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BIOCONCENTRATION, BIOTRANSFORMATION, AND CHRONIC TOXICITY OF SODIUM LAURATE TO ZEBRAFISH (*DANIO RERIO*)

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Abstract—The chronic toxicity of sodium laurate (dodecanoic acid, sodium salt; CAS 629-25-4) to zebrafish (*Danio rerio*) was determined in a 28-d growth rate study. The laurate did cause lethal effects, but a reduction in growth was not observed at sublethal exposure concentrations. The 15-d median lethal concentration was 7.6 mg/L, which is similar to the theoretical and measured solubility limit of laurate under the test conditions. The 28-d no-observed-effect concentration (lethality) was 2 mg/L, whereas growth was not impaired at 6.4 mg/L in survivors after 28 d. Laurate was extensively biotransformed to metabolites, including less polar compounds, possibly triacylglycerols. At the end of the exposure period, which was considered long enough for steady state to be achieved, the bioconcentration factor of laurate was estimated to be 255 L/kg. Body burdens of nonmetabolized laurate for surviving fish were up to 10-fold higher (2.7–8.7 mmol/kg) than the chronic critical (sublethal) body residues (0.2–0.8 mmol/kg) proposed for nonpolar or polar narcotics.

Keywords—Sodium laurate Metabolism Zebrafish Subchronic toxicity

INTRODUCTION

Historically, soaps have been regarded as surfactants with properties that present little environmental concern. They are the oldest surfactants used today. Soaps are produced by forming the alkali salts of fatty acids, with the alkyl chain consisting, on average, of 12 to 18 carbons [1]. Soaps are readily biodegradable and extensively removed in sewage treatment plants [1,2] and are generally considered to be of similar or lower aquatic toxicity compared with other commercial surfactants because of their low water solubility [1]. A review of environmental data on soap assessed the relevant acute toxicity data, but no data from chronic fish tests were found [1,2]. A risk assessment using these acute data and their correspondingly large application factor did not produce a favorable safety margin in the aquatic environment, and the authors concluded that additional chronic toxicity data should be obtained to refine the risk assessment [2].

Many toxicity tests on soap have been carried out using concentrations that exceed the water solubility (estimated solubility of 3 mg/L for C_{12} to 0.0006 mg/L for C_{18} in hard water) of the soap in the test medium [1]. In tests in which concentrations exceed the solubility limit, interpretation of effects may be difficult because undissolved material is unlikely to contribute significantly to toxicity [3]. This article presents toxicity data from a test designed to assess the subchronic effects of laurate on the growth and survival of zebrafish (*Danio rerio*). Difficulties presented by the low solubility of laurate are addressed by relating effects and body residues to estimates of soluble concentrations.

The potential for laurate to accumulate in fish may be estimated from consideration of the log octanol water partition coefficient (log K_{ow}). For lauric acid, log K_{ow} is estimated to be 4.7 for the undissociated acid [1]. The pK_a for this acid has been experimentally determined to be approx. 5 (5.3 [4]; 5 [5]) and so at pH 7 should be completely dissociated and therefore less hydrophobic than the undissociated species. An experimentally determined log K_{ow} of 3 was determined using the shake flask method [6], which, although less than the predicted value, would indicate that bioaccumulation might still occur. However, like other organisms, fish biotransform fatty acids via β-oxidation [7]; therefore, accumulation could be lower than expected from its chemical behavior. The extent of accumulation and metabolism of the laurate in the fish from the subchronic test was therefore investigated.

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MATERIALS AND METHODS

Chemicals

Lauric acid (dodecanoic acid; 98% pure) was obtained from Aldrich Chemical (Gillingham, Dorset, UK). Lauric acid (^{14}C, 3.7 μCi/mg, carbonyl carbon-labeled, >97% pure) and trilaurin (glycerol trilaurate; ^{14}C, 0.86 μCi/mg, carbonyl carbon-labeled on lauric acid, 95% pure) were synthesized and purified in house. Octadecyl (C_{18}) silica gel (40 μm, 60 Å bulk liquid chromatography packing), hexane, toluene, ethyl acetate, methanol, acetonitrile (all high-performance liquid chromatography [HPLC] grade), and phosphoric acid (American Chemical Society grade) were purchased from J.T. Baker (Deventer, The Netherlands). Soluene-350 was obtained from Packard (Groningen, The Netherlands).

Stock preparation

Stocks of sodium laurate were prepared daily by warming (above the Kraft point of 35–40°C) equimolar quantities of sodium hydroxide and lauric acid in distilled water in a water bath to keep the soap in solution (stock solutions of 5.7, 3.2, 1.82, 1.03, and 0.57 g/L). Radiolabeled lauric acid in dimethyl sulfoxide (DMSO) was added to each stock (final concentration, 0.5 μl DMSO/L) to give specific activities of 0.57, 1.02,

1.79, 3.16, and 5.71 μCi/g, respectively. The concentrations of sodium laurate were measured in the stocks on six occasions during the experiment (n = 25; concentrations 99 ± 2% of nominal) by liquid scintillation counting (LSC). Control fish were placed only in carbon-filtered tap water, which contained the same amount of DMSO as the stock solutions. Dilution water was kept at 21.5°C, and a 16-h-light/8-h-dark photoperiod was used. Fish disturbance was kept to a minimum by screening aquaria from external movement.

Growth rate study

The design of the test was based on that reported by Crossland [8], except that zebrafish were used and individuals were not marked. Zebrafish is commonly used in toxicity tests because it has been shown to be sensitive to toxicants. It is recommended for use in fish juvenile 28-d growth tests by the Organisation for Economic Cooperation and Development [9].

Groups of 16 juvenile fish (~2 months old) were temporarily anesthetized using 3-aminobenzoic acid ethyl ester (300 mg/L, <1 min duration), gently blotted to remove excess moisture, and weighed on a four-figure balance. The mean weight of the batch at the start was 68.9 mg with a standard deviation (SD) of 9.8 mg (14%). Fish were allowed to recover in clean water and then exposed to sodium laurate (final concentration, 0, 2.0, 3.6, 6.4, 11.2, and 20 mg/L) for 28 d under flow-through conditions. This concentration range was selected after a review of the published acute toxicity values to fish for sodium laurate (11 and 63 mg/L [1]; >27 mg/L, Unilever Research, unpublished results).

Fish were exposed in 5-L vessels, and the total flow rate was 170 to 180 ml/min. One volume replacement occurred every 28 min. The high flow rate was deemed necessary to maintain stable concentrations of the test substance, which is easily biodegraded. Continuously flowing test media were supplied by pumping sodium laurate stocks (made up in distilled water and kept at 35–40°C to keep the test substance in solution) with peristaltic pumps and diluting them with carbon-filtered tap water, which was gravity fed. Sodium laurate stock solutions were mixed with dilution water in sidearm flasks (stirred with magnetic stirrer) before flowing into the test vessels.

During the week, fish were fed 2% of their wet weight with a proprietary fish food (Tetramin®) and with *Artemia* (each once daily). On weekends, fish were fed once with only Tetramin at 4% of their body weight. Feeding levels were adjusted for mortalities that occurred during the test. Before feeding, surface scum (precipitated calcium laurate) was removed to minimize uptake of laurate via dietary sources. Fish were not fed on day 13 and were reweighed on day 14 (under anesthesia) to recalculate the food ratio for each exposure vessel. Fish were not fed on day 27 and were reweighed on day 28 after terminal anesthesia. Fish were frozen and stored at −20°C until tissues were analyzed, which was within 1 week. It was assumed that storage did not significantly alter lauric acid levels in fish tissue.

Mean weights and pseudospecific growth rates were compared using nonparametric analysis of variance (Kruskal-Wallis one-way ANOVA) followed by Dunn's test (two-tailed test, comparison against a control group), as a test for normality (Shapiro-Wilk) indicated some of the data were not normally distributed. The pseudospecific growth rate expresses the individual growth rate compared to the mean initial weight of the tank population. Statistical tests were carried out using Unistat (version 4). Toxicity data were analyzed by nonlinear interpolation or, if the data allowed, by the probit method.

Water and fish tissue analysis

A radiometric method was used to allow frequent sampling of test media. A more limited sampling program was carried out to allow analysis by gas chromatography using flame ionization detection (GC–FID) to verify that nonmetabolized laurate was measured using the radiometric method.

Concentrations of laurate in all the test media were determined frequently throughout the test (n = 21; one sample per test concentration on each sampling occasion) using LSC. Ten milliliters of sample were added to 10 ml of Ultima Gold scintillant (Packard, Pangbourne, Berkshire, UK) and activity determined by counting in a Packard liquid scintillation counter (2500 TR liquid scintillation analyzer). A limited number of samples (three to six per concentration) were also centrifuged to estimate crudely the water-soluble fraction. Initial results showed there was little difference between total and water-soluble fractions after centrifugation for 30 or 60 min (results not shown). Samples were therefore centrifuged at maximum speed (40,000 g in 50-ml polypropylene tubes) for 30 min, and the measured water-soluble fraction was compared to total laurate concentrations. The radiolabeled lauric acid stock (in DMSO) was kept at room temperature. Its integrity was checked at the end of the experiment and was found to be unaltered from intact lauric acid using thin-layer chromatography (TLC) and radiometric scanning (Bioscan Imaging Scanner System 200, Bioscan, Washington, DC).

Lauric acid was extracted (n = 16, one sample from each concentration with surviving fish on four occasions) from the water samples (preserved with 3% formalin [v/v]; 40% formaldehyde) to determine if biodegradation products significantly contributed to total radioactivity. Five ml of sample was put in a 50-ml separating flask, and 50 μl of 1-M sulphuric acid was added. The acidified sample was then extracted by shaking three times with 5 ml of diethyl ether (5 min each time), after which the combined extracts were back-extracted with 10 ml of deionized water. The water was removed from the ether extract with anhydrous sodium sulfate, and the extract was then evaporated to dryness in a sample concentrator under nitrogen and redissolved in 500 μl of acetonitrile before analysis. Removal efficiency of ^{14}C from the 3.6-mg/L test medium was 99 ± 1% (n = 6) using the extraction procedure described above.

Samples were analyzed by GC–FID using a Perkin Elmer GC 8700 chromatograph. On-column injection was used to load the samples on to a wall coated open-tubular (WCOT)-fused silica FFAP-CB column (length = 25 m, inner diameter = 0.32 mm, film thickness = 0.30 μm; Chrompack, Middelburg, The Netherlands) that was suitable for the analysis of fatty acids without derivatization. Under the GC conditions used (injection volume, 2 μl; helium carrier gas pressure, 9 psi; and thermal gradient, 50 to 240°C at 30°C/min followed by 7 min isothermally), lauric acid was eluted with a retention time of approx. 10.5 min. Quantitation of lauric acid was carried out from its integrated peak area, using the calibration equation obtained from the direct injection of standards containing 0 to 200 mg/L lauric acid in acetonitrile.

Total radioactivity in fish surviving to the end of the test (0, 2, 3.6, and 6.4 mg/L) or to day 16 (11.2 and 20 mg/L) was determined in about five fish at each concentration by dissolving the tissue in Soluene-350 tissue solubilizer (Packard).

Two milliliters of Soluene-350 was added to each scintillation vial, which contained one fish. Vials were incubated at 50°C for 3 h or left at room temperature overnight. After cooling, sample coloration was reduced by addition of 50 or 300 μl of hydrogen peroxide. Samples were counted in 10 ml of Hionic-fluor (Packard). Quenching was corrected for using an extended quench range of standards (Packard).

Lauric acid was extracted from whole fish by matrix solid-phase dispersion extraction. This technique has been used to extract a range of chemicals from fish tissue [10,11]. Briefly, fish were minced with scissors in a glass mortar, octadecyl silica gel was added (four times the weight of tissue), and the mixture was homogenized to a smooth paste. The paste was added to a disposable 10-ml plastic syringe (rinsed with methanol and hexane before use) and compacted using gentle pressure. Glass beads (1–1.3 mm in diameter) were placed on top of the column to minimize disturbances to the silica gel. A 200-μl disposable pipette tip containing a glass-wool plug was attached to the end of the column to control the solvent elution rate. If there was insufficient time to extract the fish tissue immediately, columns were stored at <5°C until the next day. Columns were extracted using 10 ml (2 × 5 ml) of a number of solvents successively (hexane, toluene, ethyl acetate, and methanol) to elute lauric acid and possible metabolites or biosynthesis products. After elution of each solvent had ceased, positive pressure (nitrogen gas) was used to remove excess solvent. Each of the four fractions was evaporated to dryness under nitrogen in a sample concentrator (40°C). Residues were solubilized using hexane for the hexane and toluene fractions and methanol (containing 0.012 M phosphoric acid) for the ethyl acetate and methanol fractions. The latter two were filtered through a Pasteur pipette containing a glass-wool plug to remove protein precipitates and dried down. The dried ethyl acetate and methanol fractions were then resolubilized in acetonitrile.

Extraction efficiency was determined by extraction from spiked fish tissue (unexposed fish, 0.3–0.6 g wet weight). Blanks or spiked tissue were prepared by adding 5 μl of methanol or methanol containing lauric acid to the minced tissue. Six individual fish were spiked with solvent only (control) or lauric acid at 100, 250, 500, 750, or 1,000 mg/kg (wet weight; one fish per concentration) and 500 mg/kg (five fish). The same procedure was used to extract lauric acid from fish tissue at the end of the growth rate test. Tissue was pooled from several fish (three to seven, depending on weight) to provide sufficient material (0.4–0.6 g wet weight) to quantify nonmetabolized lauric acid. The concentration of lauric acid was determined in fish that were alive after 28 d (0, 2, 3.6, and 6.4 mg/L) and in fish that had died after 4 to 8 d (11.2 or 20 mg/L). Fish that died earlier during the test (3.6 and 6.4 mg/L) were not analyzed because our primary interest was to determine body residues after 28 d in survivors.

Fractions were analyzed by GC–mass spectrometry (MS) on a Fisons Instruments MD800 bench-top GC–MS using the same column, similar oven conditions, and a helium carrier gas pressure of 5 psi. Sample injection (2 μl) was via a split–splitless injector operated in the splitless mode at 150°C. Mass spectrometric detection was based on electron ionization (70 eV) and total ion recording, although only the $m/z = 60$ chromatogram was selected for quantitation. The lauric acid–integrated peak areas were determined from standards and used to obtain a calibration equation, from which the lauric acid content of each fraction (both spiked standards and samples) was quantified.

Additionally, radioactivity recovered in each fraction was determined for some samples. Because most of the radioactivity was not associated with intact lauric acid (see Results), hexane extracts (from pooled tissue of fish exposed to 2 and 3.6 mg/L for 28 d) that contained most of the ^{14}C activity were also analyzed by TLC, followed by radiometric scanning. Thin-layer chromatography plates were developed using the method of Bilyk et al. [12]. Samples (hexane fish extracts) and standards (^{14}C lauric acid and trilaurin prepared in the hexane fraction of control fish) were spotted onto Whatman silica gel 60Å plates with preadsorbent zones. Plates were scanned for 90 min on a radiometric scanner (Bioscan) after drying.

RESULTS

Exposure concentrations

Mean measured exposure concentrations (with SDs and number of analyses in brackets) determined by LSC were 0 (0; 21), 2.2 (0.4; 21), 3.7 (1.1; 21), 6.6 (2.2; 21), 12.9 (2.5; 12), and 20.1 (5.2; 11) mg/L. On average, these concentrations were within 100 to 115% of nominal concentrations. During the test, precipitates were visible, particularly at the higher concentrations (11.2 and 20.0 mg/L). As can be seen in Figure 1, there was little difference between total and soluble laurate at concentrations of 2, 3.6, and 6.4 mg/L, but only about 80 and 40% of the total laurate was present as soluble material in the 11.2- and 20-mg/L test media, respectively. Therefore, an approximate solubility limit for laurate in the test medium was 8 to 9 mg/L. The estimated water solubility in hard water (concentration of $CaCO_3$ not specified) for sodium laurate is 3 mg/L [1] or 7.3 mg/L in water with a hardness of 100 mg/L $CaCO_3$ (G. Welch, personal communication).

Figure 2 shows the correlation between the concentration of sodium laurate determined using LSC and GC–FID. If concentrations >10 mg/L are excluded, a very good correlation between the two methods is observed. Concentrations >10 mg/L exceed the limit of solubility, and discrepancies using the two methods may be expected. No shorter-chain carboxylic acids (<12 carbons) were observed by GC–FID, indicating that the test substance in aqueous media was present as intact sodium laurate throughout the exposure period.

Tissue analyses

Hexane and methanol extracted only a small percentage of lauric acid spiked onto unexposed fish tissue (4.3 ± 2.1% and 0.5 ± 0.3%, respectively), the vast majority eluting in the toluene (71.0 ± 10.1%) and ethyl acetate (25.3 ± 4.9%) fractions. Mean recovery (from all concentrations) was 100.3 ± 9.3% ($n = 10$) and ranged from 85 to 114%. The unspiked tissue of fish from the same batch as those used in the test contained only small amounts of lauric acid (0.02 mmol/kg). Low levels were also seen in control fish tissue from the growth rate test (0.03 and 0.04 mmol/kg) and are believed to be of natural origin. Lauric acid is a minor fatty acid that makes up <2% of the fatty acids present in fish lipids [7].

The results of the lauric acid extraction are presented in Table 1. Surprisingly, pooled tissue from fish exposed to 20 mg/L sodium laurate that died after 4 to 5 d showed a lower body residue of nonmetabolized lauric acid (500 mg/kg or 2.5 mmol/kg) than those that died 5 to 8 d after the start of the test (1,298 mg/kg or 6.5 mmol/kg). Fish exposed to 11.2 mg/

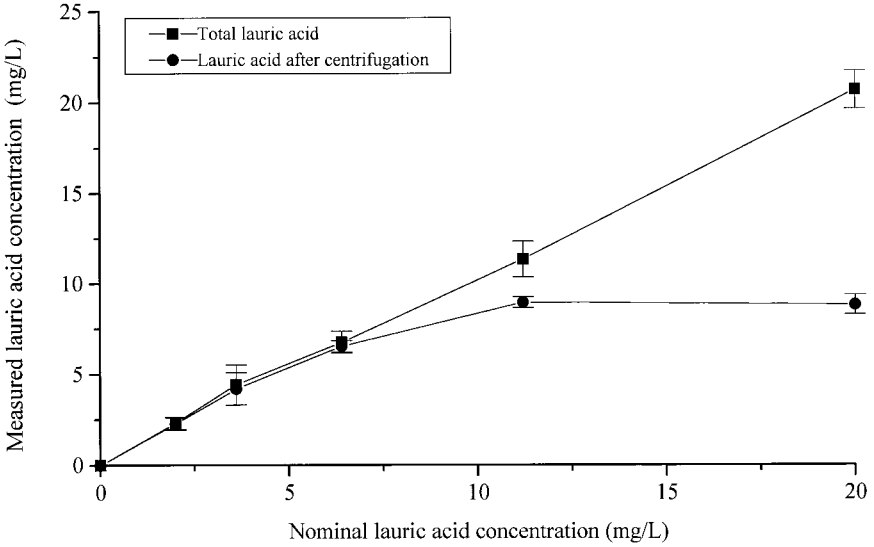

Fig. 1. Relationship between total and soluble lauric acid concentration estimated using centrifugation.

L laurate that died after 4 to 6 d of exposure had a body burden of 754 mg/kg (3.8 mmol/kg). Fish that survived to the 28-d test showed increasing body burdens with increasing exposure concentrations. Body burdens were 535 mg/kg (2.7 mmol/kg), 970 mg/kg (4.8 mmol/kg), and 1,735 mg/kg (8.7 mmol/kg) for fish exposed to 2.0, 3.6, and 6.4 mg/L sodium laurate (nominal concentrations), respectively. Table 2 shows the estimated bioconcentration factors derived from these body burdens and average test media concentrations.

Only a small fraction of the radioactivity (3–15%) recovered from fish was associated with nonmetabolized lauric acid (Table 1). This fraction was calculated as the ratio between the amount of lauric acid as determined by GC–MS and that determined in an aliquot of the same extract by LSC (assuming all ^{14}C was lauric acid). Figures 3 and 4 show the solvent extracted distribution of the radioactivity and nonmetabolized lauric acid recovered from fish exposed to 11.2 and 20 mg/L (data combined) for 4 to 8 d and to lower concentrations (2–6.4 mg/L; data combined) for 28 d, respectively. In fish exposed to the highest concentrations that died early on in the test, most of the radioactivity was recovered in the hexane (53%) and toluene fractions (32%). In fish surviving at the end of the test, most of the radioactivity was recovered in the hexane fraction (74%), with only 14% in the toluene fraction. Total radioactivity levels recovered from fish at the end of the experiment (adjusted for specific activity) were 0.016, 0.032, and 0.055 μCi/g tissue for fish exposed to 2, 3.6, and 6.4 mg/L sodium laurate, respectively. Fish exposed for 14 to 16 d to 11.2 mg/L and for 16 d to 20 mg/L contained 0.039 μCi/g.

Hexane extracts obtained from fish exposed to 2 and 3.6 mg/L laurate were analyzed by TLC. R*f* values (migration distance of substance relative to solvent front) were compared

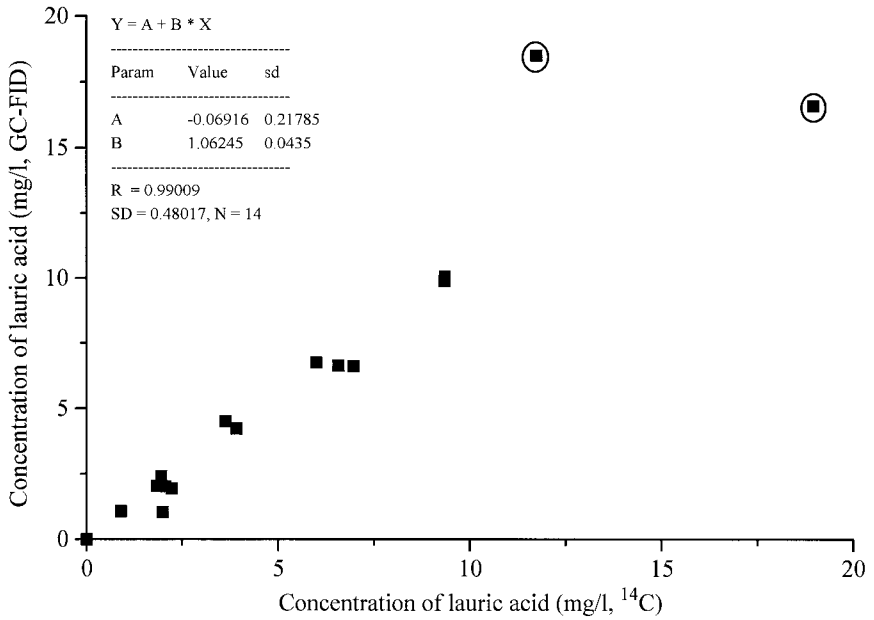

Fig. 2. Correlation of lauric acid concentration determined using liquid scintillation counting and gas chromatography with flame ionization detection (GC–FID) in aqueous samples (circled values not used in linear regression).

Table 1. Extraction of lauric acid from fish tissue

Nominal laurate concn. (mg/L)	No. fish	Tissue weight (mg)	Hexane extract (μg)	Toluene extract (μg)	Ethyl acetate extract (μg)	Methanol extract (μg)	Total lauric acid (μg)	Lauric acid in fish (mg/kg)	Intact lauric acid (% ^{14}C)	Days after start
20.0	5	396	9.1	151	23.4	14.8	198	500	6.5	4–5
	7	577	33.7	521	188	6.3	749	1,300	15.1	5–8
11.2	6	486	24.3	264	71.8	7.0	367	754	6.8	4–6
0	6	557	0.2	0.7	1.9	0.1	2.9	5	—	28
	5	480	0.2	0.6	3.1	0.3	4.2	9	—	28
2.0	3	405	14.8	164	38.6	0.2	218	538	3.0	28
	4	489	17.3	174	61.5	0.3	253	517	—	28
	4	471	15.5	190	53.3	0.3	259	551	3.7	28
3.6	3	382	21.3	289	86.7	0.2	397	1,040	3.4	28
	3	417	30.2	288	97.0	0.3	416	996	3.9	28
	4	458	26.7	291	80.0	0.7	399	871	—	28
6.4	3	391	42.7	493	78.0	0.4	614	1,570	3.1	28
	3	336	39.9	505	91.8	0.7	638	1,900	4.3	28

with those obtained for intact lauric acid and trilaurin. One major peak was visible in both extracts and had an R*f* value of 0.51. Lauric acid and trilaurin spiked into control fish hexane extract had R*f* values of 0.44 and 0.54, respectively. When fish hexane extracts (from fish exposed to 2 and 3.6 mg/L sodium laurate) were spiked with lauric acid, two peaks were visible and had R*f* values of 0.44 and 0.52. When trilaurin was added to the fish hexane extract, only one peak was observed, with an R*f* value of 0.53.

Mortality

Exposure to sodium laurate caused mortalities at total concentrations of 3.6 (6%), 6.4 (14%), 11 (75%), and 20 mg/L (75%). Fish surviving 15 d in the top two exposure concentrations were not further exposed. The estimated 4-, 8-, 15-, and 28-d median lethal concentrations (LC50s) were >20, 12, 9.9, and 9.8 mg/L, respectively. Based on soluble concentrations of laurate, LC50 values for the above time intervals were >10, 7.6, 7.6, and 7.6 mg/L.

Growth

Control fish increased in weight by 18 and 47% after 14 and 28 d, respectively (Fig. 5). In fish exposed to 2, 3.6, and 6.4 mg/L (nominal concentrations), the respective increases were 29 and 64%, 33 and 75%, and 24 and 60% after 14 and 28 d (Fig. 5). The fish that survived for 14 d at the highest concentrations had grown much less (3 and 13% at 11.2 and 20 mg/L, respectively). Unfortunately differences existed between the mean weights at day 0 ($p = 0.03$, ANOVA), which prevented comparison of the mean weights of control fish with exposed fish after 14 and 28 d. However, the pseudospecific growth rate could be calculated [9] and the different treatments compared. Growth rates were higher in laurate-exposed fish than in control animals (Table 3), although only at 3.6 mg/L (days 0–14) and at 2 and 3.6 mg/L (days 0–28) were these significantly different ($p < 0.05$). Between days 14 and 28, no significant differences existed in growth rate of control and exposed fish.

Table 2. Estimated lauric acid bioconcentration factors in zebrafish

Nominal laurate concn. in water (mg/L)	Mean measured water concn. (mg/L)	Lauric acid body burden (mg/kg) (no. fish)	Bioconcentration factor L/kg
2.0	2.21	538 (3), 517 (4), 551 (4)	243, 234, 249
3.6	3.69	1,040 (3), 996 (3), 871 (4)	282, 270, 236
6.4	6.60	1,570 (3), 1,900 (3)	238, 288
			255 ± 22[a]

[a] Mean ± SD.

Water quality

During the test, the mean (SD) temperature (°C), pH, dissolved oxygen (mg/L), and total hardness (as mg/L $CaCO_3$) were 21.4 (0.5), 7.6 (0.2), 8 (0.7), and 96.5 (4.5), respectively.

DISCUSSION

Tissue analysis

A recent review of surfactant bioconcentration [13] suggests that surfactants are rapidly metabolized in fish. Work on anionic surfactants suggests that there is a common metabolic process involving ω- and β-oxidation that leads to progressive shortening of the alkyl chain with subsequent excretion of a short-chain derivative [14]. Fatty acids also have a hydrophobic alkyl chain and a hydrophilic head group and are metabolized by the same metabolic route, although fatty acids can also be incorporated into lipids [7].

Radiometric techniques to determine lauric acid concentrations in zebrafish would have overestimated the amount of material present because of biotransformation. A specific method, GC–MS, was therefore used to analyze for nonmetabolized lauric acid following extraction from fish. As expected, increasing exposure concentrations of laurate led to increasing concentrations of lauric acid in fish tissue. After 28 d, fish exposed to 2, 3.6, and 6.4 mg/L laurate had accumulated 535, 970, and 1,735 mg/kg lauric acid (wet weight). This equates to 2.7, 4.8, and 8.7 mmol/kg. In comparison, critical body burdens for chronic effects of nonpolar and polar narcotics have been estimated to be 0.2 to 0.8 mmol/kg [15]. It is unclear why the zebrafish exposed to 2 and 3.6 mg/L accumulated laurate up to 10 times the expected effective body burden but did not show any clear signs of chronic toxicity. It is possible that, like other fatty acids, the lauric acid is stored in small quantities in organs such as liver or adipose tissue or is carried in the blood bound to albumin [7], where it is unavailable to cause a toxic response.

Although this study was not designed to measure the bio-

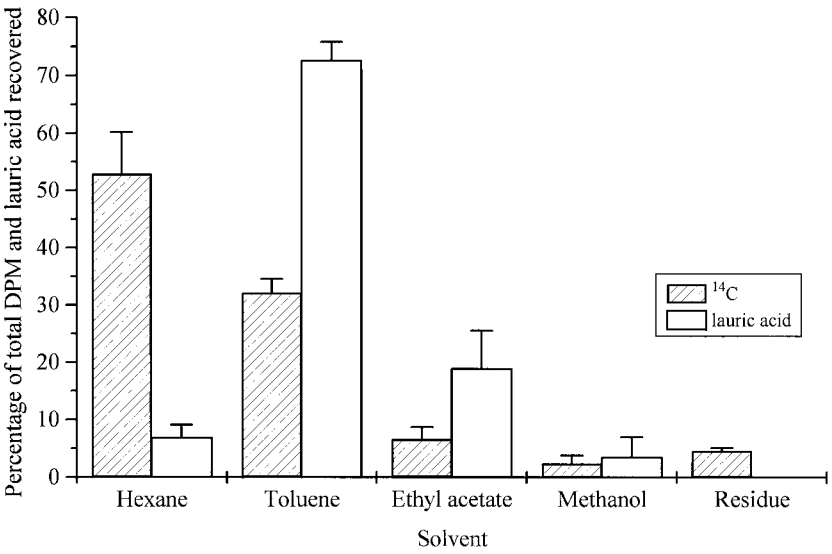

Fig. 3. Radioactivity and lauric acid recovered by matrix solid-phase dispersion extraction from fish exposed for 4 to 8 d to lauric acid (11.2 and 20 mg/L) by different solvents. DPM = disintegrations per minute.

concentration of lauric acid, the estimated time to reach 95% of steady state (assuming a log K_{ow} of 3), 40 h [16], is well within the duration of the test. In comparison, the anionic surfactant 2-*n*-(*p*-sulfophenyl)dodecane had reached steady state within 100 h [17] in bioconcentration studies using fathead minnows (*Pimephales promelas*). This suggests that the dissociated form of lauric acid would have reached steady state in the present study. The mean 28-d bioconcentration factor (BCF) was 255 L/kg with an SD of 22 based on intact lauric acid (Table 2). Growth dilution is unlikely to have affected the BCF given the relatively slow growth rate over the 28-d study period and the rapid time for lauric acid to reach a steady-state concentration. This BCF is greater than that for other surfactants with a similar chain-length hydrophobe [13]. For example, the reported BCFs for sodium dodecyl sulphate (SDS) range from 2.6 to 7.15, although the authors suggest that surfactant hydrolysis make these findings difficult to interpret [13]. The toxicity (96-h LC50) of sodium laurate and SDS to fish have been reported as 9.9 mg/L [6] and 11 mg/L [1], respectively. The 42-d no-observed-effect concentration (NOEC, toxicity) for fathead minnows for SDS was reported as >1.36 mg/L [18], which is comparable to the NOEC for sodium laurate obtained in this study (2 mg/L). Assuming both surfactants have the same mode of toxic action, this suggests that the hydrophobicity of these substances is similar. Like sodium laurate, SDS is readily metabolized by fish [19]. A possible explanation for the difference in the extent of bioaccumulation between these surfactants may lie in the relative extent and rates of metabolism. Lauric acid may remain within the body longer than SDS because the former can be used as an energy source or as a precursor of storage products, which might lead to a reduction in metabolism and excretion and therefore a higher BCF.

Fish that died at the higher exposure concentrations (11.2 and 20 mg/L) accumulated 754 and 1,298 mg/kg, respectively (4–6 and 5–8 d of exposure). Although estimates of soluble

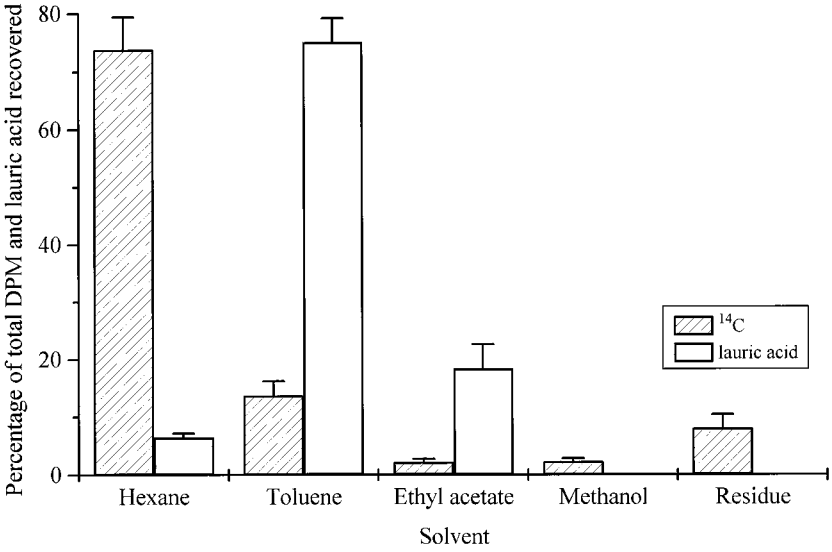

Fig. 4. Radioactivity and lauric acid recovered by matrix solid-phase dispersion extraction from fish exposed for 28 d to lauric acid (2–6.4 mg/L) by different solvents. DPM = disintegrations per minute.

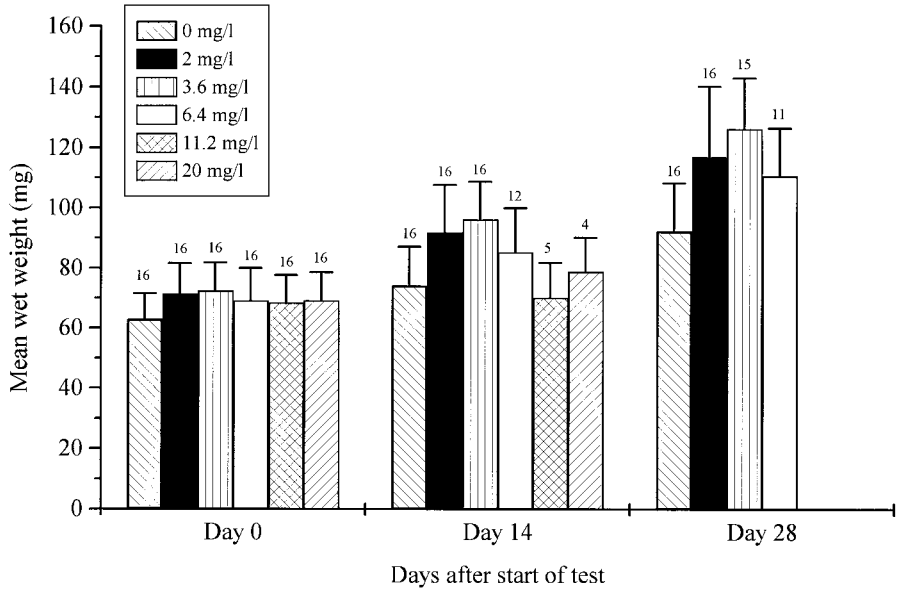

Fig. 5. Mean weight increases for *Danio rerio* exposed to increasing sodium laurate concentrations. Figures above the error bars indicate the number of fish in the sample.

laurate concentrations were similar for both the 11.2- and 20-mg/L test media, the higher tissue concentrations in fish exposed to 20 mg/L may have been due to additional uptake of particulate laurate (e.g., ingestion of small particles with food). Analysis of fish exposed to 20 mg/L that died after 4 to 5 d surprisingly showed much lower concentrations of lauric acid (500 mg/kg). The reasons for this are unclear but may be due to natural variation in the sensitivity of zebrafish to lauric acid. Other possibilities include toxic effects not directly related to body burdens, such as loss of gill integrity or loss of ions. Reduced respiration upon initial exposure may also have reduced the uptake of laurate at the highest concentration.

Extraction of fish tissue with various solvents clearly showed that the radioactivity recovered from fish was not associated with intact lauric acid. In fish that died shortly after the start of the test, the bulk of the intact lauric acid eluted in the toluene fraction, but the majority of the radioactivity was found in the hexane and toluene fractions (Fig. 4). In survivors at the end of the exposure period, a much larger proportion of the radioactivity (>70%) eluted in the hexane fraction (Fig. 3). These findings indicate that lauric acid was rapidly metabolized to more hydrophobic molecules, especially in fish that survived to day 28. It is unclear whether the enzymes involved in fatty acid esterification are inducible, although in rainbow trout it was found that the rate of palmitic acid esterification by adipose tissue increased with increasing dietary lipid [20]. In the present study, a small percentage of radioactivity could not be eluted with even the most polar solvent, indicating the label may also have become incorporated into water-soluble structures such as proteins or carbohydrates.

Henderson and Sargent [20] found that radiolabeled palmitic acid was incorporated into triacylglycerols when rainbow trout were exposed to this fatty acid. In our study, radiolabeled TLC analysis indicated that the bulk of the radioactivity was associated with substances other than lauric acid, possibly triacylglycerols. This suggests that lauric acid could have been used as an energy source via β-oxidation but also could have entered an anabolic pathway, leading to their incorporation into larger lipids.

Table 3. Pseudospecific growth rates for zebrafish exposed to increasing concentrations of sodium laurate

Time period (d)	Pseudospecific growth rate ± SD			
	Control	2 mg/L	3.6 mg/L	6.4 mg/L
0–14	1.14 ± 1.10	1.75 ± 1.31	2.04 ± 0.89	1.50 ± 1.26
0–28	1.36 ± 0.54	1.72 ± 0.79	1.99 ± 0.46	1.70 ± 0.50
14–28	1.57 ± 1.08	1.69 ± 1.58	1.94 ± 0.92	1.90 ± 0.99

Effects on survival and growth

Soaps such as lauric acid are difficult substances to test in natural water because of the formation of calcium salts, which have low solubility products. As the calcium ion concentration increases, the concentration of soluble laurate decreases. It is likely that soluble laurate causes almost all the toxic effects, whereas particulate laurate makes an insignificant contribution because of its low bioavailability [3]. Effect concentrations in the growth rate test were therefore based on soluble laurate estimated after separation by centrifugation. These estimates (8–9 mg/L) were in reasonable agreement with the calculated solubility of sodium laurate (7.3 mg/L) at the water hardness used in the test (97 ± 5 mg/L; $CaCO_3$).

The 28-d NOEC for mortality was 2 mg/L. The 96-h LC50 (>10 mg/L) is comparable to the 11 mg/L reported for *Oryzias latipes* [6], although the latter was tested at a lower hardness (51 mg/L $CaCO_3$) than in this study. Data for other soaps [1] showed significant variation in acute toxicity due to differences in water hardness and the method of preparation of test media.

The mean weight of zebrafish increased at all concentrations except 11.2 and 20 mg/L (total concentrations). Statistical analysis of the data was difficult because the control fish at the start of the test were significantly smaller than those exposed to laurate. It is clear, however, that fish exposed to 11.2 and 20 mg/L that survived 14 d showed little increase in mean weight (3 and 13%, respectively). Fish exposed to the lower

concentrations (2–6.4 mg/L) all increased in weight, and this increase was greater than that seen in control animals (Fig. 5). There appeared to be a significant increase in the growth rate in fish exposed to 3.6 mg/L compared with control fish, and, although the significance was marginal, this may have been due to the fact that pseudospecific growth rates, which by their nature have a large variance, were compared. Although there was no replication of tanks at each concentration, it has been recognized that between-tank variability is very small compared with between-fish variability [9]. It is known that fatty acids formed de novo and those originating from dietary lipids are easily esterified into neutral lipids and phospholipids [7]. Once absorbed, laurate could perhaps be used as an additional food source by these fish, resulting in slightly enhanced growth compared to that of the control fish. The 28-d NOEC for growth was 6.4 mg/L. It is unclear why growth was not reduced in fish exposed to 3.6 and 6.4 mg/L but mortality (14%) was observed.

CONCLUSIONS

The 28-d NOEC (survival) for juvenile zebrafish exposed to dispersions of laurate was 2 mg/L. Analysis of fish tissue indicated that laurate was extensively metabolized. Metabolites were more hydrophobic than the parent material, which could have been incorporated into larger lipids. This study demonstrates the importance of measuring the intact test chemical rather than total radiolabeled activity when assessing bioconcentration in fish.

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REFERENCES

1. Nederlandse Vereniging van Zeepfabrikanten. 1994. Environmental data review of soap. Final Report. BKH Consulting Engineers, Delft, The Netherlands.
2. Association Internationale de la Savonnerie et de la Détergence, Comité Européen des Agents de Surface et leurs Intermédiaires Organiques. 1996. Environmental risk assessment of detergent chemicals. *Proceedings,* AIS/CESIO Limelette III Workshop, Brussels, Belgium, November 28–29, 1995, pp 16–35.
3. Marshall SJ, van Egmond RA. 1996. Aspects of bioavailability in the ecotoxicological testing of poorly soluble substances. In Hooftman R, Manon V, Herremans J, eds, Report on the seminar, performing aquatic toxicity tests with poorly soluble substances. RIVM 679102 031. Rijksinstituut voor Volksgezondheid en milieu, Bilthoven, The Netherlands, pp 33–48.
4. Howard PH, Meylan WM. 1997. *Handbook of Physical Properties of Organic Chemicals*. CRC, Boca Raton, FL, USA.
5. Smith SW, Anderson BD. 1993. Salt and mesophase formation in aqueous suspensions of lauric acid. *Pharmacol Res* 10:1533–1543.
6. Onitsuka S, Kasai Y, Yoshimura K. 1989. Quantitative structure–activity relationship of fatty acids and sodium salts to aquatic organisms. *Chemosphere* 18:1621–1631.
7. Henderson RJ, Tocher DR. 1987. The lipid composition and biochemistry of freshwater fish. *Prog Lipid Res* 26:281–347.
8. Crossland NO. 1985. A method to evaluate effects of toxic chemicals on fish growth. *Chemosphere* 14:1855–1870.
9. Organisation for Economic Cooperation and Development. 1997. Guidelines for testing of chemicals: Proposal for fish juvenile growth test. Draft Document. Paris, France.
10. Long AR, Crouch MD, Barker SA. 1991. Multiresidue matrix solid phase dispersion (MSPD) extraction and gas chromatographic screening of nine chlorinated pesticides in catfish (*Ictalurus punctatus*) muscle tissue. *J Assoc Off Anal Chem* 74:667–670.
11. Walker CC, Lott HM, Barker SA. 1993. Matrix solid-phase dispersion extraction and the analysis of drugs and environmental pollutants in aquatic species. *J Chromatogr* 642:225–242.
12. Bilyk A, Piazza GJ, Bistline RG Jr, Haas MJ. 1991. Separation of cholesterol, fatty acylglycerols, and amides by thin-layer chromatography. *Lipids* 26:405–406.
13. Tolls J, Kloepper-Sams P, Sijm DTHM. 1994. Surfactant bioconcentration—A critical review. *Chemosphere* 24:693–717.
14. Newsome CS, Howes D, Marshall SJ, van Egmond RA. 1995. Fate of some anionic and alcohol ethoxylate surfactants in *Carassius auratus*. *Tenside Surfact Det* 32:498–503.
15. McCarty LS, Mackay D. 1993. Enhancing ecotoxicological modelling and assessment. *Environ Sci Technol* 27:1719–1728.
16. Organization for Economic Co-operation and Development. 1993. Guidelines for testing of chemicals, Vol 2, Sect 3—Bioaccumulation: Flow-through fish test. Guideline 305E. Paris, France.
17. Belanger SE, Rupe KL, Bausch RG. 1995. Responses of invertebrates and fish to alkyl sulfate and alkyl ethoxylate sulfate anionic surfactants during chronic exposure. *Bull Environ Contam Toxicol* 55:751–758.
18. Tolls J, Haller M, de Graaf I, Thijssen MATC, Sijm DTHM. 1997. Bioconcentration of LAS: Experimental determination and extrapolation to environmental mixtures. *Environ Sci Technol* 31: 3426–3431.
19. Tovell PWA, Howes D, Newsome CS. 1975. Absorption, metabolism and excretion by goldfish of the anionic detergent sodium lauryl sulphate. *Toxicology* 4:17–29
20. Henderson RJ, Sargent JR. 1981. Lipid biosynthesis in rainbow trout, *Salmo gairdnerii,* fed diets of differing lipid content. *Comp Biochem Physiol C* 69:31–37.