RESPONSES OF PERIPHONY AND INVERTEBRATES TO A TETRADECYL-PENTADECYL SULFATE MIXTURE IN STREAM MESOCOSMS

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Abstract—Alkyl sulfates (AS) are anionic surfactants widely used in household and personal cleansing applications. Aquatic toxicity of AS under laboratory conditions indicated effects at relatively low concentrations (50–230 µg/L) for some sensitive species. A comprehensive stream mesocosm study of an AS mixture composed of tetra- (C₁₄) and pentadecyl (C₁₅) chain lengths was conducted to better understand effects on microbial and macroinvertebrate populations and communities. A 56-d exposure of AS was performed at concentrations ranging from 57 to 419 µg/L (analytically confirmed exposures) and was accompanied by detailed investigations of periphyton community function (autotrophy, heterotrophy, and metabolism of test chemical), periphyton structure (algal population and community dynamics based on taxonomic identity), and invertebrate structure (benthic abundance, drift, and insect emergence patterns based on taxonomic identity). A no-observed-effect-concentration (NOEC) of 222 µg/L was concluded for several individual algal and invertebrate species based on univariate statistical analyses. An apparent energetic subsidy from C₁₄₋₁₅AS at the highest concentrations of 222 to 419 µg/L was observed and tied to changes in microbial community processing of AS when added at these high concentrations. A multivariate analysis based on principal response curves (PRC) indicated that communities in streams exposed to 222 to 419 µg/L were significantly different from the controls leading to an overall (multivariate and univariate) conclusion that 106 µg/L was the ecosystem NOEC. Exposure to AS in the environment has been demonstrated to be in the range of 5 to 21 µg/L in 100% wastewater treatment plant effluent. Potential environmental effects are at least 5 to 20 times above worst-case environmental exposures; therefore, C₁₄₋₁₅AS does not pose a risk to the aquatic environment due to normal use patterns.

Keywords—Anionic surfactant   Alkyl sulfate   Principal response curve   Experimental stream   Toxicity

INTRODUCTION

Alkyl or alcohol sulfates (AS) are anionic surfactants commonly used in numerous consumer products including laundry products, shampoo, and toothpastes. The typical structure of AS consists of monosulfated alkyl chain lengths ranging from C₁₂ to C₁₈. Both C₁₄ (tetradecyl) and C₁₅ (pentadecyl) chain lengths commonly are used in laundry applications. Alcohol sulfates are relatively difficult surfactants to test due to exceptionally rapid biodegradation through desulfation and oxidation of the alkyl chain [1] and, in some instances, low aqueous solubility of chain lengths above 14 carbons [2]. Little chronic toxicity or other higher tier data are available for this class of compounds. Removal in typical municipal wastewater treatment plants exceeds 99.5% [3,4]. As with other anionic surfactants, alkyl sulfate sorption is dominated by a hydrophobic mechanism of association and thus is chain length-dependent. Sorption coefficients to river water solids (Kₒ) range from 5 to 14,000 L/kg for C₁₄AS and 20 to 60,000 L/kg for C₁₅AS (McAvoy and Kerr, Procter & Gamble, unpublished data using methods in Belanger et al. [5]).

Dyer et al. [2] developed a chronic toxicity structure-activity relationship for C₁₂ to C₁₅ AS using Ceriodaphnia dubia. Toxicity of alkyl sulfates increased with increasing chain length. Due to low solubility for chain lengths above C₁₄, toxicity was reduced resulting in a parabolic structure-activity relationship. The trough of the structure-activity relationship where toxicity was greatest was at C₁₄. A common commercial mixture of alkyl sulfate is a near 50:50 mixture of C₁₄ and C₁₅ chain lengths. (Such a mixture would be designated by an average chain length designation as C₁₄₋₁₅AS by conventional terminology.) Chronic aquatic toxicity no-observed-effect-concentrations (NOECs) for C₁₅AS generally are <100 µg/L for invertebrates and fish based on reproduction, growth, and survival ([1,2] Procter & Gamble, unpublished data). For example, the NOECs for C₁₄₋₁₅AS were 81 µg/L for Ceriodaphnia dubia and 110 µg/L for fathead minnow (Pimephales promelas). Higher tier microcosm and mesocosm studies have been performed on C₁₅AS [6–9]. The NOEC for C₁₅AS preserving aquatic ecosystem structure and function was 224 µg/L. Stream mesocosms exposed to AS adapted quickly to biodegrade the surfactant at concentrations as low as 20 µg/L and invertebrate communities were subsidized energetically at concentrations exceeding 224 µg/L.

C₁₄₋₁₅AS is a high-volume, anionic surfactant with measurably greater toxicity under laboratory conditions and possesses a smaller chronic toxicity database than C₁₅AS. Therefore, a long-term stream mesocosm study of a mixture of tetradecyl and pentadecyl sulfate (C₁₄₋₁₅AS) was proposed and executed at the Procter & Gamble Experimental Stream Facility ([ESF], OH, USA) to gain additional understanding of environmental responses to this chemical mixture and to re-assure the environmental safety of this high production volume surfactant. A holistic assessment of microbial and macroinvertebrate population and community structure over the course of a 56-d exposure was performed with the goal of determining the concentration of C₁₄₋₁₅AS that would not adversely impact biota.
by electrospay ionization/mass spectrometry; therefore, this specific mixture hereafter will be referred to as C_{14,4} AS.

Stock solutions of C_{14,4} AS were prepared every 4 d by diluting an initially prepared stock solution. This was done to achieve a stable dispersion of C_{14,4} AS. C_{14,4} AS neat chemical was heated to 49 ± 2°C in a constant-temperature room. An appropriate aliquot (4.4 kg) of heated chemical was diluted immediately in high-quality water (17 megaohm) at 66 ± 2°C. The solution was mixed for 5 min and brought to a volume of 6.0 L in a large glass beaker on a stir plate held at 82 ± 2°C. The super stock solution was diluted in 45.8 L of high-quality water at an equivalent temperature and mixed for an additional 2 h. The mixture was then cooled for 24 h and appropriate amounts were transferred to individual ESF test chemical feed tanks (described in [8,11]). Final stock solution concentrations in the 62.5-, 125-, 250-, and 500-μg/L stream channels were 0.17, 0.35, 0.69, and 1.39 g/L, respectively. Two 456-L stainless steel tanks were dedicated for delivery, and confirmation of water column exposures (Table 1).

Determination of C_{14,4} AS exposure concentrations were made on samples preserved using 3% formalin (v/v) and prepared with solid phase extraction for analysis by high-performance liquid chromatography/mass spectrometry. A deuterated d25 dodecyl sulfate internal standard was spiked into the preserved sample prior to extraction. The C_{14,4} AS was isolated by electrospay ionization/mass spectrometry; therefore, this specific mixture hereafter will be referred to as C_{14,4} AS.

Table 1. Summary of measured stream exposure data for the C_{14,4} AS study

<table>
<thead>
<tr>
<th>Stream</th>
<th>Nominal concentration (μg/L)</th>
<th>Headbox</th>
<th></th>
<th>Tailpool</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control</td>
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<td>0.6</td>
<td>1.9</td>
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<td>8</td>
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<td>13</td>
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<td>250</td>
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<td>25</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>14</td>
<td>419</td>
<td>24</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

ESF description

The ESF houses 8 12-m-long stream channels that draw unfiltered river water from the Lower East Fork of the Little Miami River (LEFR) in southwest Ohio, USA. The LEFR is rated as an exceptional warm water fishery by the Ohio Environmental Protection Agency and contains a diverse invertebrate fauna [10]. Each stream channel receives 167 L/min and has an average velocity of 50 cm/sec consistent with invertebrate and periphyton requirements for a riffle community dominated by diatoms, mayflies, stoneflies, and caddisflies. Belanger et al. [11,12] described the physical structure of these streams in detail. Stream channels contain five distinct sampling regions: A 126-L upstream headbox, an upstream region 4.3 m long and 0.3 m wide (the periphyton reach), a tapered gravel-f®lled trays (2 cm average diameter cobble, tray of 0.0043 m³). Stream channels received illumination from 30 daylight-equivalent 1,000 W halide arc lamps. Photoperiod is automatically adjusted daily using a Suntracker® (Paragon Electric, Two Rivers, WI, USA) programmed to match the natural photoperiod of the site (39°7’7”N, 85°15’13”W) over the period of the study. Light intensity at the water surface was measured at 95 μM/s/m². Dawn and dusk were simulated over a 2.1-h interval.

Experimental design

Streams were colonized for 55 d from May 27 to July 21, 1997. Streams were dosed continuously with C_{14,4} AS beginning on July 21 and extending to September 15 or a period of 56 additional days. Nominal in-stream concentrations were control (0), 62.5, 125, 250, and 500 μg/L. Water quality, specific analytical determinations, and periphyton and invertebrate communities were evaluated during the course of the study to develop spatial and temporal patterns of ecological responses.

Streams were dosed with a mixture of sodium tetradecyl (C_{14}) and pentadecyl (C_{15}) sulfate (28.0% active as determined by CatSO4 titration [13]). The neat material contained 0.35% unreacted 45 Neodol. Neodols are a family of commercially available (Shell Chemical, Houston, TX, USA) linear alcohols used to make various alcohol-based surfactants. The C_{14,15} AS (CAS 68081-98-1) mixture was produced in the Procter & Gamble Ivorydale Technical Center Falling Film Reactor (Cincinnati, OH, USA) for the study. The relative C_{14}C_{15} ratio was 56:44 for an average of 14.4 carbons per chain as determined by electrospray ionization/mass spectrometry; therefore, this specific mixture hereafter will be referred to as C_{14,4} AS.

Stock solutions of C_{14,4} AS were prepared every 4 d by diluting an initially prepared super stock solution. This was done to achieve a stable dispersion of C_{14,4} AS. C_{14,4} AS neat chemical was heated to 49 ± 2°C in a constant-temperature room. An appropriate aliquot (~4.4 kg) of heated chemical was diluted immediately in high-quality water (17 megaohm) at 66 ± 2°C. The solution was mixed for 5 min and brought to a volume of 6.0 L in a large glass beaker on a stir plate held at 82 ± 2°C. The super stock solution was diluted in 45.8 L of high-quality water at an equivalent temperature and mixed for an additional 2 h. The mixture was then cooled for 24 h and appropriate amounts were transferred to individual ESF test chemical feed tanks (described in [8,11]). Final stock solution concentrations in the 62.5-, 125-, 250-, and 500-μg/L stream channels were 0.17, 0.35, 0.69, and 1.39 g/L, respectively. Two 456-L stainless steel tanks were dedicated for delivery, and confirmation of water column exposures (Table 1).

Determination of C_{14,4} AS exposure concentrations were made on samples preserved using 3% formalin (v/v) and prepared with solid phase extraction for analysis by high-performance liquid chromatography/mass spectrometry. A deuterated d25 dodecyl sulfate internal standard was spiked into the preserved sample prior to extraction. The C_{14,4} AS was isolated from water using a series of two C2-bonded phase cartridges, then eluted with 15 ml methanol/ethyl acetate (50/50, v/v) followed by 5 ml of ethanol. Extracts were combined, taken to dryness, and reconstituted in 5 ml methanol. All work was performed on a Micromass Quattro II triple quadruple tandem mass spectrometer (Micromass, Manchester, UK) utilizing negative ion electrospray ionization. The HPLC systems used were an HP 1090 (Hewlett-Packard, Avondale, PA, USA) and a Waters Alliance 2690 (Waters, Milford, MA, USA), both equipped with variable volume autosamplers. Exposures were confirmed from samples in the headbox and tailpool at a minimum of once per week, but most often twice.

Periphyton studies

Periphyton is a complex assemblage of bacteria, algae, fungi, and protozoa. Methods for assessments of periphyton in the ESF have been published extensively and re-applied [9,11,14]. Periphytic community structure and function were assessed using intact communities on terra cotta clay tile substrates. Tiles (five per stream on each sample day) were harvested on days 0, 14, 28, 42, and 56. Randomizations of tiles...
to be sampled were conducted a priori using a customized routine written in SAS® [15]. Radiolabel studies of C_{14}AS (tetradeyl only) mineralization by microbial heterotrophs were performed based on methods developed initially by Lee et al. [9] and Belanger et al. [5]. The $^{14}$C-C_{14}AS (specific activity of 25 mCi/mmol) was labeled uniformly on the alkyl chain and was 99% pure (determined via HPLC reverse separation). Tiles with intact periphyton communities were sealed in foil-lined, heat-sealable bags (Kapak Products, Minneapolis, MN, USA) with approximately 400 ml of water from the appropriate stream. Foil-lined bags were used because they exclude light (preventing algal $^{14}$CO$_2$ incorporation) and prevent gas exchange. Bags were injected with a tracer level of 1 nCi/ml of $^{14}$C-C$_{14}$AS. Incubations were carried out at ambient river temperatures in water tables receiving source water identical to that sent to the streams. At the end of 4 and 24 h of incubation, aliquots of water were removed from the bags to vials containing 25% H$_2$SO$_4$ or 0.5 M NaOH. Mineralization of the radiolabeled C$_{14}$AS was determined by difference of the radioactivity remaining in the base and acid vials. The difference in radioactivity was equivalent to the amount of $^{14}$C-C$_{14}$AS mineralized by the periphyton. The $^{12}$CO$_2$ evolution was converted to $\mu$g C$_{14}$AS mineralized/m$^2$/h based on stream exposure concentrations and reported as percent mineralized to CO$_2$. Incorporation of label into biomass or remaining as degradation intermediates was not included in this study, so estimates are based on parent mineralization only.

Ecotoxicological responses of periphyton were assessed using tiles from the periphyton reach as described above. Determination of responses included community function as well as structure. As with mineralization studies, five tiles per stream on each sample day (days 0, 14, 28, 42, and 56) were collected for evaluation. For function, a dual incubation of tritiated ($^3$H) amino acids for assessment of heterotrophic activity and $^{14}$C-labeled bicarbonate to assess autotrophic activity was performed. Tiles were removed from each stream and placed in a pre-assigned location within a circular poly-carbonate chamber (37 cm i.d., 5 cm deep) containing 1.5 L of stream water (from the stream sampled). The chambers were placed into a flowing water table to maintain chambers within 1 to 2°C of stream water temperatures. During this time, Masterflex pumps were used to recirculate the water within each chamber at a rate of 1,000 ± 100 ml/min.

Radiolabel incubation for heterotrophic activity analysis used an amino acid mixture of 19 different $^3$H amino acids (Amersham mixture TRK.440; Piscataway, NJ, USA). The $^3$H amino acids were added to the incubation chambers for a radioactive concentration of 31 $\mu$Ci/L (average specific activity of 40 mCi/mmol). In addition to the $^3$H amino acid mixture, 31 $\mu$Ci/L $^{14}$C–sodium bicarbonate (ICN, Irvine, CA USA, specific activity = 56 mCi/mmol) was added to provide a dual-label incubation for simultaneous assessment of both the heterotrophs (bacteria) and photoautotrophs (algae). Radiolabel incubation was stopped after 80 to 90 min when tiles were removed to a bath of 3% glutaraldehyde. Periphyton cell suspensions were prepared from each tile by removing periphyton using a razor blade. Heterotrophic and autotrophic periphyton effects were assessed using total radioactivity uptake on an aerial and biomass-normalized basis [9]. In addition to functional measurements described above, algal periphyton were evaluated for population and community structure using a subsample of the cell suspensions prepared from the aforementioned radiolabel incubations [11]. Taxonomy analyses were performed at the species level for diatoms and, where possible, for soft algae (greens, cyanophytes, browns, and reds) using well-established methods [5,11,14]. Population abundances were expressed as cell or biovolume density where biovolume density is the cell density multiplied by the average volumetric size of the taxon. Volumes were determined during taxonomic identifications. Total abundance (cell density and biovolume) for the community, taxa richness, and species diversity were calculated based on population level data. Algal species were designated as dominant populations if their proportional contribution to the community based on cell density or biovolume density exceeded 5% of the community total.

**Invertebrate studies**

Invertebrate responses to C$_{14}$AS exposure were evaluated in a comprehensive fashion employing the methods published previously [6,12]. Five replicate cobble-filled trays were selected randomly from each stream channel on a given sampling day. Randomizations were conducted a priori using a customized SAS routine [15]. Trays were removed 3 d prior to dosing (herein referred to as day 0 for convenience) and after 14, 28, and 56 d of exposure. Contents of each tray were sieved through a series of graded mesh sieves terminating at 250 $\mu$m. Organisms removed from sieves were preserved in 10% buffered formalin and then transferred to 70% ethanol for sample processing. At least 250 organisms from each tray were identified to the lowest practical taxon (usually genus for immature insects and varied for other phyla and orders). Population level abundance and community level indices (richness, diversity) were calculated [6].

Drift of invertebrates was assessed throughout both the colonization and exposure periods of the investigation because previous studies in the facility indicated this was sometimes an important phenomenon structuring benthic communities early in the dosing of the streams [6,12,16]. Nets were placed into the stream channel at the transition zone between the periphyton tiles and invertebrate trays and at the end of the invertebrate reach. This effectively isolated the invertebrate-dominated stream reach. The upstream net collected invertebrates caught in drift from the source river, which were being transported through the system, and the downstream net collected only those organisms drifting from the cobble-filled trays. Drift nets were made of 243-$\mu$m nylon netting terminating in a detachable cod end. Baseline drift into the ESF stream channels was determined at the upstream location in two streams during the colonization period on days –55, –41, –27, and –13 prior to dosing. On day 0 of dosing, drift was assessed at the upstream and downstream location of all streams 0.5 h prior to initiation of exposure and 0.5 and 2 h after the onset of C$_{14}$AS exposure. Drift in all streams also was assessed on days 15, 36, 43, 55, and 56 of exposure. Taxonomic identification of drift was performed identically to that used for benthic trays. Drift was expressed as organisms/L based on population level identifications [16].

Emergence sampling of adult insects was conducted to gain insight into additional potentially relevant mechanisms for invertebrate loss other than those reflected in benthic abundance and drift [5]. Baseline emergence in the ESF was evaluated on day –55, –41, –34, –26, –19, –10, –5, 5, 10, 17, 26, 33, 34, 39, 44, and 54 of exposure using black lights set overnight above pans containing alcohol at the transition section of stream channel 4 in the middle of the building. These samples should be viewed as an integrated general assessment of
the emergence phenomena for all streams during the study. Second, specific sampling of the control and each dosed stream was performed on days -40, -24, -11, 6, 18, 34, 46, and 55 of the study. Stream channels were isolated by covering each stream with black-out cloths suspended on cables as a support. Black lights and alcohol pans were placed under the netting overnight. Sampling began after dusk and collections were made before dawn. Samples were identified to species based on published and unpublished taxonomic keys by experts specializing in the various taxonomic orders (e.g., Ephemeroptera, Coleoptera, Diptera, etc.). Community abundance, taxa richness, and population abundances were summarized for each sample.

**Water quality**

Information on water quality was gathered using a variety of techniques. The ESF computerized data acquisition system was used to collect and analyze dissolved oxygen, pH, specific conductance, and temperature information on a 5-min basis that provides a detailed view of prevailing conditions in the ESF and LEFR [11]. Figure 1 provides temperature, conductivity, pH, and dissolved oxygen profiles of the ESF stream water based on daily averages (288 data points/day) during the 56 d of colonization and subsequent 56 d of exposure. Additional ESF building and water quality conditions are monitored, including stream flows, turbidity, and light intensity. Comprehensive water quality assessments were performed based on grab samples collected from the tailpool of the control stream on a weekly to biweekly basis over the 112-d period. Total suspended solids were monitored throughout the study because this is an important variable affecting bioavailability, water quality, and habitat development (Fig. 2). The LEFR discharge data was downloaded from the U.S. Geological Survey [17] (http://water.usgs.gov/oh/nwis/current/?type=flow). Table 2 provides means and averages for monitored parameters including major cations, anions, nutrients, and organic carbon.

Confirmation of low or no levels of pollutants in ESF source water and sediment was accomplished by a scan for the 137 priority and conventional pollutants listed by the U.S. Environmental Protection Agency [18] prior to colonization. Grab water and sediment samples were collected at the ESF Intake on the LEFR above the adjacent wastewater facility operated by Clermont County (OH, USA). Priority pollutant scans consist of general water quality parameters, volatile compounds, pesticides, polychlorinated biphenyls and other semivolatile compounds, and metals/metalloids.

### Data analysis and summarization

Endpoints were analyzed statistically using parametric analysis of variance (ANOVA) or nonparametric one-way layout Kruskal-Wallis as appropriate where C$_{14}$AS was considered the main effect. Appropriate descriptive univariate statistics (e.g., arithmetic mean, geometric mean, mode, standard deviation, standard error, coefficient of variation, confidence interval estimation, rank order, correlation) were calculated for various analytical measurements or biological response endpoints. Principal response curve (PRC) analysis [19] was employed for taxonomic data sets. The PRC also is known generically as redundancy analysis, which is a constrained form of principal component analysis. It has the advantage of allowing the effects of explanatory variables (in this case all observed species abundances) to be expressed and were combined with a Monte Carlo permutation test for statistical anal-

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**Fig. 1.** Water quality in Procter & Gamble Experimental Stream Facility (Clermont County, OH, USA) streams during the C$_{14}$AS study measured by automated sensors in the control stream tailpool at 5-min intervals. Data are compiled as a 24-h average for each parameter in this figure.

**Fig. 2.** Relationship of the Lower East Fork of the Little Miami River (LEFR) discharge (in cubic feet per second [CFS] or ft$^3$/s; U.S. Geological Survey daily flow data, Perinton, OH) and total suspended solids (TSS) in Procter & Gamble Experimental Stream Facility streams (OH, USA).
ysis. The permutation test concludes with a one-way ANOVA of canonical coefficients derived from the integrated population abundance data (all species) with C_{14}AAS as the main effect on each sampling day. Van den Brink and ter Braak [19] termed the inferred no-observed-effect concentration as NOE-C_{ecosystem} (ecosystem NOEC) that is used in this paper as well. Inference of statistical significance was made at = 0.05 for univariate and multivariate analyses. Univariate statistical analyses were conducted within a specialized application developed using SAS [15], known as a Data Warehouse [20], that facilitates all portions of data analysis, association, and numerical manipulations. Canonical Community Ordination (Ver 4.0) was used to perform the PRC [21].

Polynomial least squares regression analysis was used to detect significant trends for all analyzed endpoints (structure and function) as a function of C_{14}AAS concentration [22]. This method allowed for consideration of a variety of responses that might be ecologically relevant, including curvilinear (e.g., quadratic) as well as linear response models. Regression analysis on each parameter was performed separately for a linear, quadratic, and combined (linear and quadratic terms combined) model. The model that best described the response data (highest statistical significance and highest r-square) was then used to predict effect concentrations that altered the response parameter by 20% (EC20). Point estimates and 95% confidence intervals were generated using SAS (Ver 8.2, Cary, NC, USA). Significance was inferred at α = 0.05. These analyses were used to support interpretations from ANOVA and NOEC determinations described above given the pseudo-replicated experimental design (the exception to this being day 0 of dosing analyses prior to C_{14}AAS treatment).

Guckert [23] provides a detailed description and discussion of the compromises in experimental design between pseudo-replicated tests (several streams, one concentration each) versus replication with fewer treatments. Although the statistical arguments presented by Hurlbert [24] and Kosinski [25] fundamentally are correct, there are benefits to increasing exposure concentrations at the expense of replication if certain principles are followed. A primary concern of pseudo-replicated studies is the identification of a response that is not associated with the treatment resulting in a conclusion of an effect where there is none. In the studies at this facility, great care has been taken to develop a water distribution system that reduces influential stochastic phenomena [11]. Research was conducted to determine the long-term interstream variability and replicability [26], Belanger et al., unpublished data). Lasty, day 0 of dosing analyses (or after 56 d of colonization) prior to treatment provides a true indication of interstream replication. Similar analysis of experimental stream data for a like-constructed system was given by Wong et al. [27], which demonstrated that between-stream variation is minimal and community similarity remains high through time. Eleven similar studies in the ESF, encompassing eight different surfactants and effluents, have been analyzed in a similar manner with a significant effect indicated in only 0.2% of analyzed endpoints on day 0. To increase the rigor of using ANOVA on these pseudo-replicated stream studies, the following decision framework was developed to identify the NOEC for ecosystem level responses [12,16,23]: Determine if a change in an endpoint is statistically significant; determine if an effect, regardless of concentration, is ecologically consistent with other related endpoints within each stream; determine if the effect displays an exposure-response behavior; determine if the effect is consistent, transient, or progressive over the exposure period; conclude ecologically relevant effects for endpoints that are statistically significant, ecologically consistent, display exposure-response, and consistent or progressive; and, using best professional judgment, conclude the concentration at which no-adverse-effects of exposure are expected. This step concludes a relevant predicted no-effect-concentration for the model ecosystem. The approach was applied to all the statistical tools, including univariate ANOVA, regression analyses, and the PRC analysis.

<table>
<thead>
<tr>
<th>Parameter (mg/L)</th>
<th>Mean</th>
<th>SD</th>
<th>Maximum</th>
<th>Minimum</th>
<th>n</th>
<th>Values above MDL</th>
<th>Values below MDL</th>
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<td>10.0</td>
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<tr>
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<td>6.1</td>
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<td>5.0</td>
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<td>0</td>
</tr>
<tr>
<td>Total residual chlorine</td>
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<td>0</td>
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<td>Carbonaceous biological</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen demand</td>
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<td>1.6</td>
<td>6.4</td>
<td>2.0</td>
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<td>12</td>
</tr>
<tr>
<td>Methylene blue active</td>
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<td>0</td>
<td>0.1</td>
<td>0.1</td>
<td>17</td>
<td>1</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 2. Water quality measurements from grab water samples taken from the tailpool of the control stream channel. Mean and standard deviations (SD) were computed on values above the detection limit. MDL = method detection limit.
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Fig. 3. Mineralization of tetradecyl alkyl sulfate over a 4-h incubation period by microbial communities exposed to C_{14.4}AS in situ. Treatments that are significantly different from the control are indicated by an asterisk (*, $\alpha = 0.05$). TSS indicates total suspended solids in mg/L.

RESULTS

C_{14.4}AS analytical

Exposure of streams to C_{14.4}AS was highly consistent and showed low levels of temporal variability (Table 1). The travel time through the stream channel itself is approximately 20 s during which relatively little loss was expected to occur. Tailpool samples were taken at the outlet from the tailpool and include an additional 2.5-min residence in this location. Upstream–downstream differences existed for all exposure concentrations indicating the potential for loss via biodegradation and sorption-settling. In any case, samples taken at the head of the stream were 84 to 91% of the expected nominal concentration consistent with historic analytical confirmation of exposure at the ESF [6,11,12]. Therefore, for the purposes of expressing C_{14.4}AS exposure to stream communities, upstream (measured headbox) exposures will be used.

Response of periphyton to C_{14.4}AS

The C_{14.4}AS was mineralized rapidly at all exposure concentrations; however, data from only the initial 4-h incubation are given because trends remained the same after 24 h (Fig. 3). Fourteen days of C_{14.4}AS exposure resulted in near-equal levels of mineralization during the 4-h incubation regardless of exposure concentration. Control mineralization activity indicated a baseline level of about 20% of $^{14}$CO$_2$ recovered. With the exception of day 28, levels of mineralization in all dosed streams were statistically higher than those observed in the control stream throughout the study. Mineralization in the dosed streams approximated 50% of the $^{14}$CO$_2$ recovered during the dosing period. A midstudy storm influenced the process, as indicated by a reduction in the level of mineralization, and stream total suspended solids levels were high (180 mg/L). Figure 2 shows the increase in LEFR discharge at this time from approximately 40 cubic feet per s ([CFS] ft$^3$/s) to 300 CFS. Total suspended solids declined to baseline levels following the storm and C_{14.4}AS mineralization returned to the level observed on day 14 early in the study.

Periphytic community function was evaluated via incorporation of radiolabeled bicarbonate (for autotrophy) and triitated mixed amino acids (for heterotrophy). Bicarbonate incorporation was fairly consistent throughout the study and showed a lack of statistically significant temporal and dose-response trends (Fig. 4A). However, C_{14.4}AS-exposed streams demonstrated consistently higher levels of bicarbonate incorporation than the control from day 14 onward. On day 28 the highest exposure of 419 mg/L was significantly greater than the control. By day 56, the three highest treatments were elevated above the two lowest treatments and control, but not significantly. Incorporation of mixed amino acids was more variable than for bicarbonate incorporation (Fig. 4B). As with bicarbonate, the control and low-exposure stream (57 µg/L)
usually had the lowest rate of incorporation but on only one occasion was this significant (day 28).

Algal abundance was evaluated as algal biovolume density (μm³/mm²). By day 42, total algal biovolume density was reduced in an apparent exposure-dependent manner and by day 56, the 419-μg/L exposed stream was significantly less than the control (Fig. 5A). Regression analysis indicated the concentration-response was not significant, and, therefore, the ANOVA results can be viewed cautiously as conservative. Biovolume was relatively variable due to the community composition. The very large centric diatoms, *Pleurosira laevis* (10,600 μm³/cell) and *Melosira varians* (900 μm³/cell), were among the most abundant taxa at 42 and 6% of total community biovolumes during the study, respectively. Small shifts in cell numbers/mm² of these species result in large overall variations or changes in the community. By day 56, *Pleurosira* and *Melosira* were absent from the 419-μg/L treatment and the third most-dominant taxon, *Navicula symmetrica*, was unaffected based on one-way ANOVA (Fig. 5B). Regressions of biovolume densities on day 56 for *P. laevis*, *M. varians*, and *N. symmetrica* were not significant despite the former two taxa not being observed at the highest concentration (Table 3). In part, this is attributable to the relatively low abundance (and therefore variable) of algae at this point in the study. *Navicula cryptcephala var. veneta* also was affected in a similar manner to *Pleurosira* and *Melosira*, and all three had NOECs of 222 μg/L. Of the 131 algal taxa observed in the study during the four sampling days from day 14 to 56, only 2% of the time did species have NOECs less than the highest concentration of 419 μg/L (Fig. 6).

A PRC analysis of algal population biovolumes observed over the course of the 56-d study was conducted and indicated a strong influence of *Pleurosira* and *Melosira* (Fig. 7). The stream exposed to 419 μg/L had significantly lower canonical coefficients compared to the control on day 56. Using the definition of Van den Brink and ter Braak [19], the NOEC_eco (ecosystem NOEC) based on this analysis would be 419 μg/L. *Pleurosira* was clearly the most influential taxon with the largest species score in terms of absolute value. Very few taxa had even marginally positive species scores that would indicate a pattern of increasing abundance with increasing C14.4 AS concentration. *Cocconeis placenta*, an early invading taxon that dominated algal identifications early in the study, had the highest species score of the algae observed. *Navicula saintecrusis* and *N. symmetrica* (Fig. 5B) possessed the third and fourth most negative scores, respectively, although neither was affected significantly based on population level ANOVAs.

**Response of invertebrates to C14.4 AS**

Emergence of aquatic insects was evaluated in the ESF building as a whole and from individually covered stream channels approximately every other week. A total of 142 insect species were collected in these sampling events during the colonization and dosing period of the study. In general, whole building samples were richer and collections consisted of greater numbers of individuals than individual stream samples (Fig. 8A). This likely is due to the nature of the collections where whole building samples integrate emergence from all streams in the ESF (8 channels total) in one collection. Richness peaked early in the study (July 30, day 12 of exposure; Fig. 8A). Numerical abundance was more variable with three distinct peaks for whole building collections (Fig. 8B) with peak abundance occurring 11 d before dosing began (July 10). Species richness and total numbers of emergent insects displayed no apparent and sustainable exposure-response pattern (Fig. 8A and B; Table 3). Emergence samples are unreplicated and one-variable linear regression was used to confirm the presence or absence of exposure-response patterns. No consistent relationships were found between species richness and C14.4 AS exposure in emergence samples. Only day 34 emergence displayed such a relationship and the EC20 exceeded the highest exposure concentration. Similarly, no significant relationships existed between abundance of adults in emergence samples and C14.4 AS exposure concentration.

Insect drift was assessed at several time points during the C14.4 AS exposure. A total of 111 different taxa were collected. An initial drift response (increased drift density measured as total numbers of organisms/L) occasionally has been observed.
Table 3. Linear regression model results for significant linear (L), quadratic (Q), and mixed linear and quadratic (M) models. Models that indicated increases in the parameter are indicated with a plus sign (+). EC20 = 20% effective concentration

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Day</th>
<th>F-statistic</th>
<th>p-Value</th>
<th>r-Square</th>
<th>Model</th>
<th>EC20 (µg/L)</th>
<th>95% Lower and upper CI (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physella density</td>
<td>14</td>
<td>13,771</td>
<td>&lt;0.001</td>
<td>0.505</td>
<td>Q</td>
<td>105 (+)</td>
<td>28–180</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>41,239</td>
<td>&lt;0.001</td>
<td>0.596</td>
<td>Q</td>
<td>94 (+)</td>
<td>15–130</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>17,964</td>
<td>&lt;0.001</td>
<td>0.571</td>
<td>Q</td>
<td>201 (+)</td>
<td>–8–655</td>
</tr>
<tr>
<td>Ferrissea density</td>
<td>14</td>
<td>13,771</td>
<td>&lt;0.001</td>
<td>0.505</td>
<td>Q</td>
<td>105 (+)</td>
<td>28–180</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>41,239</td>
<td>&lt;0.001</td>
<td>0.596</td>
<td>Q</td>
<td>94 (+)</td>
<td>15–130</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>17,964</td>
<td>&lt;0.001</td>
<td>0.571</td>
<td>Q</td>
<td>201 (+)</td>
<td>–8–655</td>
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<tr>
<td>Total invertebrate density</td>
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<td>18,030</td>
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<td>0.392</td>
<td>L</td>
<td>79 (+)</td>
<td>63–95</td>
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<td>Invertebrate richness</td>
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<td>0.244</td>
<td>Q</td>
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<td>Invertebrate diversity</td>
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<td>29,647</td>
<td>&lt;0.001</td>
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<td>L</td>
<td>&gt;419</td>
<td></td>
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<tr>
<td>EPT abundance</td>
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<td>0.260</td>
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<td>Emergence richness</td>
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<td>&gt;419</td>
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<tr>
<td>Emergence abundance</td>
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<td>L</td>
<td>216</td>
<td>188–247</td>
</tr>
</tbody>
</table>

*EPT = Ephemeroptera, Plecoptera, and Trichoptera.

during surfactant exposure [12,16], therefore, drift sampling immediately following the initiation of dosing was conducted at 1 and 2 h (Fig. 9A). Nearly the entire drift was composed of insects at these times (>99% of organisms) and ranged from approximately 0.008 to 0.016 organisms/L with no exposure-dependent increase or decrease. Nocturnal drift also was evaluated on days 15, 36, 43, and 55 proximate by 1 or 2 d to emergence sampling. Nocturnal drift density generally was higher than that during the daytime during the initial stages of dosing (Fig. 9B). Drift density was lowest in the 419 mg/L concentration on drift abundance (0.16, 0.77). Com-

Fig. 6. Distribution of algal and invertebrate population no-observed-effect concentrations (NOECs) on day 56 only of the C14,4 AS mesocosm study.

![Figure 6](image)

Fig. 7. Principal response curve analysis of algal communities during the C14,4 AS exposure. Treatments that are significantly different from the control are indicated by an asterisk (*, α = 0.05). Taxa most highly associated with either high positive or high negative canonical coefficients are indicated in the species score plot.
and 11A). Samples on day 14 and 28 indicated NOECs less than 57 µg/L and 106 µg/L, but by day 56 the NOEC had risen to 222 µg/L. Based on the decision framework presented earlier, the NOEC of 222 µg/L at 56 d is concluded for the population in the study. Turbellaria comprised 2% of all observed invertebrates in the trays. Dominant taxa included the gastropod mollusks Physella and Ferrissee, which increased in abundance with increasing C₁₄₄₄ AS-exposure concentration (Fig. 11). Corbicula, a pelecypod mollusk historically sensitive to surfactant exposure, also increased in abundance although it was not a dominant taxon. Significant increases above the control abundance for these taxa occurred at 419 µg/L (based on ANOVA) for Corbicula and Physella and at 222 to 419 µg/L for Ferrissee. Regression analysis suggested an EC20 for Turbellaria density reduction was 78 µg/L. Physella and Ferrissee EC20s for increased densities were 201 and 12 µg/L, respectively (Table 3). These broad patterns of selected organisms increasing or decreasing also were reflected in a PRC analysis of invertebrate abundance data from cobble-filled trays (Fig. 12). The predominant response was of increased abundance with the 222- to 419-µg/L exposed streams being consistently greater than the control. Because the multivariate PRC represents an integration of responses for all taxa, this would indicate a NOECₑₒ of 106 µg/L using the definition of Van den Brink et al. [19]. Physella and Ferrissee possessed the highest positive species scores and Turbellaria the largest negative species score. These statistical observations from the PRC are consistent with the population-level ANOVAs described for these taxa.
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Fig. 10. Community-level responses of macroinvertebrates during the C_{14.4} AS study. Taxa richness (A) across the entire study and richness, total invertebrate community abundance, and Ephemeroptera, Plecoptera, and Trichoptera (EPT) density (B) on day 56 are presented. Prior to day 56 there were no treatment-related differences. Day 56 data were normalized to the control values to assist comparisons. Treatments that are significantly different from the control are indicated by an asterisk (*, α = 0.05).

Fig. 11. Abundance of several invertebrate taxa that experienced significant decline (Turbellaria, A) or increased (Corbicula, Ferrissea, and Physella, B-D) relative to the control. Treatments that are significantly different from the control are indicated by an asterisk (*, α = 0.05).

DISCUSSION

Responses of ESF stream communities exposed to C_{14.4} AS included both significant declines and increases in various endpoints. Three of approximately 264 algal and invertebrate species collected on tile and cobble substrata had population level NOECs of 222 µg/L at the end of the 56-d exposure period. Exposure-dependent, short- and long-term responses with respect to drift were not observed during the study. Emergence of adult insects also was not affected. Principal response curves for both algal and invertebrate abundance on tiles and in trays, respectively, indicate a strong similarity between adverse responses of several aquatic taxa including Pleurosira, Melosira, and Turbellaria, detected by multivariate and univariate statistical techniques. Because of the exceptional influence of Pleurosira and Melosira size on overall algal community biovolumes, the biovolumes of the entire algal community was reduced significantly at the highest exposure concentration of 419 µg/L, resulting in a NOEC of 222 µg/L.

Ecological or energetic subsidy initially was defined by Odum et al. [28] as an intermediate state of ecosystem condition that lies between the nominative (normal operating range) and toxicologically stressed state. Odum et al. [28] coined the phrase subsidy-stress gradient in describing this phenomenon. The responses of interest in most assessments of model ecosystems during exposure to potentially toxic chemicals are those directly related to toxicity [29]. In the case of C_{14.4} AS, heterotrophic periphyton in ESF streams quickly adapted to the presence of the linear alkyl sulfate and utilized it as a source of carbon and energy during the exposure period. We observed that C_{14.4} AS mineralization in the dosed streams was approximately 2.5 times that seen in the control streams for the duration of the study representing a substantial difference in carbon and energy input versus that of the control. Consistent with this observation was the fact that on most occasions, mixed amino acid incorporation as an index of microbial heterotrophic function was elevated above control levels, as was algal uptake of bicarbonate. These observations were not statistically significant due to overlapping variability across treatments. However, given the mineralization levels in the dosed streams, it must be assumed that a fraction of the carbon and energy derived from C_{14.4} AS metabolism was passed up the food chain. Density of several key taxa in ESF stream channels clearly increased with increasing C_{14.4} AS concentration. The most dramatic of these were gastropod mollusks. The PRC analysis for invertebrate abundance indicated the two highest exposure concentrations of 222 and 419 µg/L.
L were significantly different from the control as a result of increased abundances of *Physella*, *Ferrissia* (both gastropods), and the chironomids *Cardiocladius* and *Cricotopus*. Most observations involving deviations from the nominative (control) state were indicative of subsidy (~2% of invertebrate population ANOVAs), *Physella* and *Ferrissia* both are periphytic grazer-scrapers and likely benefited somewhat from additional heterotrophic biomass present in the ESF stream channels at the higher C14.4 AS concentrations. In regard to these responses, apparent shifts important to the ecology of the system were observed at 222 μg/L and higher. However, note that these responses are not toxicological in nature, but they do represent a shift in trophic dynamics. The combination of both reduced algal abundance and altered invertebrate abundances lead to ecotoxicological NOEC and lowest-observable-effects concentration (LOEC) conclusions of 106 and 222 μg/L, respectively, using the a priori decision framework presented earlier in this paper and in Belanger [16]. The ANOVA results in this study are presented using a pseudo-replicated experimental design. Regression analysis identified the same ecological and toxicological drivers. For example, Turbellaria by day 56 had a predicted EC20 for reduced population abundance of 78 μg/L (95% confidence interval 54–102 μg/L). *Physella* and *Ferrissia* both increased with EC20s that reflect their combined influence in the PRC. All three techniques (univariate ANOVAs, regression, and multivariate PRC) provide equivalent trends and guidance although the numerical results differ slightly. The PRC and population-level one-way ANOVAs in particular were consistent with each other; however, given the limitations of the experimental design of these types of studies, the multivariate summarization of the data likely is more faithful to all the observations that were made. The PRC methods initially developed by Van den Brink and colleagues [19,30] have become a preferred method to express results from mesocosm studies because contributions of even rare or uncommon species can be valued by the technique [31] that essentially are ignored by univariate analyses. Further, these analyses provide equal weight to both species that increase or decrease in abundances, a traditional problem in assessing the significance of such observations. In this sense, the PRC result indicating the overriding influence of increased abundances of gastropods is being interpreted as adverse and the NOEC conclusion of 106 μg/L is an acknowledgement of this outcome.

Organic subsidies have been shown to be important in structuring stream invertebrate communities and production of fish. A study of sewage effluent fed to experimental streams at 25 and 50% (v:v) resulted in increased nutrient and organic load (~2- to 4-fold) [32]. The snail *Lymnaea peregra* increased four- to eight-fold in density at 50% effluent during the course of a nine-month exposure. Synthetic enrichment of experimental streams with sucrose has been used to assess potential for altering food habits of trout through a cascade of underlying responses [33]. Sucrose additions (~1–4 mg/L) into a low productivity, softwater, first order stream encouraged prolific filamentous bacterial growth that in turn led to increased tendipid chironomid abundance. Trout production cued into tendipids and increased proportionately to the abundance of this chironomid group.

Other higher tier data are available for alkyl sulfate. Belanger et al. [6] summarized ecotoxicological responses of macroinvertebrates to dodecyl (C12) AS exposure in the Procter & Gamble Experimental Stream Facility. A NOEC of 224 μg/L was estimated based on reduction of mayfly taxa. As in this study, periphyton rapidly acclimated to C12 AS and biodegraded the AS resulting in detectable upstream–downstream loss in the stream channels [9,14]. Microbial communities were not toxicologically impacted at 224 μg/L, but experienced a shift to more heterotrophic conditions that supported increased abundance of oligochaetes and mollusks similar to that observed for C14.4 AS. Lack of toxicity to algae was confirmed at >553 μg C12 AS/L in a flow-through microcosm study by Belanger et al. [34] including no effect on taxonomic richness, diversity, and abundance. One taxon out of the top 12 dominants had a NOEC of 55 μg/L, but the species change did not affect higher level community structure nor intercommunity similarity across the exposure concentrations tested. Steber et al. [35] tested a mixture of C16 and C14 AS in a microcosm fed sewage effluent from a laboratory sewage treatment system. A NOEC of 550 μg/L was derived although this is difficult to compare to microcosm or mesocosm data for C12 and C14 AS. The chain length used has very low solubility and would most likely be sorbed to sewage solids.

McAvoy et al. [3] monitored AS in five U.S. wastewater treatment plant effluents. The average chain length of AS in effluent was 13.4 alkyl carbons and the total mass of AS, all chain lengths combined, was 21 μg/L in 100% effluent. Removal was greatest in the one activated sludge plant that was monitored with the balance being trickling filter plants. Removal of surfactants typically is greater in activated sludge suggesting these monitoring results provide conservative estimates of exposure of surface waters to AS. This hypothesis is supported by AS monitoring reported by Matthijs et al. [4] of several activated sludge plants in Holland where the total AS mass in effluent averaged 5.7 μg/L and the average alkyl chain length was 12.3. Because the environmentally monitored chain lengths are one to two alkyl carbons less than that used in the C14.4 AS stream mesocosm study, an additional margin of safety is indicated because these lower chain length distributions would have even lower inherent toxicity. Monitored levels in the environment (5–21 μg/L) are approximately 10- to 50-fold below levels of ecotoxicological effects indicated by ESF mesocosm studies of C12 and C14 AS. Chronic single-species NOECs range from 50 to approximately 230 μg/L for C12 to C15 chain lengths and 770 to 12,000 μg/L for C14 AS (summaries provided in HERA 2002 and at http://www.heraproject.com [36]). Ratios of most-sensitive chronic single species to stream mesocosm NOECs for surfactants usually are in the range of 1 to 5 [37,38]. The stream mesocosm conclusions for C14 AS are in the same range and, when combined with monitored AS concentrations in the environment, support an overall conclusion of low risk to the aquatic environment.

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REFERENCES

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