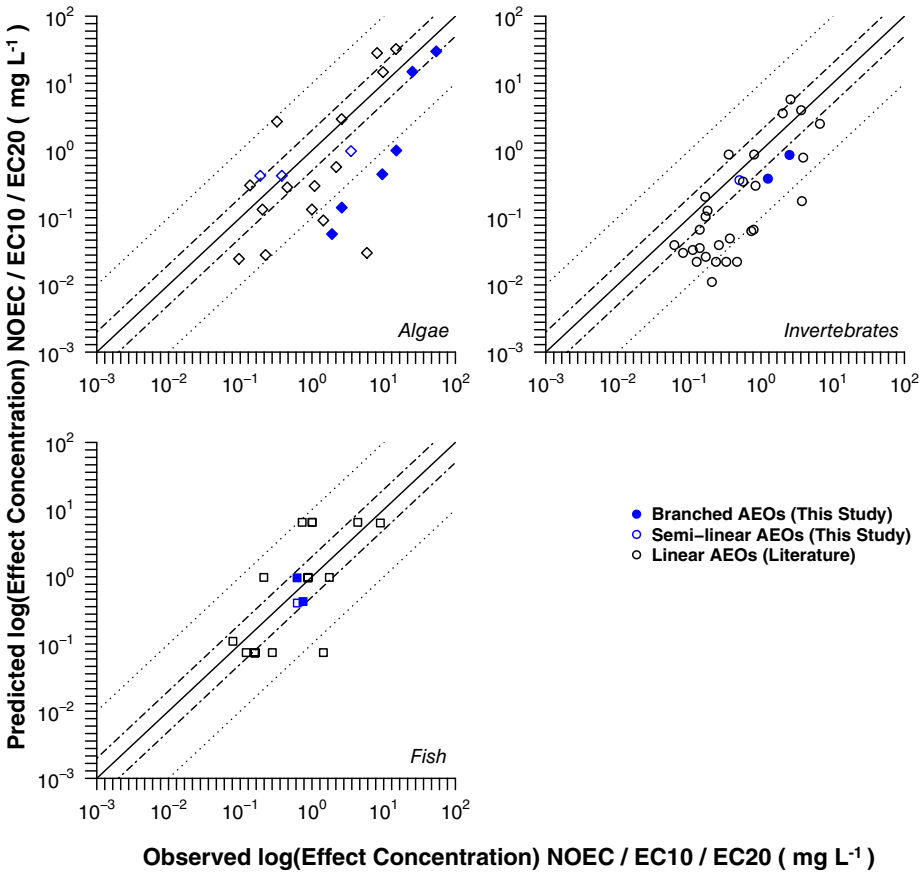

Fig. 3 Comparison of TLM-predicted *versus* observed chronic toxicities using representative AEO structures. Colored symbols indicate experimental data for branched AEO (filled) or semilinear AEO (open) from this study. Open black symbols indicate literature data. Solid lines represent 1:1 agreement, semidashed lines represent a factor of 2× predictive error, and dotted lines represent a factor of 10× predictive error. RMSE errors (Eq. (7)) for the individual species are tabulated and are summarized in Table S2

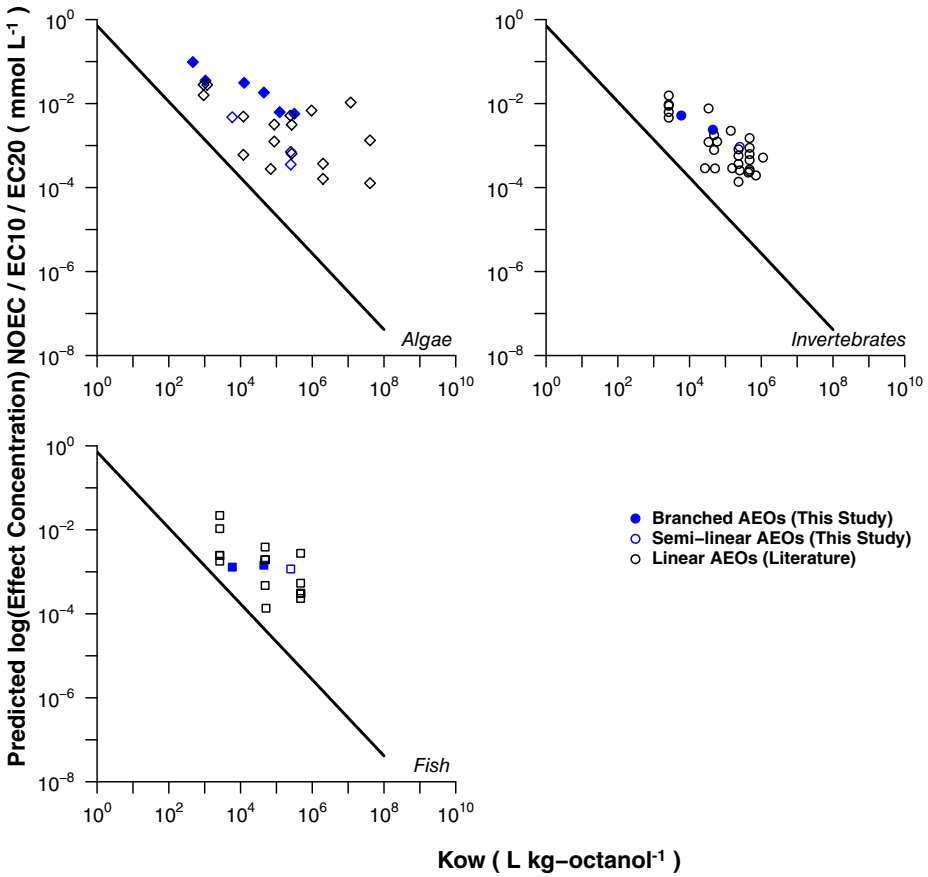

Fig. 4 Comparison of TLM-predicted HC5 *versus* observed chronic toxicities as a function of octanol—water partition coefficient (K_{ow}). Colored symbols indicate experimental data for branched AEO (filled) or semilinear AEO (open) from this study. Open black symbols indicate literature data. Solid lines represents the TLM-predicted HC5 (Eq. (6)) as a function of K_{ow}, protective of 95% of species

Despite these uncertainties, for fish, the prediction accuracy is generally comparable to the acute predictions (chronic RMSE of 0.507 *versus* acute RMSE of 0.491), while chronic RMS errors for invertebrates and algae were 0.26 and 0.37 log-units higher (approximately 1.8× and 2.5× higher) than acute RMS errors, respectively.

Comparison of Chronic Toxicity Data to TLM-Predicted HC5s

To confirm that a TLM-derived HC5 value (Eq. (6)) is sufficiently protective, the chronic AEO toxicity data were compared to the baseline narcotic HC5 value, and the results were plotted as a function of the octanol–water partition coefficient. The results of this are illustrated in Fig. 4.

There were 79 chronic effects data points representing the most sensitive chronic endpoint observed for the three general taxa (algae, invertebrates, fish) including 15 unique species and several life stages (see Table S2). For the 79 observations, four or fewer chronic endpoints falling below the HC5 baseline would constitute a threshold level value that is protective of ≥95%. As there were zero observed chronic effects below the baseline HC5, the TLM-derived threshold values for AEO can be considered to demonstrate adequate protection of sensitive species.

Acute and Chronic Classification of AEO Using TLM

A comparison of the GHS classification assignment from experimental data to TLM predicted values is presented in Table 6. Assignment of acute aquatic toxicity classification from TLM predictions is in good agreement with experimental values, with "correct" assignments in 63% of cases. For remaining cases, classification is overpredicted in 21% of cases but is underpredicted in only 16% of cases. Although the correct assignments are slightly lower for branched AEO (56%), the TLM is conservative, with only 4% of cases (n = 1) underpredicted. For 9 of the 15 cases (60%) of overprediction by TLM for a given species, the predicted values were consistent with both measured and predicted toxicity

Table 6 Prediction matrix for GHS acute and chronic ecotoxicity classification

Experimental	TU TLM predicted: all data [branched AEO]			
Acute	Acute 1 xC50 ≤ 1	Acute 2 1 < xC50 ≤ 10	Acute 3 10 < xC50 ≤ 100	Not acute xC50 > 100
Acute 1; xC50 ≤ 1	11 [0]	3 [0]	0 [0]	0 [0]
Acute 2; 1 < xC50 ≤ 10	10 [6]	23 [7]	6 [0]	0 [0]
Acute 3; 10 < xC50 ≤ 100	0 [0]	3 [3]	9 [6]	2 [1]
Not Acute; xC50 > 100	0 [0]	0 [0]	2 [2]	2 [2]

Chronic[a]	Chronic 1 CE ≤0.01	Chronic 2 0.01 < CE ≤0.1	Chronic 3 0.1 < CE ≤1	Not chronic CE > 1.0
Chronic 1; CE ≤0.01	0 [0]	0 [0]	0 [0]	0 [0]
Chronic 2; 0.01 < CE ≤0.1	1 [0]	4 [0]	1 [0]	0 [0]
Chronic 3; 0.1 < CE ≤1	1 [0]	20 [0]	20 [2]	3 [0]
Not Chronic; CE > 1.0	0 [0]	4 [1]	10 [4]	14 [3]

	Acute		Chronic	
	All	Branched	All	Branched
Correct:	45 (63.4%)	15 (55.6%)	38 (48.7%)	5 (50%)
Overpredicted	15 (21.1%)	11 (40.7%)	36 (46.0%)	5 (50%)
Underpredicted	11 (15.5%)	1 (3.7%)	4 (5.1%)	0 (0%)

[a] GHS chronic classification/toxicity ranges for rapidly biodegradable substances.

levels for other species (i.e., does not result in an over-classification of the substance). Many (64%) of the under-predictions by the TLM occur when either the predicted or measured values are close to a classification threshold.

Prior reports have shown a lower inherent aquatic toxicological hazard attributable to highly branched AEO relative to linear or semilinear analogs. These findings are also reflected in the Recommendations for the Harmonized Classification and Labelling developed by the European Committee of Organic Surfactants and their Intermediates (CESIO), which recommends relatively less severe GHS classification for an alternative chemical abstract services number commonly used to describe butene trimer-based branched C13 AEO (CESIO, 2017). The results presented here similarly support decreased environmental hazard classification for the branched ethoxylates tested.

None of the branched AEO tested fall under the criteria for classification as "very toxic to aquatic life" (LC50 ≤ 1 mg L^{-1}). Both 3-mol and 5-mol ethoxylates of the C13-rich oxo-alcohol were evaluated specifically to address whether acute toxicity may be overestimated if based on read-across to linear or semi-linear AEO of similar carbon chain length. Data for three trophic levels confirm that acute toxicity for C13br-3EO and C13br-5EO fall within the 1–10 mg L^{-1} acute toxicity range and that read-across is not appropriate, and TLM predictions are overly conservative.

Based on an evaluation of the underlying literature data used in the TLM evaluation (see Table S2), linear and semilinear alcohols of carbon chain length of C12-13, C12-15, C13/15, and C14-15 AEO with a range of 3, 4.5/6, 5, 6.5, and 7 mol of EO would all be classified as GHS Acute aquatic category 1, with LC50 values ≤ 1 mg L^{-1} for the most sensitive species. In contrast, however, the branched AEO of similar carbon chain length and level of EO (C12br-7EO, C13br-3EO, C13br-5EO, C13br-7EO) are less toxic to aquatic organisms (with LC50 values between 1 and 10 mg L^{-1}) and would be classified as GHS Acute 2. Similarly, C12br-12EO and C13br-12EO data support classification as GHS Acute 3 (LC50: 10–100 mg L^{-1}) *versus* a semi-linear hydrophobe-based C12-15-12EO, which would be classified more stringently as Acute 2. At a lower alcohol chain length, the C10br-9EO meets GHS Acute 3 criteria.

Conclusions

A wide range of AEO surfactants derived from branched C8-rich, C9-rich, C10-rich, C11-rich, and C13-rich oxo-alcohols with 1–20 mol of ethoxylate were tested and found to meet the OECD readily biodegradable criteria and thus are expected to undergo rapid degradation in the environment. In addition, preliminary results indicate that, similar to their parent compounds, AEO derived from branched oxo-alcohols exhibit a low potential to bioaccumulate in aquatic organisms.

Aquatic toxicity of branched AEO has been overestimated when read-across was applied from similar chain

length linear or semilinear AEO. In agreement with previously published reports, this study observed lower inherent toxicity for highly branched AEO relative to semilinear analogs, which may result in a lower GHS classification and labelling requirement than previously predicted from read-across from linear AEO.

The TLM has been demonstrated to predict the acute toxicity of branched and linear AEO mixtures and individual homologues, with accuracies comparable to those observed for other neutral organic narcotic chemicals. TU addition of individual constituent AEO was shown to improve the performance of the TLM for acute toxicity, as well as chronic toxicity predictions for AEO mixtures. Furthermore, the TLM-derived HC5 threshold levels have been shown to be sufficiently protective when compared to available chronic toxicity data for AEO. This supports the use of the TLM model in risk assessment for novel AEO and mixtures.

While the current study and TLM analysis has focused on aquatic risk assessment, the TLM framework provides the platform to extend predictive effects thresholds to sediment and soil organisms using the principles of equilibrium partitioning theory and building on previous work for petroleum hydrocarbons (Redman et al., 2014b) and for single AEO homologues (Droge et al., 2008), accounting for sorption properties of AEO to organic carbon and clay minerals in sediments (Droge et al., 2009). Validation and application of the TLM framework in this capacity could allow for a more robust assessment of ecological hazard and risk, particularly where experimental data are limited or where a large number of candidate substances must be assessed.

Conflict of Interest The authors declare that they have no conflict of interest.

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