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Identification of methyl triclosan and halogenated analogues in male common carp (*Cyprinus carpio*) from Las Vegas Bay and semipermeable membrane devices from Las Vegas Wash, Nevada

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ABSTRACT

Methyl triclosan and four halogenated analogues have been identified in extracts of individual whole-body male carp (*Cyprinus carpio*) tissue that were collected from Las Vegas Bay, Nevada, and Semipermeable Membrane Devices (SPMD) that were deployed in Las Vegas Wash, Nevada. Methyl triclosan is believed to be the microbially methylated product of the antibacterial agent triclosan (2, 4, 4'-trichloro-4-hydroxydiphenyl ether, Chemical Abstract Service Registry Number 3380-34-5, Irgasan DP300). The presence of methyl triclosan and four halogenated analogues was confirmed in SPMD extracts by comparing low- and high-resolution mass spectral data and Kovats retention indices of methyl triclosan with commercially obtained triclosan that was derivatized to the methyl ether with ethereal diazomethane. The four halogenated analogues of methyl triclosan detected in both whole-body tissue and SPMD extracts were tentatively identified by high resolution mass spectrometry. Methyl triclosan was detected in all 29 male common carp from Las Vegas Bay with a mean concentration of 596 $\mu\text{g kg}^{-1}$ wet weight (ww) which is more than an order of magnitude higher than previously reported concentrations in the literature. The halogenated analogs were detected less frequently (21%–76%) and at much lower concentrations (<51 $\mu\text{g kg}^{-1}$ ww). None of these compounds were detected in common carp from a Lake Mead reference site in Overton Arm, Nevada.

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1. Introduction

Triclosan (Fig. 1A, 2, 4, 4'-trichloro-2'-hydroxydiphenyl ether, Chemical Abstract Service Registry Number 3380-34-5) is an antibacterial and antimicrobial agent that has been in daily use for more than 30 years in many households and personal-care products. It is the primary or secondary active agent in liquid detergents, liquid hand soaps (used in both

homes and in hospitals), deodorants, cosmetics, creams, lotions, mouthwash, toothpaste, and is impregnated in fabrics, plastics, carpets, plastic kitchenware, and toys (Adolfsson-Erici et al., 2002). Methyl triclosan (Fig. 1B, 2, 4, 4'-trichloro-2'-methoxydiphenyl ether, Chemical Abstract Service Registry Number 4640-01-1) is believed to be the microbially methylated (Boehmer et al., 2004; Balmer et al., 2004) product of triclosan.

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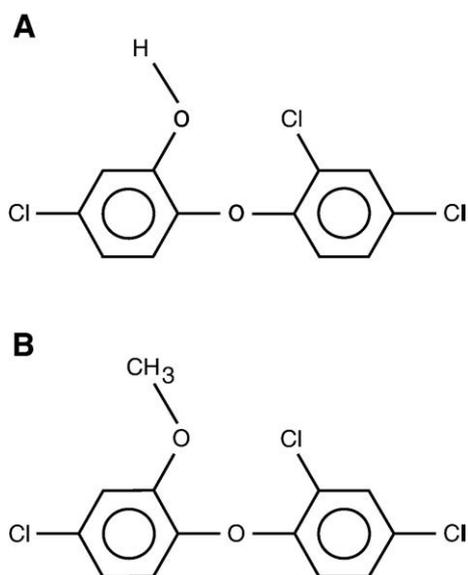


Fig. 1 – (A) Structure triclosan; and (B) structure methyl triclosan.

In recent years, controlled laboratory experiments have been conducted with triclosan and some of its higher chlorinated analogues in efforts to determine how exposure to these chemicals might affect biological organisms. Several studies suggest that triclosan might inhibit fatty acid and lipid biosynthesis (McMurry et al., 1998; Levy et al., 1999). Other studies suggest that some strains of bacteria have become resistant to triclosan (McNaughton et al., 1995; Heath and Rock, 2000; Chuanchuen et al., 2001; Meade et al., 2001). In marine bivalves it was shown to alter activity of several kinase enzymes, reduce membrane stability of immune cells and interfere with redox balance in organs (Canesia et al., 2007). It has been shown to be an endocrine disruptor of the thyroid system at environmentally relevant concentrations ($0.03 \mu\text{g L}^{-1}$) in amphibians (Veldhoen et al., 2006) as well as mammals (Crofton et al., 2007). There is evidence that triclosan not only can disrupt the thyroid system but has both estrogenic and androgenic activity from *in vitro* assays using MCF-7 human breast cancer cells (Gee et al., 2008). Another study suggests that triclosan and some of its higher chlorinated analogues might potentially function as a nonspecific depressant on the central nervous system in mice (Miller et al., 1983). Detoxification of triclosan has been shown in small mammals by elevated cytochrome P-450 enzymes in mice (Miller et al., 1982).

Several studies have reported the presence of triclosan and methyl triclosan in wastewater (Lindstrom et al., 2002; McAvoy et al., 2002), and described the fate of triclosan in activated sludge during wastewater treatment (Federle et al., 2002). Although triclosan is relatively insoluble in water it has been measured in wastewater at concentrations that range from 0.03 to $0.059 \mu\text{g L}^{-1}$ (Okumura and Nishikawa, 1996; van Stee et al., 1999; Hua et al., 2005), has been shown to have a high removal rate (90–95%) during wastewater treatment (Sabaliunas et al., 2003; Paxéus and Gryaab, 2004; Thomas and Foster, 2005)

and has a short $1/2$ life of 2.1–3.3 h in shallow surface waters (Sabaliunas et al., 2003). A recent USGS study of pharmaceutical and emerging contaminants of 139 streams in the United States found triclosan at 57.6% of the sites with a median concentration of $0.14 \mu\text{g L}^{-1}$ and a maximum concentration of $0.83 \mu\text{g L}^{-1}$ (Kopin et al., 2002). Up to 12% of triclosan in surface water can be converted by photolysis to several compounds including 2,4-dichlorophenol, and the dioxin 2,8-dichlorodibenzo-*p*-dioxin (Latch et al., 2005). During chlorination of treated finished drinking water triclosan readily reacts with free chlorine to form chloroform and a number of chlorinated phenolic compounds (Rule et al., 2005). Triclosan has been reported in human milk and in bile of fish exposed to wastewater (Adolfsson-Erici et al., 2002). Orvos et al. (2002) determined acute toxicity of triclosan for algae, *Scenedesmus sp.* (96 h $EC_{50}=1.4 \mu\text{g L}^{-1}$), an invertebrate *Daphnia magna* (48 h $EC_{50}=390 \mu\text{g L}^{-1}$) and rainbow trout *Onchorynchos mykiss* (lowest-observed-effect-concentration = $71.3 \mu\text{g L}^{-1}$). Although algae were the most sensitive taxa to triclosan toxicity other more sensitive endpoints like endocrine disruption were not measured.

Methyl triclosan has been reported in aquatic biota samples with a mean concentration of $11.6 \mu\text{g kg}^{-1}$ and maximum of $38 \mu\text{g kg}^{-1}$ (Miyazaki et al., 1984) up to $35 \mu\text{g kg}^{-1}$ in several species of fish from various Swiss lakes (Balmer et al., 2004) and in fish from three major German rivers up to $33 \mu\text{g kg}^{-1}$ (Boehmer et al., 2004). In North America the first report of methyl triclosan in fish was from the Detroit River where it was found in plasma at a very low level of $0.0132 \mu\text{g kg}^{-1}$ (Alaee et al., 2003). The estimated bioconcentration factor (BCF) of methyl triclosan in fish was on the order of $1\text{--}2.6 \times 10^5$ on a lipid basis (Balmer et al., 2004) which is considerably higher than the BCF for triclosan on a ww basis which ranged from 2532 to 4157 in zebrafish, *Danio rerio* (Orvos et al., 2002). Another study has reported the presence of triclosan and the hydroxylated forms of several chlorinated analogues in aquatic biota (Okumura and Nishikawa, 1996). The compounds identified in that study were 2,3,4,4'-tetrachloro-2'-hydroxydiphenyl ether, 2,4,4',5-tetrachloro-2'-hydroxydiphenyl ether, and 2,3,4,4',5-pentachloro-2'-hydroxy diphenyl ether, where the concentrations of the triclosan analogues ranged from $0.89\text{--}2.5 \mu\text{g kg}^{-1}$. Since there are very few studies in North America pertaining to the presence, distribution, concentration range, and potential biological effects of methyl triclosan and its analogues in aquatic organisms the results from this study will be an important contribution.

In this study, the presence of 2,4,4'-trimethyl-2'-methoxydiphenyl ether, 2,3,4,4'-tetrachloro-2'-methoxydiphenyl ether, 2,4,4',5-tetrachloro-2'-methoxydiphenyl ether, 2,3,4,4',5-pentachloro-2'-methoxydiphenyl ether, and bromo-2,4,4'-trichloro-2'-methoxydiphenyl ether tentatively are identified in SPMD and male common carp (*Cyprinus carpio*) extracts collected in Lake Mead, Nevada, by capillary gas chromatographic mass spectrometry (GC/MS). The research described herein confirms the identity of the methyl triclosan and tentatively identifies four halogenated analogues of methyl triclosan in aquatic biota based on capillary gas chromatography with low resolution electron ionization and electron-capture negative ionization mass spectrometry, high-resolution mass spectrometry, and discusses their potential formation and pathway into the environment.

2. Materials and methods

2.1. Chemicals

Glass-distilled GC²-grade acetone, anhydrous diethyl ether, hexane, and dichloromethane were obtained from Burdick and Jackson (Muskegon, MI). Analytical grade sodium hydroxide and anhydrous sodium sulfate were obtained from JT Baker (Phillipsburg, NJ). Alumina and silica gel adsorbents were obtained from Scientific Adsorbents (Atlanta, GA). Analytical standards decafluorobiphenyl, α -HCH-*d*₆, *p,p'*-DDT-*d*₈, 2,2',3,3',4,4',5,6,6'-nonachlorobiphenyl, 4,4'-dibromooctafluorobiphenyl, and naphthalene-*d*₈ were obtained from Protocol Analytical Supplies (Middlesex, NJ) at stock concentrations of 100 ng μ L⁻¹. Commercial triclosan (purity >98%) was obtained from KIC Chemicals (Armonk, NY). *N*-Methyl-*N'*-nitrosoguanidine (97%) was obtained from Aldrich Chemical Company (Milwaukee, WI).

All glassware was cleaned with soap and water, rinsed with deionized water, heated at 450 °C for 10 h, and solvent rinsed with dichloromethane prior to use. Alumina and silica gel for adsorption chromatography were prepared according to the procedure of Leiker et al. (1995). Sodium sulfate was heated at 450 °C for 8 h prior to use.

2.2. Lake Mead study area

A portion of Las Vegas Wash and both Las Vegas Bay and Overton Arm are part of the U.S. National Park Services' Lake Mead National Recreation Area (Fig. 2). Las Vegas Bay is located at the

western edge of Lake Mead and receives inflow from Las Vegas Wash. Overton Arm is in the northern most part of Lake Mead and receives inflow from the Virgin and Muddy Rivers. Lake Mead is used extensively for recreation, including fishing and water sports, for public water supply, and is a critical habitat for three populations of the endangered razorback sucker (*Xyrauchen texanus*). Las Vegas Wash is the only surface-water drainage outlet for the Las Vegas metropolitan area, which has one of the fastest population growth rates in the United States with a 2006 population of over 1.7 million (U.S. Census Bureau, 2008). Interest in this area has developed since a 1995 U.S. Geological Survey (USGS) study found evidence of endocrine and reproductive impairment in male fish from Las Vegas Bay (Bevans et al., 1996) which was further confirmed in a more comprehensive study in 1999/2000 (Patino et al., 2003). A number of other studies have documented the presence of a complex variety of environmental contaminants (Covay and Leiker, 1998; Snyder et al., 2001; Boyd and Furlong 2002; Osemwengie and Gerstenberger, 2004; Goodbred et al., 2007) in Las Vegas Bay.

In 2001, the mean flow entering into Las Vegas Bay from storm water runoff, groundwater, and three major tertiary wastewater-treatment facilities that discharge into Las Vegas Wash was about 606,000 m³d⁻¹ (Rosen et al., 2006). Waste water effluent discharges into Lake Mead are projected to increase substantially as population increases over the next 20 to 25 years. The domestic water intake for the greater Las Vegas area is at Saddle Island, about 10 km downstream of Las Vegas Wash (Fig. 2). Because the Las Vegas area receives almost all of its drinking water from Lake Mead, it has been suggested that water-quality issues in Las Vegas Bay potentially could impact the Las Vegas area drinking-water supply

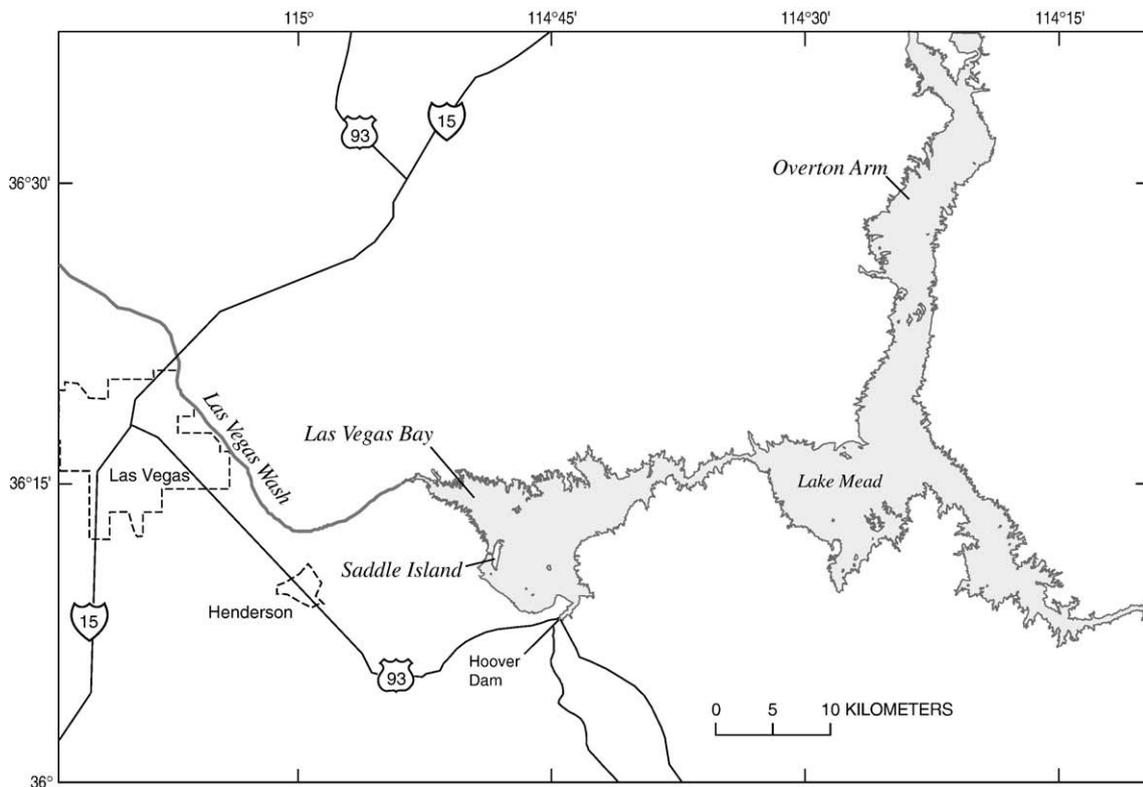


Fig. 2 – Map of Lake Mead and sampling sites.

(LaBounty and Horn, 1997). Overton Arm on the other hand receives minimal input of wastewater effluent and is generally used as a reference site in Lake Mead.

As part of the USGS 1999/2000 study to assess potential endocrine disruption in Las Vegas Bay, fish samples were collected for routine chemical analysis in both Las Vegas Bay and the Overton Arm in Lake Mead. The Overton Arm was selected as a reference site because of its distance from Las Vegas Bay (>52 km), and minimal input of wastewater effluent. While screening for unknown compounds, methyl triclosan and four halogenated analogues were detected in SPMD and fish tissue extracts from Las Vegas Bay and not detected in fish from the Overton Arm justifying its use as a reference site where fish would be minimally exposed to environmental contaminants.

2.3. Samples (fish)

Twenty-nine individual male common carp were collected by electroshocking from both Las Vegas Bay and Overton Arm during May and June 1999 (Goodbred et al., 2007). After fish were collected, internal and external examinations were performed, where one gonad was removed for histology (Patiño et al., 2003), and samples were wrapped in solvent-washed aluminum foil, and shipped frozen to the USGS' National Water Quality Laboratory in Denver, Colorado.

Individual whole body male carp, minus one gonad, were thawed at room temperature and then thoroughly homogenized with a Hobart meat grinder (Model No. 4146). Samples were prepared for analysis using previously described methodology (Leiker et al., 1995). Briefly, the samples were treated as follows. A 10 g aliquot of homogenate was added to 100 g of anhydrous sodium sulfate. The sodium sulfate and tissue mixture was frozen at -20°C . While frozen, the mixture was homogenized into a free-flowing powder using a blender and then quantitatively transferred into a fritted disk glass Soxhlet extraction thimble. Surrogate standards (1 ng each) consisting of decafluorobiphenyl, α -HCH- d_6 , p,p' -DDT- d_8 , and nonachlorobiphenyl were added to the sample prior to extraction. The tissue/sodium sulfate and tissue mixture were extracted overnight with a Soxhlet apparatus using dichloromethane.

The extract was concentrated in a Kuderna–Danish (K–D) apparatus to a volume of 5.0 mL. A 1 mL aliquot was removed for lipid determination. Two Waters Envirogel (Milford, MA) gel permeation chromatographic (GPC) preparative columns (19×150 mm and 19×300 mm) were used to remove lipid material from a 2 mL aliquot of the extract. The mobile phase was dichloromethane, and the flow rate was 5 mL/min. The fraction from 14 to 30 min was collected for analysis in a clean K–D unit, concentrated to 5 mL, exchanged into hexane, and was re-concentrated to about 1 mL.

The extract was purified further and divided into two fractions by passing it through a column dry packed (from top to bottom) with 5 g of 8.5% water-deactivated neutral alumina, 3 g of 2% water-deactivated silica, and 0.5 cm of granular sodium sulfate. The column was pre-rinsed with 50 mL of hexane and the rinse discarded. The sample extract was applied to the column and eluted with 30 mL of hexane (fraction 1). The column then was eluted with 25 mL of 50% (v/v) acetone in hexane (fraction 2). Each fraction was collected in a 35 mL K–D receiver, concentrated to a

volume of 5 mL, and then concentrated further under a gentle stream of filtered ultrapure nitrogen at ambient temperature to a volume of 0.5 mL.

The extract was transferred quantitatively, with hexane rinses, to a 1 mL GC vial. The volume of the extract was adjusted to 0.5 mL. The GC vial was sealed with a Teflon-lined cap and stored at -20°C until analysis. Just prior to analysis, injection standards consisting of 4,4'-dibromooctafluorobiphenyl (25 ng, resulting in a concentration of $50\text{ pg }\mu\text{L}^{-1}$ in the final extract) and naphthalene- d_8 (1 μg , resulting in a concentration of $2\text{ ng }\mu\text{L}^{-1}$ in the final extract) were added to each sample as injection standards.

Authentic standards of methyl triclosan and its higher chlorinated forms were not available at the time of this study. A commercial triclosan standard was derivatized with ethereal diazomethane according to the procedure of Fales et al. (1973). Briefly, ethereal diazomethane produced from *N*-methyl-*N'*-nitrosoguanidine (97%) was allowed to react with triclosan overnight at ambient temperature in a sealed conical-shaped reaction vial. Based on GC/MS analysis, the purity of the derivatized triclosan is estimated to be >95%. The molecular structure of the derivatized product was established by capillary gas chromatography under low- and high-resolution electron ionization (EI) mass spectrometry. Chromatographic and mass spectral conditions for confirming the structure of the derivative methyl triclosan and methyl triclosan in the sample extract were identical.

The response of 4,4'-dibromooctafluorobiphenyl was used to estimate the concentration of the higher chlorinated analogues of methyl triclosan during the electron-capture negative ion (ECNI) analysis, and derivatized triclosan was used to estimate the concentration of methyl triclosan during the EI analysis.

Estimates of methyl triclosan concentration in water from Las Vegas Bay were done by using the mean BCF of the range reported in fish by Balmer et al. (2004) from several Swiss lakes (mean of $1\text{--}2.6 \times 10^5 = 1.8 \times 10^5$) and dividing that by the actual mean concentrations of methyl triclosan in fish from this study. BCFs were used for fish because they have previously been reported in the methyl triclosan literature (Balmer et al., 2004) and there is no literature to suggest that methyl triclosan is taken up in food.

2.4. Samples (SPMDs)

Standard SPMDs 91.4 cm long, 2.5 cm wide made from 70–95 μm low density polyethylene tubing filled with ultrapure triolein (>99%) were constructed by Environmental Sampling Technologies (EST) Laboratory in St. Joseph, MO. Standard SPMDs were deployed at five sites in Las Vegas Wash upstream, downstream, and adjacent to the main wastewater outfalls for the cities of Las Vegas and Henderson (See Covay and Leiker, 1998 for details). Two SPMDs were placed around stainless steel carriers inside a protective metal canister, sealed in hexane rinsed airtight canisters over Argon gas and shipped overnight. The cans were kept frozen until deployment and brought chilled to the field in coolers. At each site a location was selected that was deep enough for the SPMD to remain submerged throughout the deployment period and minimized chances of vandalism. A section of steel fencepost was driven into the substrate to secure the SPMDs in

the stream. Then the SPMDs in the deployment devices were taken out of the sealed canister placed within a protective metal deployment device that was quickly placed underwater, and attached to the fencepost with a plastic tie using Nitrile gloves during all handling. The SPMDs were deployed in Las Vegas Wash for 5 weeks, from early October to mid-November 1997. During retrieval, wire cutters were used to cut the plastic tie, the deployment device was removed, cleaned of sediment and debris and then quickly placed back in its sealed can. The cans were chilled in a cooler, brought back to the lab, and frozen till overnight shipment back to EST for extraction and cleanup.

At the lab SPMDs were removed from the carrier, washed with tap water, cleaned with a soft bristled brush, then rinsed in a beaker containing 1 N HCl for 30 s. They were then rinsed with tap water followed by an acetone and 2-propanol rinse, and allowed to rapidly air dry. The SPMDs were then put in clean jars (two jars per site) with at least 125–150 ml of hexane in an incubator at 18 °C for 18 h. The hexane was then decanted into another jar and the first jar was filled again with hexane. Both jars were further incubated for 8 h after which the contents were combined into a Kuderna–Danish flask. The combined sample was concentrated then further evaporated with ultra high pure N₂ to approximately 0.5 mL, and filtered through glass fiber filter paper (Fisher, G-6) using hexane as the transfer solvent. The sample was again blown down under ultra high pure N₂ to a volume of about 0.5 mL for further cleanup with GPC. A 300 mm×21.2 mm phenogel column (Phenomenex, Torrance CA) was used with methyl chloride and 2% MeOH at a flow rate of 3.9 mL/min as the GPC eluent. The final sample was blown down under ultra high pure N₂ to a volume of about 0.5 mL then adjusted with hexane to 1 mL and quantitatively transferred to an amber ampule for chemical analysis.

2.5. Gas chromatography mass spectrometry analyses

The capillary gas chromatographic low resolution electron-capture negative ion mass spectral (GC/ECNIMS) analyses were carried out on a Hewlett-Packard-5890 gas chromatograph interfaced to HP 5989A mass spectrometer. Chromatographic separations were achieved on a 60 m×0.25 mm inside diameter 5% phenyl-95%-dimethylpolysiloxane, film thickness 0.25 μm fused-silica capillary column (FSCC) using the following temperature program: initial oven temperature was held at 50 °C for 3 min, oven temperature was ramped to 150 at 20 °C/min, from 150 to 275 at 2 °C/min, from 275 to 300 at 20 °C/min and held for 20 min at 300 °C. Splitless injections of 2 μL of each sample extract were made. The carrier gas was helium with a linear velocity of 25 cm/s. The injection port temperature was 250 °C, and the transfer line temperature was 275 °C. The GC/ECNIMS analyses were conducted under the following conditions: modifying gas, methane; source temperature, 125 °C; quadrupole temperature, 110 °C; instrument was repetitively scanned from 150 to 600 Da with a cycle time of 1.29 s per scan; emission current, 300 μA; and electron energy, 200 eV. Perfluorotributyl amine (FC-43) was used for mass axis calibration and tuning. Source pressure (0.0267 Pa) was adjusted to maximize *m/z* 633, while minimizing *m/z* 452 with methane (modifying gas), and then tuned to maximize *m/z* 452.

Low-resolution and high-resolution capillary gas chromatographic electron ionization mass spectral (GC/EIMS) analyses were conducted using a Varian 3400 gas chromatograph interfaced to a MAT-95 high-resolution mass spectrometer. Chromatographic separations for both low- and high-resolution mass spectral analyses were described above. Low-resolution EI analysis was conducted under the following conditions: resolution, 1000; electron energy, 70 eV; emission

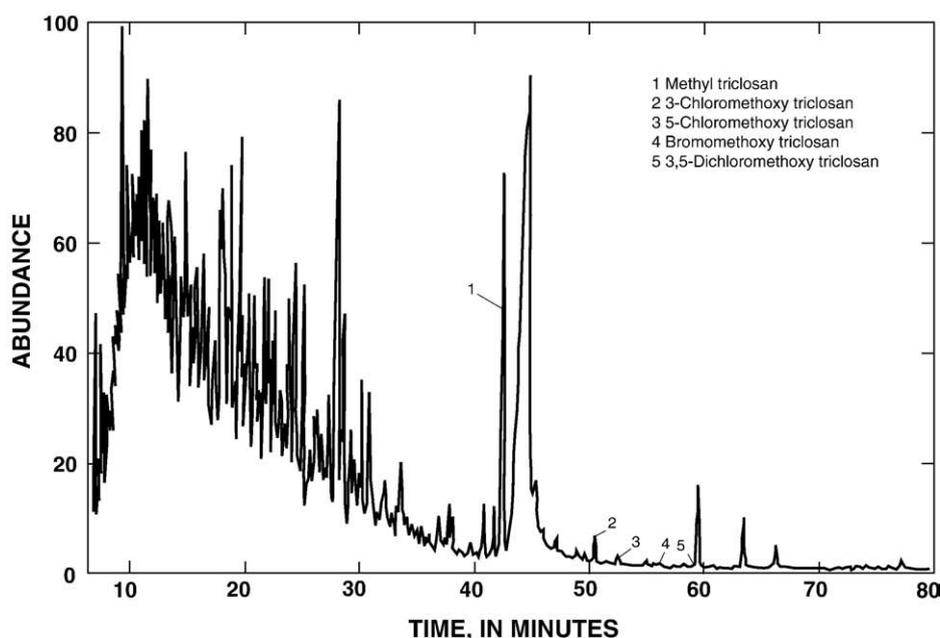


Fig. 3 – Gas chromatographic/electron ionization mass spectral (GC/EIMS) chromatogram of semipermeable membrane devices (SPMD) extract downstream of wastewater treatment facility.

Table 1 – High-resolution confirmation data for methyl triclosan and its halogenated analogues

Peak Number (Fig. 3)	Compound	Formula	Monoisotopic Mass	Observed Mass	Error (mmu)
1	Methyl triclosan ^a	C ₁₃ H ₉ Cl ₃ O ₂	301.9668	301.9696	–2.8
		C ₁₂ H ₆ Cl ₂ O ₂	251.9745	251.9768	–2.3
2	Methyl triclosan ^b	C ₁₃ H ₉ Cl ₃ O ₂	301.9668	301.9686	–1.8
		C ₁₂ H ₆ Cl ₂ O ₂	251.9745	251.9737	0.8
3	3-Chloromethoxy triclosan	C ₁₃ H ₈ Cl ₄ O ₂	335.9278	335.9263	1.5
		C ₁₂ H ₅ Cl ₃ O ₂	285.9355	285.9338	0.7
4	5-Chloromethoxy triclosan	C ₁₃ H ₈ Cl ₄ O ₂	335.9279	335.9253	2.6
		C ₁₂ H ₅ Cl ₃ O ₂	285.9355	285.9368	–1.3
5	3,5-Dichloromethoxy triclosan	C ₁₃ H ₇ Cl ₅ O ₂	369.8889	369.8900	–1.1
		C ₁₂ H ₃ Cl ₄ O ₂	319.8966	319.8933	0.2
5	Bromomethoxy triclosan	C ₁₃ H ₈ BrCl ₃ O ₂	379.8793	379.8764	0.9
		C ₁₂ H ₅ BrCl ₂ O ₂	329.8850	329.8836	–1.4

^a From derivatized triclosan.

^b From SPMD extract.

current, 100 μ A; high voltage, 5 kV; and source temperature, 200 °C. The instrument was scanned repetitively from 50 to 700 Da with a scan-rate of 1 s per decade. High-resolution EI analysis was conducted under the following conditions: resolution, 7500 (set statically); electron energy, 70 eV; emission current, 100 μ A; high voltage, 5 kV; and source temperature, 200 °C. The instrument was repetitively scanned from 50 to 600 Da with a scan-rate of 3 s per decade. Perfluorokerosene was used for both low- and high-resolution mass axis calibration and tuning for all EI analysis.

3. Results and discussion

3.1. Identification of methyl triclosan and higher chlorinated analogues

A low-resolution total ion chromatogram (Fig. 3) shows the analysis of an SPMD extract from Las Vegas Wash. The peaks

labeled 1 through 5 in the chromatogram are listed in Table 1, and their empirical formulas have been confirmed by high-resolution mass spectrometry.

The EI mass spectrum (Fig. 4) of methyl triclosan detected in the SPMD and tissue extracts and the derivatized triclosan are identical in all respects. In this spectrum an intense ion at m/z 302 contains a three-chlorine isotope cluster. A subsequent ion at m/z 252 contains a two-chlorine isotope cluster, representing a loss of 50 amu from the suspect molecular ion at m/z 302. This strongly suggests the loss of a CH₂Cl moiety, which results from a cyclization reaction in which the oxygen of an *ortho*-methoxy group, attached to the phenyl ring, attacks an *ortho*-chlorine of the other phenyl ring (Janson and Sundstrom, 1974; Tulp et al., 1977).

The derivatized triclosan, SPMD extract containing the proposed methyl triclosan, and tentatively identified analogues were reanalyzed by GC with high-resolution mass spectrometry to confirm the initial mass spectral results. The data from these experiments are listed in Table 1. The

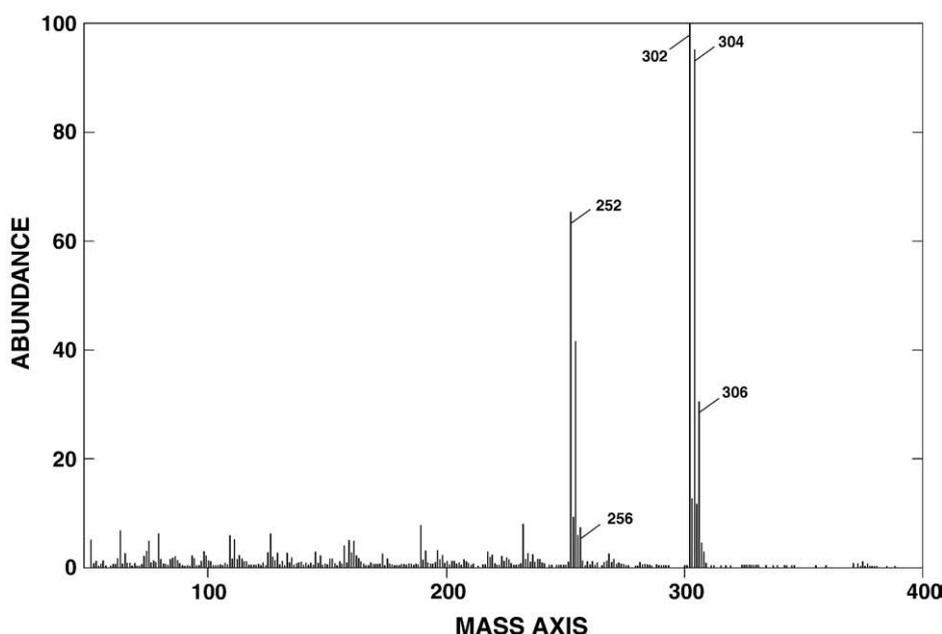


Fig. 4– Electron ionization (EI) mass spectrum of methyl triclosan in semipermeable membrane devices (SPMD) extract downstream of wastewater treatment facility.

Table 2 – Kovats retention indices for methyl triclosan higher chlorinated analogues

Compound	Indices
Methyl triclosan ^a	2145
Methyl triclosan ^b	2143
3-Chloromethoxy triclosan	2291
5-Chloromethoxy triclosan	2325
3,5-Dichloromethoxy triclosan	2449
Bromomethoxy triclosan	Not determined

^a From derivatized triclosan.
^b From SPMD extract.

exact mass for the ion at m/z 302 of the derivatized triclosan and the sample extracts are within 2.5 mmu of the theoretical value for the molecular ion. In addition, the subsequent loss of a CH_3Cl moiety at m/z 252 is supported by the high-resolution data. The empirical formula and rings plus double bonds value of eight also support the proposed structure for methyl triclosan shown in Fig. 1B. Thus, the high-resolution mass spectral data support the proposed empirical formula for the methyl triclosan. Further support for the proposed identification was provided by determining Kovats Retention Indices for the derivatized triclosan and methyl triclosan detected in the tissue extracts (Table 2). The Kovats Retention Indices data are identical.

The EI and ECNI mass spectra of the four tentatively identified halogenated analogues and their structures identified in SPMD and tissue extracts are shown in Figs. 5A–D and 6A–C, respectively. These structures are postulated on the basis of a previous study (Okumura and Nishikawa, 1996) and on the high-resolution data listed in Table 1. In the EI and ECNI spectra, molecular ions are present for each compound. The molecular ion at m/z 336 for the 3- and 5-chloromethoxy triclosan isomers contain the four-chlorine isotope cluster. The molecular ion at m/z 370 for the 3,5-dichloromethoxy triclosan contains the appropriate five-chlorine isotope cluster. As can be observed in all the EI spectra, the loss of a CH_3Cl moiety is present and is supported by the high-resolution data in Table 1. The presence of bromomethoxy triclosan also was confirmed by high-resolution mass spectrometry. The molecular ion at m/z 380 contains a single bromine atom and a three-chlorine isotope cluster. This structure also shows the loss of a CH_3Cl moiety and is supported by the high-resolution data in Table 1. All four halogenated compounds were detected in both SPMD sample extracts from Las Vegas Wash and in tissue sample extracts from Las Vegas Bay. However these compounds were not detected in samples from the reference site in Overton Arm. Authentic standards were not available at the time of the study for absolute confirmation. However, Kovats Retention Indices for the postulated structures were determined and are listed in Table 2.

3.2. Formation, source, and concentration of methyl triclosan and its higher chlorinated analogues

Several hypotheses are presented on the formation and source of methyl triclosan and its higher chlorinated analogues in Las Vegas Wash and Las Vegas Bay. First, because these com-

pounds have not been detected in the whole-body tissue samples from Overton Arm (Table 3) which is over 52 km from Las Vegas Bay, we assume that they are not ubiquitous throughout Lake Mead. Because common carp in lakes and reservoirs generally have limited movement (Miller and Crowl, 2006) the contaminant concentrations in common carp sampled within Las Vegas Bay most likely reflect sources in close proximity to Las Vegas Bay, like Las Vegas Wash, and not other distant sources into Lake Mead. Second, because these compounds are also present in SPMD sample extracts from Las Vegas Wash and as well as tissue sample extracts from Las Vegas Bay (Table 3), the data indicate that the compounds most likely originate in discharges from the three wastewater-treatment plants that discharge effluent into Las Vegas Wash. In addition, triclosan has been detected in surface-water grab samples (mean concentration of $0.40 \mu\text{g L}^{-1}$) from Las Vegas Wash (Zaugg, 2002; pers. comm.) which is well above levels ($0.03 \mu\text{g L}^{-1}$) shown to disrupt the thyroid endocrine system in amphibians (Veldhoen et al., 2006).

Methyl triclosan was detected in all 29 male common carp from Las Vegas Bay and not in fish at Overton Arm (Table 3). The mean concentration of $600 \mu\text{g kg}^{-1}$ is well over one order of magnitude higher than concentrations found in fish from Europe and Japan (Miyazaki et al., 1984; Balmer et al., 2004; Boehmer et al., 2004). Using the mean methyl triclosan concentration on a lipid weight basis of $7,400 \mu\text{g kg}^{-1}$ in fish from this study and a BCF of 1.8×10^5 the back calculated mean water concentration of methyl triclosan in Las Vegas Bay would be $0.04 \mu\text{g L}^{-1}$. This is an order of magnitude lower than the triclosan concentration of $0.4 \mu\text{g L}^{-1}$ in Las Vegas Wash (Zaugg, 2002; pers. comm.), indicating that Las Vegas Wash water derived from highly-treated sewage effluent is the likely source of the triclosan. The other chlorinated analogues were found less frequently (21%–76%) and at a much lower concentration in Las Vegas Bay ($<51 \mu\text{g kg}^{-1}$) and not detected at the reference site in Overton Arm.

Collectively our results suggest that a portion of triclosan coming into wastewater-treatment facilities is not being removed and is converted to methyl triclosan and halogenated analogs. These compounds present at low levels in the effluent are then discharged into Las Vegas Wash and transported into Las Vegas Bay where they bioaccumulate in fish. Our hypothesis is further supported by the results of a USGS national reconnaissance study that showed triclosan is commonly found downstream from wastewater-treatment facilities (Kolpin et al., 2002) and that it is present in wastewater effluent (Okumura and Nishikawa, 1996; van Stee et al., 1999; Lindstrom et al., 2002; McAvoy et al., 2002; Hua et al., 2005). In spite of finding methyl triclosan and four of its halogenated analogs in both aquatic biota and SPMDs from Las Vegas Wash and Bay we did not find the parent compound triclosan even though it does have a $\log K_{ow} > 4$ which would predict it accumulating in both tissue and SPMDs.

Boehmer et al. (2004) had somewhat similar results for two rivers in Germany where they found triclosan in only a few fish samples but methyl triclosan in every fish sample. However even though Boehmer et al. (2004) found triclosan in fish from a third river the concentrations were generally an order of magnitude lower than methyl triclosan concentrations. Based on their results and other studies they proposed that methyl triclosan is

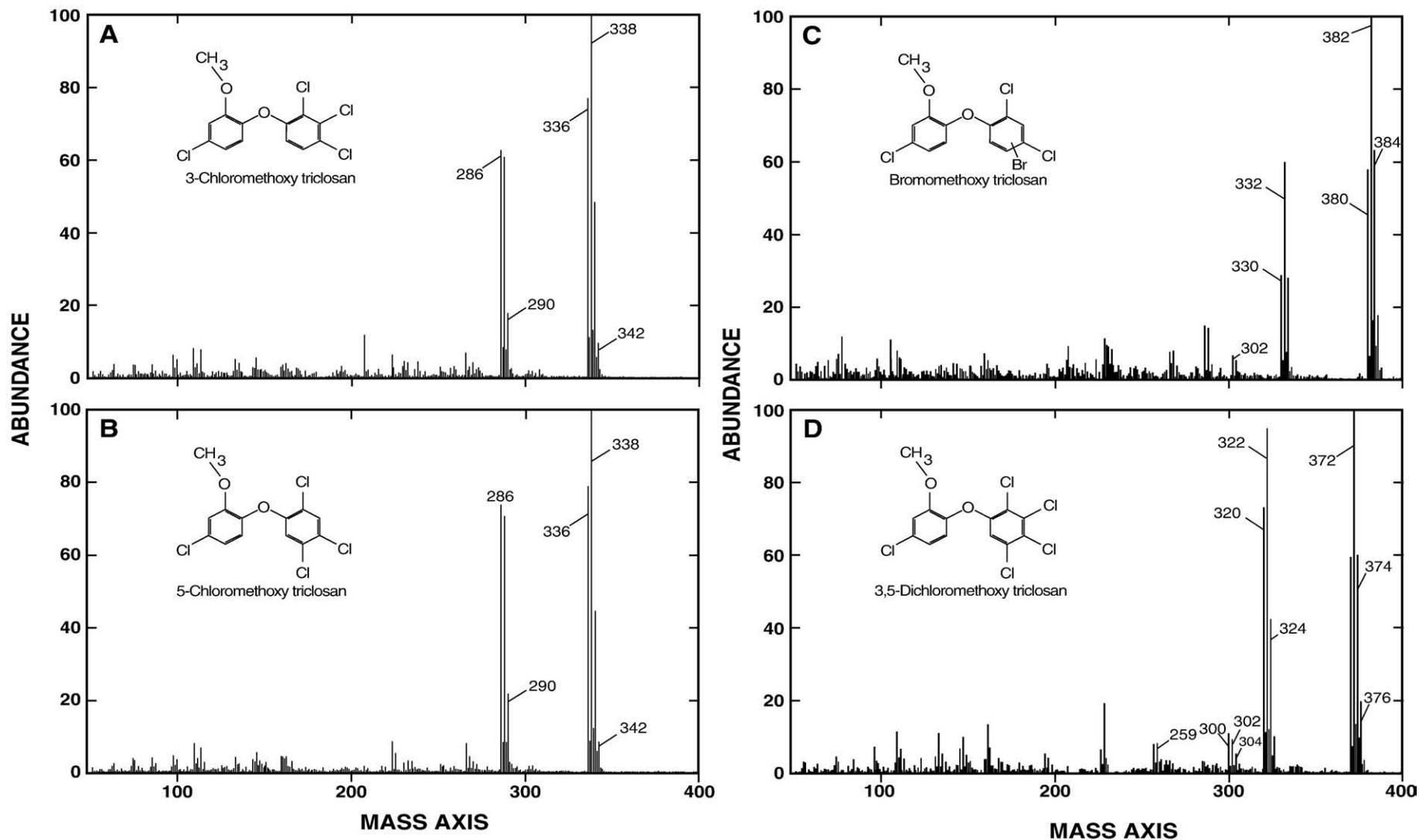


Fig. 5 – Electron ionization (EI) mass spectrum of halogenated analogues of methyl triclosan in SPMD extracts.

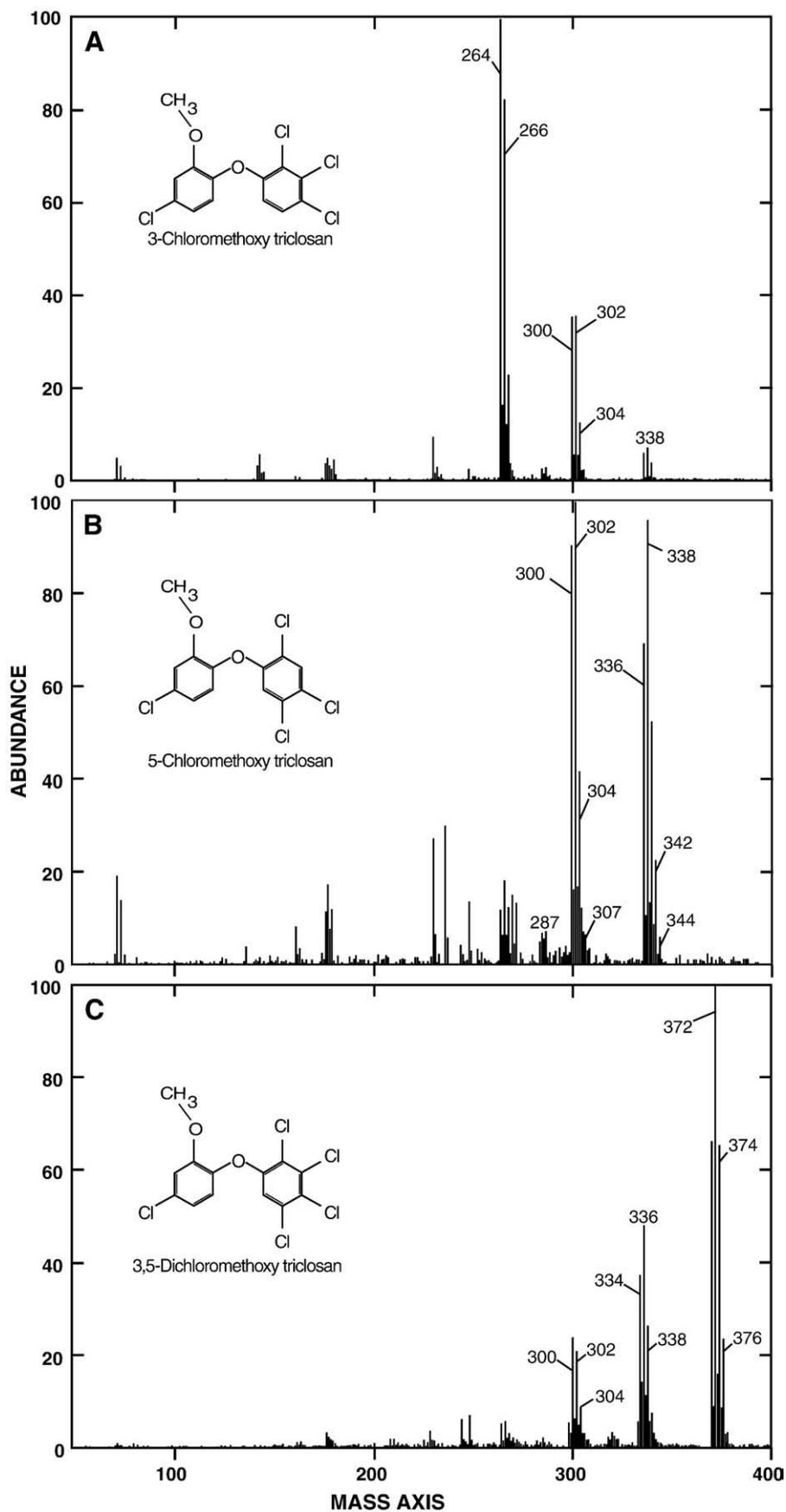


Fig. 6–Electron-capture negative ion (ECNI) mass spectrum of halogenated analogues of methyl triclosan in SPMD extracts: (A) 3-Chloromethoxy triclosan; (B) 5-Chloromethoxy triclosan; and (C) 3,5-Dichloromethoxy triclosan.

Table 3 – Summary statistics^a for estimated concentrations of methyl triclosan and analogues from male common carp sampled in May and June 1999, from Lake Mead, Nevada

Compound	Las Vegas Bay (N=29)	Overton Arm (N=29)
Methyl triclosan	600 ± 800 (20–2800)	ND
3-Chloromethoxy triclosan	13 ± 14 (<2–51)	ND
5-Chloromethoxy triclosan	0.5 ± 1.0 (<1–4)	ND
3,5 Dichloromethoxy triclosan	5 ± 6.7 (<1–24)	ND
Bromomethoxy triclosan	Not quantified	ND

ND not detected at estimated detection limit of 1 µg kg⁻¹.
^a Mean ± 1 standard deviation and range (minimum–maximum); units, µg kg⁻¹ wet weight.

more persistent in the environment and therefore will bioaccumulate more than triclosan in aquatic biota. Although Okumura and Nishikawa (1996) reported the presence of triclosan and its chlorinated analogues in aquatic biota we believe differences in how the samples were prepared for analysis can explain differences in results between their study and our study. The current study uses a combination of GPC and alumina and silica

gel adsorption chromatography for sample preparation prior to analysis. Triclosan and its chlorinated analogues would not be detected under these conditions because of the polarity of the phenolic functionality in the parent compound precludes removal from the adsorption chromatographic column because of the polarity of the second eluant. In contrast Okumura and Nishikawa (1996) extracted their samples with acetonitrile, washing the extract with hexane, isolating the compounds under aqueous basic conditions, neutralizing with acid, partitioning into hexane, and derivatizing with diazomethane. It is important therefore to consider extraction methods when comparing triclosan and methyl triclosan detection frequencies in aquatic biota between different studies.

The mechanism for conversion of triclosan into the methyl form is not clear although Balmer et al. (2004) and Boehmer et al. (2004) have proposed that triclosan is converted into the methyl form through microbial methylation of the phenolic group attached to the aromatic ring. This process is not unprecedented. A previous study has shown that the phenolic group on pentachlorophenol is microbially methylated (Cserjesi and Johnson, 1972).

The chlorinated and brominated analogues can be produced if the wastewater is treated with chlorine disinfection. For instance, the Clark County Sanitation District wastewater-treatment facility in Las Vegas, Nevada is a tertiary process

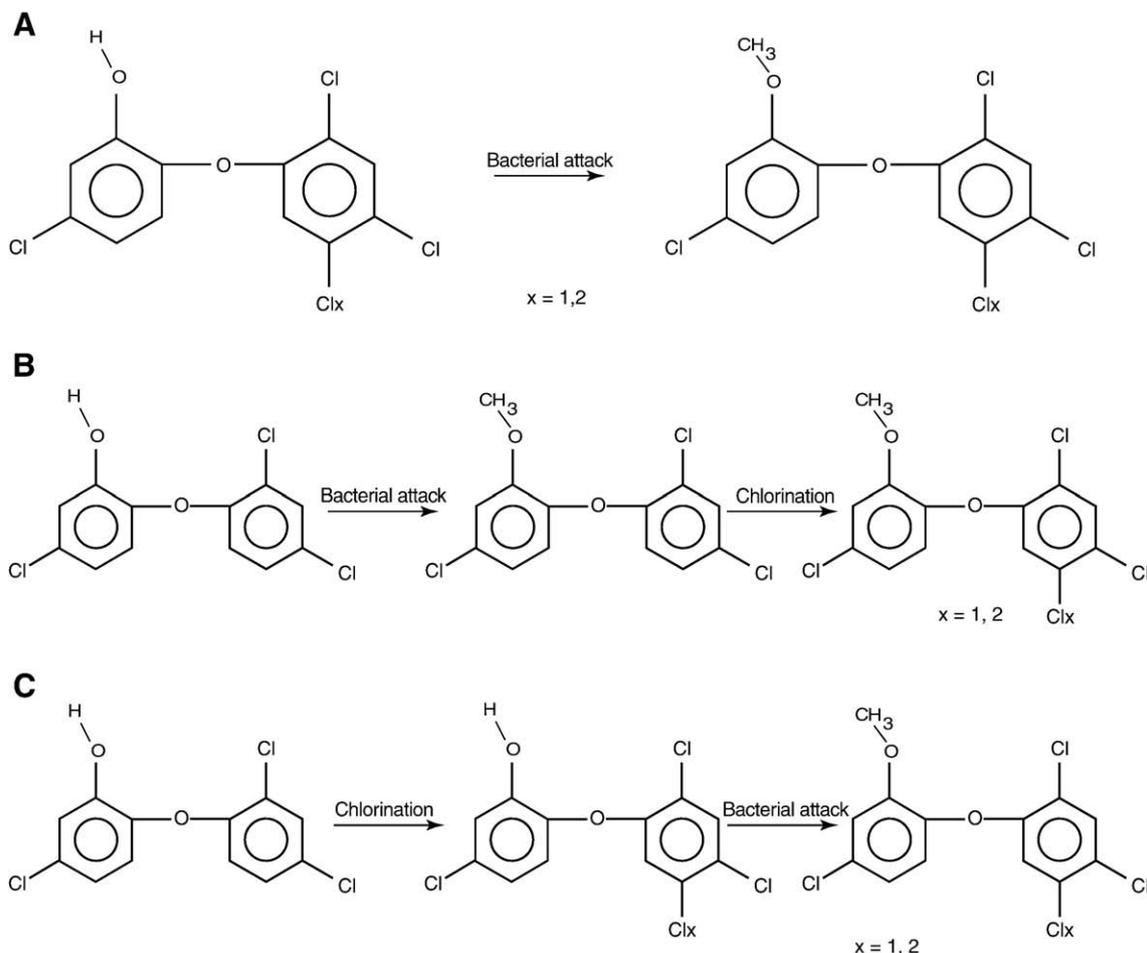


Fig. 7 – (A) Bacterial attack of higher chlorinated analogues; (B) Bacterial attack followed by chlorination; and (C) Chlorination followed by bacterial attack.

that uses sodium hypochlorite for post-treatment disinfection (Clark County Sanitation District, 2008). As a result of this post-treatment, the conversion of triclosan or methyl triclosan to the higher chlorinated analogues may be a result of three possible mechanisms (Fig. 7A–C). The first possible mechanism (Fig. 7A) assumes that the higher chlorinated phenolic analogues are impurities already in the triclosan, and that the parent compound and analogues are methylated through a bacterial attack, as discussed above. However when the commercially obtained triclosan in this study was derivatized it was found to be free of higher chlorinated analogues. These data suggest that this mechanism is not likely. The second possible mechanism (Fig. 7B) assumes that the phenolic group in the parent compound is converted to a microbially methylated product. This product is chlorinated to the higher analogues during final chlorination of the treated wastewater. The third possible mechanism (Fig. 7C) suggests that the chlorination of triclosan to the higher chlorinated phenolic analogues takes place initially. Afterwards, the phenolic functionality is methylated microbially. To support this hypothesis, a previous study reported that chlorinating triclosan with sodium hypochlorite, the same reagent that is used in the Clark County Sanitation District wastewater-treatment facility produced higher chlorinated analogues (Kanetoshi et al., 1987). The presence of the bromomethoxy triclosan most likely is caused by the presence of bromine in the sodium hypochlorite or in the treated water.

Not only is the mechanism of conversion from triclosan to methyl triclosan not well understood, but the degree of conversion of triclosan into its analogues also is unknown. The data suggest that the compounds identified in this study are environmentally derived from a parent compound, triclosan. It is not known if the methyl form is produced in the wastewater-treatment facilities or in the surface waters of Las Vegas Wash, or a combination of both. One can assume that the higher chlorinated analogues result from post-treatment chlorination. Other factors to consider are the differences in the 1/2 lives of these compounds in the environment (Sabaliunas et al., 2003) and their susceptibility to degradation in surface water by photolysis (Latch et al., 2003).

As the original triclosan is converted into its methyl form with increased chlorine content, it becomes more hydrophobic. These forms would be expected to have higher log K_{ow} s and would be bioconcentrated more readily into the lipid tissue of aquatic biota. The exact mechanism of uptake in aquatic biota is unclear. The most likely mechanisms are a combination of processing water over the gills during respiration, and ingestion of food.

4. Conclusions

Methyl triclosan and four halogenated analogs were found and identified in male common carp from Las Vegas Bay and SPMD extracts from Las Vegas Wash using a variety of analytical approaches including: low resolution GC/EIMS, and GC/ECNIMS and high resolution GC/EIMS. Methyl triclosan also was confirmed by comparison of its chromatographic Kovats Retention Index with the methyl ether derivative of commercial triclosan. The source of methyl triclosan in Lake Mead is most likely tertiary treated effluent being discharged into Las Vegas

Wash which enters Las Vegas Bay at the northern edge of Lake Mead, Nevada. Concentrations of methyl triclosan in fish are an order of magnitude higher than previously reported in the literature. Methyl triclosan is probably being formed by bacterial methylation of triclosan in the waste water treatment process. High concentrations of triclosan in Las Vegas Wash are well above levels that have been shown to disrupt thyroid endocrine systems in amphibians. However further studies would be useful to fully assess the ecotoxicological relevance of triclosan and its environmentally derived analogues to aquatic biota. Also future studies are needed to better document the distribution and concentration range of these compounds in the aquatic environment, their role as potential endocrine disruptors, and other potential adverse effects.

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REFERENCES

- Adolfsson-Erici M, Petterson M, Parkkonen J, Sturve J. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* 2002;46:1485–9.
- Alaee M, D'Sa I, Bennett E, Letcher R. Levels of triclosan and methyl triclosan in the plasma of fish from the Detroit River. *Organohalogen Compd* 2003;136:136–40.
- Balmer ME, Poiger T, Droz C, Romanin K, Bergqvist PA, Müller MD, et al. Occurrence of methyl triclosan a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland. *Environ Sci Technol* 2004;38:390–5.
- Bevans HE, Goodbred SL, Miesner JF, Watkins SA, Gross TS, Denslow ND, et al. Synthetic organic compounds and carp endocrinology and histology in Las Vegas Wash and Las Vegas and Callville Bays of Lake Mead, Nevada, 1992 and 1995. *Water-Resources Investigations Report 96-4266*. Denver, CO: U.S. Geological Survey; 1996. 12 pp.
- Boehmer W, Rudel H, Wenzel A, Schroeter-Kermani C. Retrospective monitoring of triclosan and methyl-triclosan in fish: results from the German Environmental Specimen Bank. *Organohalogen Compd* 2004;60:1516–21.
- Boyd RA, Furlong ET. Human-health pharmaceuticals compounds in Lake Mead, Nevada and Arizona, and Las Vegas Wash, Nevada, October 2000–August 2001. *Open-File Report 02-385*. Carson City, NV: U.S. Geological Survey; 2002. 18 pp.
- Canesia L, Ciaccib C, Lorusso LC, Bettib M, Galloa G, Pojana G, et al. Effects of triclosan on *Mytilus galloprovincialis* hemocyte function and digestive gland enzyme activities: possible modes of action on non target organisms. *Comp Biochem Physiol* 2007;145:464–72.
- Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP. Cross-resistance

- between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* 2001;45:428–32.
- Clark County Sanitation District. Wastewater treatment data; 2008. <http://www.cleanwaterteam.com/facility/treatment.htm> last accessed August 11, 2008.
- Covay KJ, Leiker TJ. Synthetic organic compounds in water and bottom sediment from streams, detention basins, and sewage-treatment plant outfalls in Las Vegas Valley, Nevada, 1997. Open-File Report 98-633. Denver, CO: U.S. Geological Survey, 1998. 15 pp.
- Crofton KM, Paul KB, DeVito KB, Hedge JM. Short-term *in vivo* exposure to the water contaminant triclosan: evidence for disruption of thyroxine. *Environ Toxicol Pharmacol* 2007;24:194–7.
- Cserjesi AJ, Johnson EL. Methylation of pentachlorophenol by *Trichoderma virgatum*. *Can J Microbiol* 1972;18:45–9.
- Fales HM, Jaouni TM, Babashak JF. Simple device for preparing ethereal diazomethane without resorting to codistillation. *Anal Chem* 1973;45:2302–3.
- Federle TW, Kaiser SK, Nuck BA. Fate and effects of triclosan in activated sludge. *Environ Toxicol Chem* 2002;21:1330–7.
- Gee RH, Taylor CA, Darbre PD. Oestrogenic and androgenic activity of triclosan in breast cancer cells. *J Appl Toxicol* 2008;28:78–91.
- Goodbred SL, Leiker TJ, Patino R, Jenkins JA, Denslow ND, Orsak E, et al. Organic chemical concentrations and reproductive biomarkers in common carp (*Cyprinus carpio*) collected from two areas in Lake Mead, Nevada, May 1999–May 2000. Data Series 286, U.S. Geological Survey, Data Series 286, Reston, VA; 2007. 19 pp.
- Heath RJ, Rock CO. A triclosan-resistant bacterial enzyme. *Nature* 2000;406:145–6.
- Hua W, Bennett ER, Letcher RJ. Determination of triclosan in waste and surface waters from the Detroit River using high performance liquid chromatography-electrospray-tandem quadrupole mass spectrometry. *Environ Int* 2005;31:621–30.
- Janson B, Sundstrom G. Mass spectrometry of the methyl ethers of isomeric hydroxychlorobiphenyls — potential metabolites of chlorobiphenyls. *Biomed Mass Spectrom* 1974;1:386–92.
- Kanetoshi A, Ogawa H, Katsura E, Kaneshima H. Chlorination of Irgasan DP300 and formation of dioxins from its chlorinated derivatives. *J Chromatogr* 1987;389:139–53.
- Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000; a national reconnaissance. *Environ Sci Technol* 2002;36:1202–11.
- LaBounty JF, Horn MJ. The influence of drainage from the Las Vegas Valley on the limnology of Boulder Basin, Lake Mead, Arizona-Nevada. *J Lake Reserv Manage* 1997;13:95–108.
- Latch DE, Packer JL, Arnold WA, McNeill K. Photochemical conversion of triclosan to 2,8-dichlorodibenzo-p-dioxin in aqueous solution. *J Photochem Photobiol. A* 2003;158:63–6.
- Latch DE, Packer JL, Sender BL, Van Overbeke J, Arnold WA, McNeill K. Aqueous photochemistry of triclosan: formation of 2,4-dichlorophenol, 2,8-dichlorodibenzo-p-dioxin, and oligomerization products. *Environ Toxicol Chem* 2005;24:517–25.
- Leiker TJ, Madsen JE, Deacon JR, Foreman WT. Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory — determination of chlorinated pesticides in aquatic tissue by capillary-column gas chromatography with electron-capture detection. Open-File Report 94-710W. Denver, CO: U.S. Geological Survey; 1995. 42 pp.
- Levy CW, Roujeinikova A, Sedelnikova S, Baker PJ, Stuitje AR, Slabas AR, et al. Molecular basis of triclosan activity. *Nature* 1999;398:383–4.
- Lindstrom A, Buerge IJ, Poiger T, Bergqvist PA, Müller MD, Buser HR. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environ Sci Technol* 2002;36:2322–9.
- McAvoy DC, Schatowitz B, Jacob M, Hauk A, Eckhoff WS. Measurement of triclosan in wastewater treatment systems. *Environ Toxicol Chem* 2002;21:1323–9.
- McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature* 1998;394:531–2.
- McNaughton M, Mazinke N, Thomas E. Newborn conjunctivitis associated with triclosan 0.5% antiseptic intrinsically contaminated with *Serratia marcescens*. *Can J Infect Control* 1995;10:7–8.
- Meade MJ, Waddell RL, Callahan TM. Soil bacteria *Pseudomonas putida* and *Alcaligenes xylooxidans* subsp. *denitrificans* inactivate triclosan in liquid and solid substrates. *FEMS Microbiol Lett* 2001;204:45–8.
- Miller SA, Crowl TA. Effects of common carp (*Cyprinus carpio*) on macrophytes and invertebrate communities in a shallow lake. *Freshw Biol* 2006;51:85–94.
- Miller TL, Lorusso DJ, Deinzer ML. The acute toxicity of nonachloropredioxin and 3- and 4-hydroxyonachlorodiphenyl ether in mice. *J Toxicol Environ Health* 1982;10:699–707.
- Miller TL, Lorusso DJ, Walsh ML, Deinzer ML. The acute toxicity of penta-, hexa-, and heptachlorhydroxydiphenyl ethers in mice. *J Toxicol Environ Health* 1983;12:245–53.
- Miyazaki T, Yamagishi T, Matsumoto M. Residues of 4-chloro-1-(2,4-dichlorophenoxy)-2-methoxybenzene (triclosan methyl) in aquatic biota. *Bull Environ Contam Toxicol* 1984;32:227–32.
- Okumura T, Nishikawa Y. Gas chromatography-mass spectrometry determination of triclosans in water, sediment and fish samples via methylation with diazomethane. *Anal Chim Acta* 1996;325:175–84.
- Osemwengie LI, Gerstenberger SL. Levels of synthetic musk compounds in municipal wastewater for potential estimation of biota exposure in receiving waters. *J Environ Monit* 2004;6:533–9.
- Orvos DR, Versteeg DJ, Inauen J, Capdevielle M, Rothenstein A, Cunningham V. Aquatic toxicity of triclosan. *Environ Toxicol Chem* 2002;21:1338–49.
- Patiño R, Goodbred SL, Draugelis-Dale R, Barry CE, Foott JS, Wainscott MR, et al. Morphometric and histopathological parameters of gonadal development in adult common carp from contaminated and reference sites in Lake Mead, Nevada. *J Aquat Anim Health* 2003;15:55–68.
- Paxéus N, Gryaab K. Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, beta-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment. *Water Sci Technol* 2004;50:253–60.
- Rosen MR, Goodbred SL, Patiño R, Leiker TJ, Orsak E. Investigations of the effects of synthetic chemicals on the endocrine system of common carp in Lake Mead, Nevada and Arizona, U.S. Geological Survey Fact Sheet 2006-3131; 2006. 4 pp.
- Rule KL, Ebbett VR, Vikesland PJ. Formation of chloroform and chlorinated organics by free-chlorine-mediated oxidation of triclosan. *Environ Sci Technol* 2005;39:3176–85.
- Sabaliunas D, Webb SF, Hauk A, Jacob M, Eckhoff W. Environmental fate of triclosan in the River Aire Basin, UK. *Water Res* 2003;37:3145–54.
- Snyder SA, Villeneuve DL, Snyder EM, Geisy JP. Identification and quantification of estrogen receptor agonists in wastewater effluents. *Environ Sci Technol* 2001;35:3620–5.
- Thomas PM, Foster GD. Tracking acidic pharmaceuticals, caffeine, and triclosan through the wastewater treatment process. *Environ Toxicol Chem* 2005;24:25–30.

- Tulp MThM, Olie K, Hutzinger O. Identification of hydroxyhalobiphenyls as their methyl ethers by gas chromatography mass spectrometry. *Biomed Mass Spectrom* 1977;4:310–6.
- U.S. Census Bureau. 50 fastest-growing metro areas in west and south; 2008. <http://www.census.gov/Press-Release/www/releases/archives/population/009865.html> last accessed June 2.
- van Stee LLP, Leonards PEG, Vreuls RJJ, Brinkman UATh. Identification of non-target compounds using gas chromatography with simultaneous atomic emission and mass spectrometric detection (GC-AED/MS): analysis of municipal wastewater. *Analyst* 1999;124:1547–52.
- Veldhoen N, Skirrow RC, Osachoff H, Wigmore H, Clapson DJ, Gunderson MP, et al. The bacterial agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquat Toxicol* 2006;80:217–27.