Developing a Passive Dosing Method for Acute Aquatic Toxicity Tests of Cationic Surfactant Benzalkoniums (BACs)

Anh T.Ngoc Do[1,*], Kyoshiro Hiki[1], Haruna Watanabe[1], Hiroshi Yamamoto[1], Satoshi Endo[1]

[1] Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), 305-8506 Onogawa 16-2, Tsukuba, Ibaraki, Japan

*Corresponding author: Anh T. Ngoc Do

Phone: +81-29-850-2695

E-mail: do.thingocanh@nies.go.jp/anhdn195@gmail.com

ORCID: 0000-0001-9650-1579

ABSTRACT

Benzalkonium chlorides (BACs) have been of environmental concern due to their widespread use and potential harm. However, challenges arise in defining and controlling the exposure concentration (C_w) in aquatic toxicity tests involving BACs with a long alkyl chain (i.e., #C >14). To address this, a novel passive dosing method was introduced in the 48 h-acute ecotoxicity test on *Daphnia magna* and compared to the conventional solvent-spiking method in terms of C_w stability and toxicity results. Among thirteen sorbent materials tested for their sorption capacity, polyethersulfone (PES) membrane was an optimal passive dosing reservoir, with equilibrium desorption of BACs to water achieved within 24 h. The C_w of BACs remained constant in both applied dosing methods during the test period. However, the C_w in solvent-spiking tests was lower than the nominal concentration for long-chain BACs, particularly at low exposure concentrations. Notably, the solvent-spiking tests indicated that the toxicity of BACs increased with alkyl chain length from C6 to 14, followed by a decline of toxicity from C14 to 18. In contrast, the passive dosing method displayed similar toxicity levels of BACs with C14–18, indicating higher toxicity of C16 and C18-BACs than inferred by the solvent spiking test. These findings emphasize the potential of applying this innovative passive dosing approach in aquatic toxicity tests to generate reliable and accurate toxicity data and support a comprehensive risk assessment of cationic surfactants.

KEYWORDS: aquatic toxicity, benzalkonium chlorides, cationic surfactants, *Daphnia magna*, passive dosing method, polyethersulfone (PES) membrane.

Synopsis:

A new passive dosing method using polyethersulfone (PES) membrane was successfully developed for the 48 h-acute immobilization toxicity test of benzalkonium chlorides on *Daphnia magna.*

Introduction

Benzalkonium chlorides (BACs) have been of environmental concern because of their widespread use and potential harm to ecological and human health.[1, 2] Since the registration of the first product containing BACs in the US in 1947,[3] the BACs production has risen for a broad range of applications, including biocides, antiseptics, disinfectants, personal care products, cosmetics, pharmaceuticals, and medical/building materials. Particularly, the ban of triclosan and triclocarban in antibacterial soaps by the US Food and Drug Administration (FDA) in 2016 and the global pandemic of COVID-19 beginning in 2020 have further promoted the use of BACs.[4-6] BACs are one of the most common groups of cationic surfactants, characterized by a positively charged quaternary ammonium nitrogen atom bonded with a benzyl group and an alkyl chain (C6–C18). Their amphiphilic properties enable them to adhere to solid phases that are predominantly negatively charged such as sediment, soil, sewage sludge, and laboratory glassware.[7, 8] Literature studies have extensively reported the detection of BACs in the aquatic environment, which are primarily composed of BAC homologs with alkyl chain lengths of 12−18 carbons. Surface water concentrations of BACs were typically in the µg/L range; e.g., Taiwanese rivers from 2.5 to 65 µg/L[9], U.S. stream water from 1.22 to 3.28 µg/L[10], river water in Spain > 0.1 µg/L [11] and Polish surface water from 72.8 to 331 µg/L.[12] Because of the strong sorption properties, sediment and sewage sludge concentrations can be high, reportedly up to 21 and 191 mg/kg, respectively.[13] Previous studies have documented the toxicity of BACs to aquatic organisms such as fish,[14-16] crustaceans,[8, 17-19] algae,[20, 21] and bacteria.[22, 23] Despite the findings raising concerns about the potential threat of BACs to the ecological system, the existing database on the toxicity of BACs to aquatic organisms remains unsatisfactory.

The most common experimental approach in aquatic toxicity testing is to spike the exposure medium directly with the pure substance for sufficiently water-soluble organic compounds, or with a biocompatible and water-soluble solvent as an intermediate for poorly water-soluble organic compounds.[24, 25] However, defining and controlling their bioavailable, freely dissolved concentrations (C_{free}) is a challenge for strongly sorptive compounds, including cationic surfactants.[8, 26] C_{free}, which is usually not measured in

toxicity tests, may be lower than the nominal concentration (C_{nom}) because of sorption of the chemical to dissolved organic matter, glass surfaces, and test organisms or degradation during the testing time, resulting in low test accuracy.[27-29] Therefore, such toxicity data of the chemicals might not be reliable and useful for environmental risk assessments, and there is an urgent need for new approaches to overcome these challenges in existing test protocols.

Passive dosing is an alternative approach based on the equilibrium partitioning concept introduced in toxicity testing and could solve the challenges mentioned above. [30-32] The method typically uses a polymer sorbent phase that is biocompatible, does not react with the test chemical, and acts as a chemical partitioning source to the exposure medium.[33] The passive dosing method is expected to have several advantages when applied in ecotoxicity testing. [24, 31, 34] For instance, C_{free} can be defined and controlled based on the partitioning equilibrium between the passive dosing phase and exposure medium (i.e., with a partition coefficient between the sorbent and water phases, $K_{sorbent/water}$). Moreover, there is no oversaturation/precipitation issue, and spiking solvent can be avoided. C_{free} can remain constant throughout the test if the sorbent material has a large enough sorption capacity for the test chemical (i.e., high mass or volume and high $K_{sorbent/water}$), even if some chemical loss occurs, e.g., due to degradation. C_{free} is measured directly in the medium sample or, if not possible, can be estimated from $C_{sorbent}$ and known $K_{sorbent/water}$, improving the reliability of toxicity data. Passive dosing methods have been successfully applied in toxicity testing for nonpolar hydrophobic organic compounds such as polycyclic aromatic hydrocarbons.[28, 30, 32, 35-38] However, sorption mechanisms of ionic compounds are different from those of nonpolar hydrophobic compounds [39] and a passive dosing method for ionic compounds has not been established yet. Although passive sampling methods have recently been applied in ecotoxicity tests to obtain C_{free} forBACs,[8, 40], to the best of our knowledge, there is no prior research on a passive dosing method for cationic chemicals, including cationic surfactants with a long alkyl chain (i.e., #C $\geq$14), for which a tool to control the aqueous exposure concentration is urgently required.

Herein, a passive dosing method was developed to investigate the 48 h-acute toxicity of BAC homologs on *Daphnia magna*. To develop and apply the new passive dosing approach for BACs, we focused specifically on 1) the selection of a passive dosing phase from thirteen candidate materials; 2) the loading of BACs on the dosing phase, the release to the exposure medium during the acute toxicity test, and the time to reach desorption equilibrium; and 3) the comparison of the 48 h-acute toxicity tests on *D. magna* using the conventional spiking and the newly developed passive dosing methods. Based on the results of these experiments, we discuss the effect concentrations in the ecotoxicity tests using the spiking and passive dosing methods. Finally, the relationship between toxicity and the number of carbon atoms in the alkyl chain of BAC homologs is explored.

Materials and Methods

Chemicals and Materials. Benzylhexyldimethylammonium chloride (C6-BAC, >96% purity), benzyldimethyloctylammonium chloride (C8-BAC, >96% purity), benzyldecyldimethylammonium chloride (C10-BAC, >97% purity), and benzylhexadecyldimethylammonium chlorine (C16-BAC, >97% purity) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Benzyldodecyldimethylammonium chloride dihydrate (C12-BAC, >98% purity), benzyldimethyltetradecylammonium chloride hydrate (C14-BAC, >98% purity), and benzyldimethyloctadecylammonium chloride (C18-BAC) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Three internal standards (i.e., Benzyl-2,3,4,5,6-d_5-dimethyldecylammonium chloride (d_5-C10-BAC, >99.1 atom % purity), Benzyl-2,3,4,5,6-d_5-dimethyldodecylammonium chloride (d_5-C12-BAC, >99.1 atom % purity), Benzyl-2,3,4,5,6-d_5-dimethylhexadecylammonium chloride (d_5-C16-BAC, >99.1 atom % purity) were used for quantification of BACs and purchased from CDN Isotopes Inc. (Pointe-Claire, Canada). The following thirteen sorbent materials were used: polyethylene (PE) mesh, nylon (Ny) mesh, polyethylene terephthalate (PET) mesh, polyphenylene sulfide (PPS) mesh, polyethersulfone (PES) membrane filters from two providers, Empore octadecyl (C18) SPE disk, Empore styrenedivinylbenzene-reversed phase sulfonate (SDB-RPS) disk, RP-modified silica gel high performance thin layer chromatography (HPTLC) plate, two cation exchange

membranes (Fumasep FKE and FKS membranes), C18 solid phase microextraction LC (C18 SPME) fiber, and styrene-divinylbenzene (XAD2) resin. These sorbents were considered candidates because of their relatively high surface area (meshes, porous materials), known high sorption properties for ionic chemicals (C18), and/or cation exchange properties. Additional information on the chemicals, materials and cleaning procedure can be found in the Supporting Information (SI, Section S1, Table S1 and S2). Solvents were purchased from Fujifilm Wako Chemicals (Osaka, Japan) and were of GC or LC/MS grade. Formic acid, ammonium formate, both of LC-MS grade, were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Ultrapure water of LC/MS grade (Fujifilm Wako Chemicals) or reverse-osmosis-treated tap water further purified with an Ultrapure Water System (RFU665DA, Advantec, Tokyo, Japan) was used in the loading experiment. For the ecotoxicity tests, tap water dechlorinated with activated charcoal was employed as exposure medium. All glass materials used in this study were baked at 450 °C for 4 h.

Comparing sorption capacity of sorbent materials. Batch sorption experiments were carried out to screen the sorbents for their sorption capacity as a potential passive dosing reservoir. The detailed procedure is presented in Section S2, SI. Briefly, in a 20 mL amber glass vial, sorbent was immersed in 10 mL of 100 µg/L of C12-BAC in 5 mM $CaCl_2$ solution (except a HPTLC piece, which was placed in a 50 mL glass beaker covered tightly with aluminum foil). After shaking for 24 h (i.e., for PES membrane, HPTLC plate, C18 fiber, SDB RSP membrane, C18 membrane, FKS, FKE and XAD2) or 72 h (i.e., for PE mesh, Nylon mesh, PET mesh, PPS mesh), water samples were taken and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in Section S6, SI. The concentration in the sorbents was obtained based on the mass balance calculation. The experiment was performed in duplicate.

Loading of BACs on PES membrane. Due to the cost and the large number of PES pieces required for the acute toxicity test, a roll of PES membrane (300 mm × 3 m, 0.1 µm pore size) purchased from GVS (Bologna, Italy), which has similar mechanical properties and the same pore size as the PES membranes used for the sorption experiments, was cut into pieces and used as passive dosing reservoirs for the

following tests. For loading of BACs onto the membranes, six cleaned PES pieces of 3.5 × 3.5 cm^2 each were immersed in a 20% (v/v) methanol/water mixture with defined concentrations of a single chemical in a glass beaker at 25°C. After 24 h of shaking, water was added to each beaker to enhance the loading efficiency, yielding a total loading volume of 10 mL and a final fraction of methanol in the solution of 13.33% (v/v). The loading beakers were shaken horizontally at 150 rpm for 4 days. After loading, the PES membranes were rinsed in excess water to remove adhered loading solution and then dried in the fume hood for at least 4 h to ensure that all methanol evaporated before transfer to the clean glass beakers for the desorption and ecotoxicity tests described below. PES membrane extraction was conducted for checking the loading efficiency of C14-, C16- and C18-BACs (further details in Section S3, SI).

Desorption kinetics and equilibrium. The desorption behavior of BACs from the PES membrane was examined to determine the pre-equilibration time. Dechlorinated tap water (hardness: ~80 mg-$CaCO_3$/L) was selected as the exposure medium. Note that use of Elendt M4 synthetic medium resulted in lowering of the aqueous phase concentration of C14-BAC, likely due to degradation, and thus this medium was not used. In this desorption experiment, a loaded PES piece was placed in a 50 mL glass beaker, which received 50 mL of dechlorinated water and was shaken at 135 rpm, 25°C for 5 days (C14-BAC) and 7 days (C16-BAC). Note also that a piece of polyacrylate (PA) microfiber was put in the beaker for passive sampling but the results of this will be reported elsewhere. At desired time intervals, 0.5 mL water sample was taken with a glass pipette after five times aspirating and dispensing for pre-wetting of the pipette and was further diluted with 0.5 mL of acetonitrile (ACN) containing internal standard (100 µg/L) to measure the water concentration (C_w) of BACs. After the desorption test, the PES membranes were extracted two times with 0.1% formic acid/ACN at 150 rpm and 25°C for 24 h each. Then, all the dechlorinated water was replaced by 5 mL of 0.1% formic acid/ACN mixture to extract the wall of the glass beaker (2–3 h, 25°C, 150 rpm). The PES and glass beaker extracts were diluted with ACN/internal standard before subjected to LC-MS/MS analysis (Section S6, SI).

Acute ecotoxicity tests with solvent spiking method. The water flea *D. magna* used in the ecotoxicity experiments has been subcultured at the National Institute for Environmental Studies (NIES, Japan) for more than 25 years. Daphnids were maintained in groups of 20–40 individuals/L at 21 ± 1 °C under a 16 h-light/8 h-dark cycle, bred by dechlorinated tap water (hardness: ~80 mg-$CaCO_3$/L) and fed daily by a 1 mL aliquot of the green alga *Chlorella vulgaris* at a rate of 5.0×10^8 cells/mL/day.

The acute ecotoxicity test with *D. magna* was performed following OECD Test Guideline 202. [41] Neonates less than 24 h old were exposed to a single BAC homolog at five concentrations and a control (with or without methanol), with 5 newborns in each of four replicate beakers. Test solutions were prepared by dissolving pure solid in water (C6-BAC), by diluting methanol stock solutions with water in a 300 mL flask before transferring to test beakers (C8-, C10-BACs), or by directly adding methanol stocks to water in test beakers (C12–C18-BACs), considering that longer chain BACs are more susceptible to losses during solution preparation. For all BACs except C6, the final methanol concentration in water was 0.01% (v/v). The test solutions were shaken at 150 rpm, 25°C for 24 h to dissolve the BAC in water. The preparation of the test solutions is described in more detail in Section S4, SI. The test system was set up with 50 mL dechlorinated tap water (pH: ~8) under light-dark cycles of 16:8 h at 21°C, following the culture conditions. Food and aeration were not provided throughout the acute ecotoxicity test. The test solutions were not changed during the exposure. The acute toxicity test was performed for 48 h without shaking. After 24 and 48 h, immobilization of the daphnids was determined by gently shaking the water and checking their movement for 15 s. C_w of the BAC were measured just before adding *D. magna* and at the end of the 48 h acute toxicity test. In some toxicity tests, dissolved organic carbon (DOC) was measured before adding daphnids and at the end of the ecotoxicity test (further details in Section S5, SI). Water quality was measured before and after the acute toxicity test (see Table S3). EC_{50} values were obtained based on the 2-parameter log-logistic model in the drm() function of the R-package drc (version R 4.2.2, R Core Team, 2022).[42] For each exposure level, the arithmetic mean of the measured C_w at the start and end was used for the EC_{50} estimation.

Acute ecotoxicity tests with passive dosing method. The ecotoxicity test was conducted for C14-, C16- and C18-BACs using the passive dosing approach because these long alkyl chain BACs were expected to be more susceptible to chemical loss processes such as sorption onto the glass vessels and formation of micelles in exposure solution than shorter analogs.[8, 43] The loaded PES membranes were prepared following the procedure described in the Loading experiment section. Each test beaker received a loaded PES membrane and 5 daphnids in 50 mL (C14-BAC) or 30 mL (C16- and C18-BACs) of dechlorinated water. Note that, for C14-BAC, one piece of 40 mm PA fiber was put into each test beaker for passive sampling, the results of which however will be reported elsewhere because this is beyond the scope of this article. The acute toxicity test was performed for 48 h without shaking after a 24 or 48 h pre-equilibrium period to allow the passive dosing system to reach equilibrium. Water was sampled every 24 h starting from just before adding daphnids to the end of the exposure experiment to observe the stability of C_w throughout the test period. A beaker containing a PES membrane that experienced the same loading procedure but without the test chemical was prepared as a control sample. The loaded PES membranes were either immediately extracted before the toxicity test or used for the toxicity test, retrieved from the beaker after 48 h exposure, and extracted to check for loss of BACs from the PES membranes to the exposure system. Beaker wall extraction was also conducted for C14-, C16- and C18-BACs after the acute ecotoxicity test, following the procedure described above.

Results and Discussions

Sorbent selection. To be applied in the acute toxicity test, the passive dosing format should satisfy several prior requirements (i.e., inert, biocompatible, high sorption capacity for the test chemicals).[24] As shown in Figure 1, the partitioning of C12-BAC from water to XAD2 resin, cation exchange membranes (FKS, FKE), C18 fiber, and all polymer meshes exhibited low log $K_{sorbent/water}$ (<3). Empore disks (SDB-RPS, C18) and HPTLC sorbed C12-BAC relatively well, but the small particles detached from these materials and suspended in the aqueous phase during the experiment, which would lead to undefined exposure conditions. PES membrane appears to be a suitable candidate as a passive dosing reservoir (log $K_{sorbent/water}$ ~4) for defining and controlling C_w of BACs by the equilibrium partitioning in the ecotoxicity test.

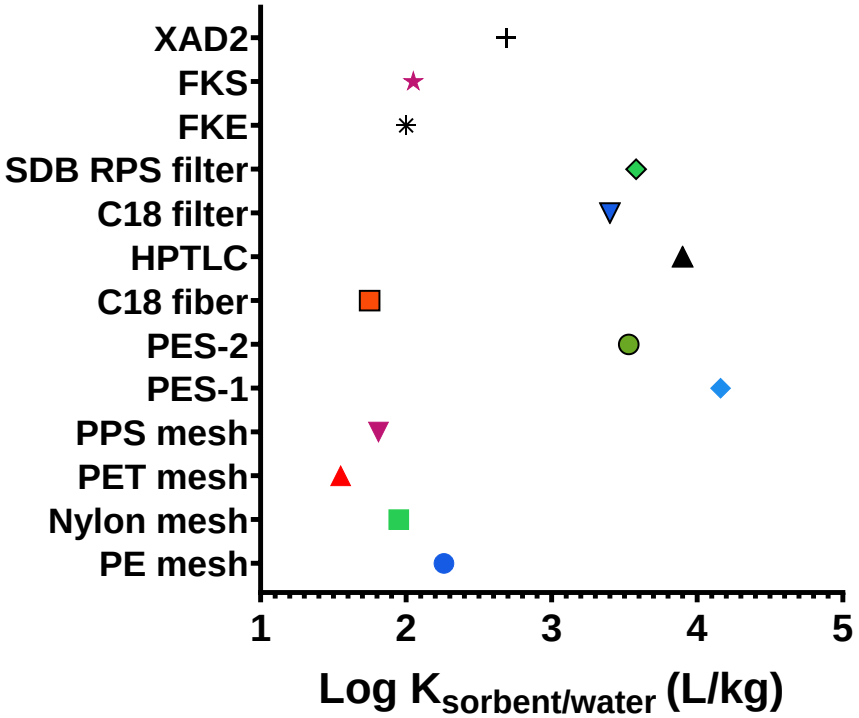

Figure 1. Partition coefficients of C12-BAC between sorbent materials and water ($K_{sorbent/water}$) with 5 mM $CaCl_2$.

Loading of PES membrane. Efficient and uniform loading of the chemical on polymer material is a prerequisite step for the passive dosing approach. Because passive dosing would be particularly

advantageous for long-chain BACs, the target substances were changed from C12-BAC to C14-, C16- and C18-BACs at this point. The actual mass of C14-, C16- and C18-BACs loaded on the PES membrane ranged from 70 to 148 % of the mass added initially to the loading solution (Figure S1). Some BACs (1-30 %) remained in the loading solution, particularly when the BAC concentrations in the loading solution were high. The distribution between PES membrane and the loading solution followed the nonlinear Freundlich isotherm model (Figure S2) with a Freundlich exponent of 0.23–0.43, indicating relatively weak sorption at high loading levels. C14- and C16-BACs on the glass beakers were less than 2 % of the added mass in the loading experiment (Figure S3). In a preliminary loading experiment for two different exposure concentrations, the masses of C14-BAC in replicated loaded PES membranes were similar (Figure S4), indicating uniform distribution to the PES membrane pieces in the loading step.

Desorption of BACs from PES membrane to water. Equilibrium between PES membrane and water was achieved for both C14- and C16-BACs within 24 h under gentle shaking (Figure S5). Afterwards, the C_w of C14- and C16-BACs remained stable, demonstrating that the chemical supply from PES membrane is sufficient to overcome any possible mass loss (e.g., sorption onto glass beakers). The calculated logarithmic partition coefficients of BACs between PES membrane and water (K_{PESw}) for both chemicals are greater than 4 (Table S5), confirming a high sorption affinity of PES membrane for these BACs. Separate experiments indicated that the presence of clean PES membrane in dechlorinated tap water had no effect on the immobility of *D. magna* (Table S6).

228

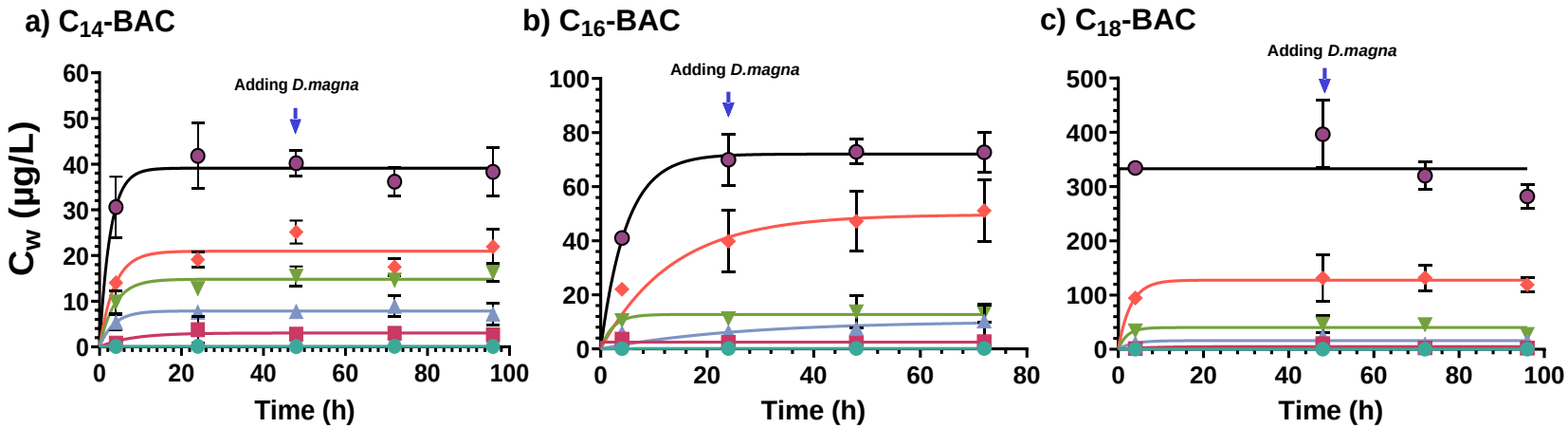

Figure 2. The water concentration (C_w) of a) C14-BAC, b) C16-BAC and c) C18-BAC during the *D. magna* 48 h toxicity test using passive dosing method from PES membrane. The C_w of C14-BAC, C16-BAC and C18-BAC were fitted using a first-order equation, $C_w = C_{w,eq}(1 - e^{-kt})$, where $C_{w,eq}$ (µg/L) is the aqueous concentration at equilibrium, t (h) is the time from the start of desorption, and k (1/h) is the rate constant of the first-order equation. The start and end of the exposure were at t_{start} = 48 h and t_{end} =96 h, respectively, for C14-BAC and C18-BAC and were t_{start} = 24 h and t_{end} = 72 h, respectively, for C16-BAC. Error bars represent standard deviations (n = 4).

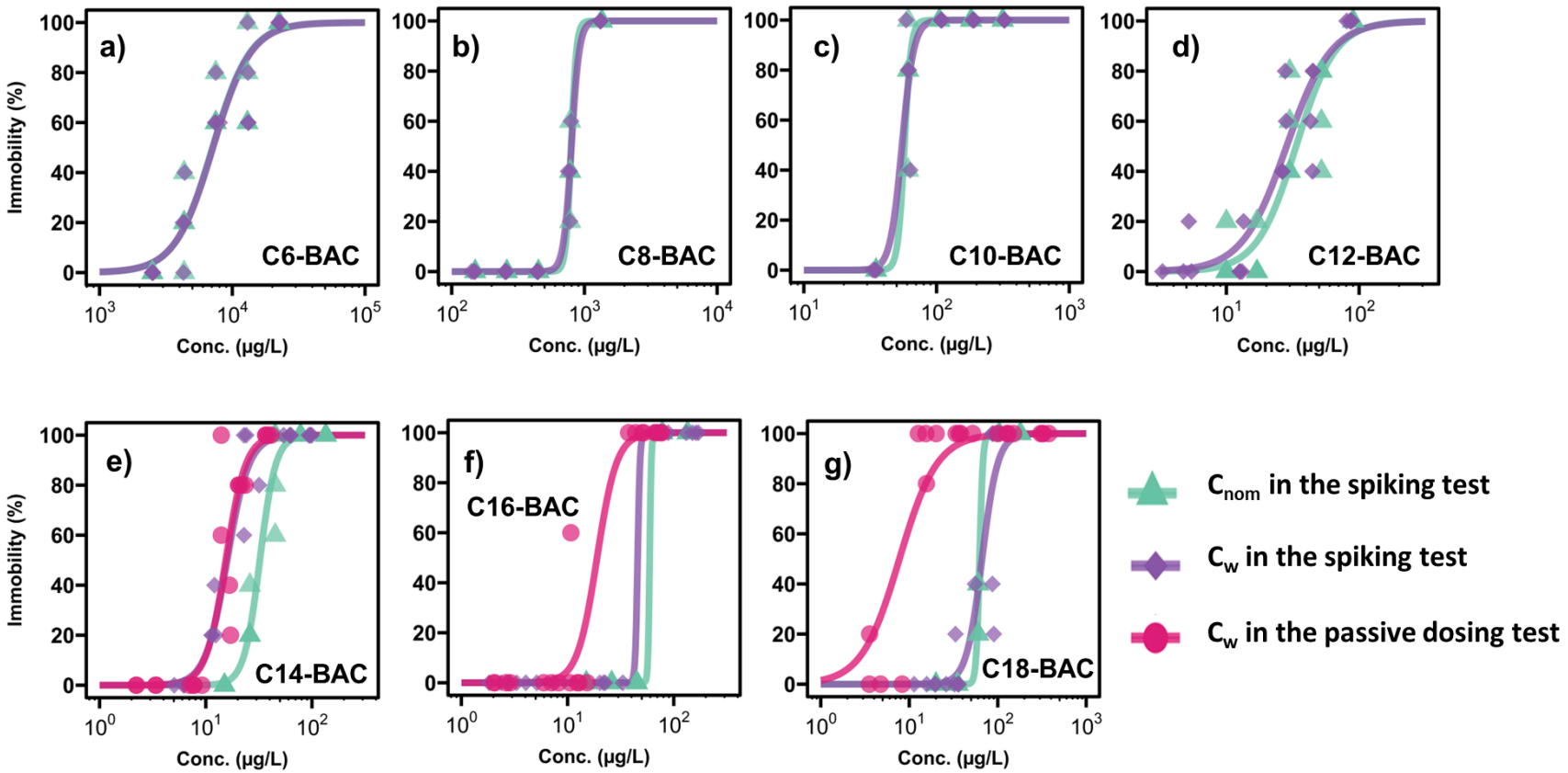

Figure 3. Concentration-response curves of a) C6-BAC, b) C8-BAC, c) C10-BAC, d) C12-BAC, e) C14-BAC, f) C16-BAC and g) C18-BAC from 48 h acute toxicity tests on *D. magna*. The curves were drawn using C_{nom} in the solvent spiking test (green line), measured C_w in the solvent spiking test (purple line) and measured C_w in the passive dosing test (pink line). The measured C_w in the solvent spiking and passive dosing tests were the average of the concentrations before adding daphnids and at the end of the ecotoxicity test. Data from all four replicates are shown. The data of the control beakers are not shown.

229

Table 1. Comparison of EC_{50} values (± standard error) in the acute toxicity test on *D. magna* of BACs.

Chemicals	EC_{50} (µg/L) in this study (in 48 h test duration)		EC_{50} (µg/L) in literature
	Spiking test	Passive dosing test	Spiking test
C6-BAC	7100 ± 700		
C8-BAC	800 ± 30		
C10-BAC	55 ± 3		
C12-BAC	28 ± 3		130 (C_w in 24 h test duration) [17] 16 (C_{free} in 48 h test duration) [8] 6.61 (C_w) in 48 h test duration) [19]
C14-BAC	16 ± 2	15 ± 1	130 (C_w in 24 h test duration) [17] 8.27 (C_w in 48 h test duration) [19]
C16-BAC	46*	19 ± 3	220 (C_w in 24 h test duration) [17] 180 (C_w in 24 h test duration) [44]
C18-BAC	66 ± 6	8 ± 1	

* The model fitting showed a large standard error due to the obtained mortality data being only 0 and 100 %, therefore the standard error was not shown here.

Acute ecotoxicity tests using solvent spiking and passive dosing methods.

Exposure concentration. In the solvent spiking test, the measured C_w agrees with C_{nom} of BACs with the alkyl chain lengths from C6–C10. For these short chain BACs, the exposure concentrations are under control and there may be no need for a passive dosing method.

C_w were lower than C_{nom} for BACs with alkyl chain lengths of C12–16, particularly at low concentrations (Figure S6, Table S7). The lower C_w compared to C_{nom} for C12–16-BACs is partially due to sorption of the chemicals to glass vessels. Figure S7 shows the measured recoveries for BACs from water and the glass beaker in the spiking toxicity test. Losses from 7% to 60% of the total mass due to sorption to the glass beaker were observed for C12–16-BACs. The sorption affinity for the glass beaker increases with increasing alkyl chain length of BACs, which agrees well with previous studies on sorption of BACs to glass/plastic surface.[26, 43] The greater loss to the glass surface at lower concentrations suggests limited sorption sites on the glass surface, which may be saturated at relatively high concentrations. At low concentrations of C12–16-BACs, 20–40% of the added mass was not recovered from either the water or glass wall, indicating the presence of another loss process. It is possible that the loss was due to the glass pipette [43] during water sampling, even after five times aspirating and dispensing in order to equilibrate the pipette surface. Furthermore, up to 140% recoveries (water + beaker wall) of C16- and C18-BACs at high concentrations were observed for an unknown reason.

Interestingly, the agreement between the measured and nominal concentrations was better for C18-BAC than C16-BAC. Moreover, the chemical loss of C18-BAC was not particularly high at low concentrations, in contrast to C12–16-BACs. We speculate that C18-BAC was not completely dissolved into its free form and that colloidal association might have occurred with C18-BAC in water in the presence of methanol (0.01% v/v). Thus, the measured C_w may include a fraction of C18-BAC that was not available for sorption. Note however that the critical micelle concentration (CMC) of C18-BAC in deionized distilled

255 water at 25°C was reported to be 24 mg/L in a previous study,[45] which is higher than the concentrations
256 used in the current study.

257 All in all, there remain several challenges to define the exposure concentration in the solvent
258 spiking toxicity testing of BACs with a long alkyl chain. Using C_{nom} to calculate the effect concentrations
259 of BACs with a long alkyl chain (#C ≥ 14) in solvent spiking tests may not provide reliable data to assess
260 their toxicity, and even measured C_w may not represent the true bioavailable exposure concentration for
261 C18-BAC.

262 In the passive dosing tests, measured C_w values remained constant during the test period, notably,
263 even after the addition of daphnids (Figure 2). DOC concentrations in the solvent spiking test (15–24 mg/L)
264 were higher than those in the passive dosing method (0.8-4.3 mg/L) (Table S8). The presence of methanol
265 (0.01 %, v/v) in the exposure medium caused an increase in DOC in the spiking test, whereas the exposure
266 water in the passive dosing test contained a low concentration of DOC. Therefore, the binding of C14-18
267 BACs to dissolved organic components in the test medium is considered negligible and the measured C_w is
268 regarded as C_{free} in this study. Desorption equilibrium was reached within the 24 h pre-equilibration time
269 for C14- and C16-BACs and 48 h for C18-BAC (Figure 2). Sorption to the glass beaker ranged from 0.1 to
270 6 % of the total measured mass (i.e., the sum of masses from water, PES membrane, and glass beaker) and
271 increased with alkyl chain length (Figure S8). The PES membrane retained >91% of the loaded BACs,
272 indicating that the PES membrane served as a partitioning source. The calculated mean log K_{PESw} for C14-,
273 C16-, and C18-BACs within the tested concentration range were 4.61–4.80, 4.45–5.41 and 4.14–5.33,
274 respectively (Table S9). Sorption isotherms of C14–C18-BACs on PES membrane (Figure S9) indicate that
275 BACs with longer alkyl chain lengths have higher Freundlich coefficients and lower Freundlich exponents,
276 showing their strong nonlinear sorption to PES membrane. Notably, concentration-dependent sorption of
277 C18-BAC on PES membrane is slightly stronger than C16-BAC (Figure S9). Thus, the obtained K_{PESw}
278 values in the investigated concentration range for C18 are not higher than those for C16, although there are
279 two more CH_2 groups in the hydrocarbon chain.

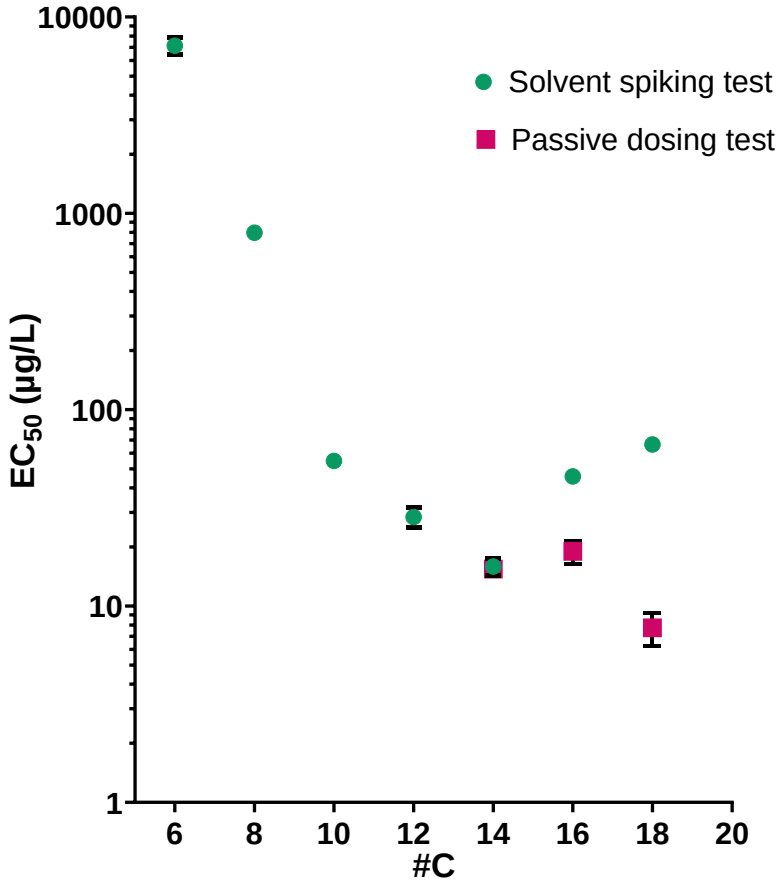

Figure 4. Relationship between EC_{50} values and carbon numbers in the alkyl chain of BACs. The error bars present the standard errors. See Table 1 for the details.

Concentration–response curves and EC_{50} values. The concentration–response curves in the 48 h acute toxicity test on *D. magna* using the solvent spiking and passive dosing approaches of BACs are compared in Figure 3. Neither mortality nor immobilization of *D. magna* was observed in the blank controls, solvent controls or controls with clean PES membrane. The experimental median effective concentration (EC_{50}) values, which were calculated using the arithmetic mean of measured C_w at adding daphnids and at the end of the experiment for each beaker, of the studied BACs are listed in Table 1. Notably, there was no significant difference between the arithmetic mean and the geometric mean here. *D. magna* 48 h-EC_{50} values have been reported for only C12- and C14-BACs in the literature, and they agree with the EC_{50} values obtained in this study within a factor of 2–4.[8, 19] EC_{50} values from the solvent spiking and passive dosing methods from this study agree well for C14-BAC (Figure 4). However, the EC_{50} values from the

passive dosing method for C16-BAC and C18-BAC are lower than those from the solvent spiking method. Thus, according to the solvent-spiking tests, the toxicity of BACs increases with increasing alkyl chain length up to 14 carbon atoms, beyond which the trend apparently decreases. In contrast, the EC_{50} values are similar with the alkyl chain length ≥ 14 carbon atoms in the passive dosing tests (Figure 4). It is commonly known that toxicity of chemicals increases with increasing hydrophobicity.[46] However, a "cutoff effect" has often been observed in toxicity of amphiphile homologous series, including cationic surfactants; that is, toxicity increases with alkyl chain length up to a certain point, above which toxicity remains more or less constant or even decreases.[20, 47-52] Sorption losses to glass beakers do not explain the cutoff in this study, because we used the measured C_w to calculate the EC_{50}. There are several possible explanations for this behavior. For long-chain cationic surfactants in the spiking tests (e.g., C18-BAC), there may be difference between the freely dissolved and total concentrations due to association with dissolved organic components or formation of micelles,[26, 53] resulting in low bioavailability. As mentioned, this difference is considered negligible in passive dosing methods, thereby mitigating a cutoff effect. Another possible explanation for the cutoff point could be that the time required to reach organism/water equilibrium is longer for BACs with a long chain compared to those with a short chain, and the former do not reach equilibrium within the experimental time.[20, 26, 54]. This could also explain why a slight cutoff effect was observed for C14–18-BACs in the 48 h toxicity test even using the passive dosing method.

Implications for the application of the newly developed passive dosing method in aquatic toxicity of cationic surfactants

A new passive dosing method with PES membrane was developed for the acute toxicity test on *D. magna* of BACs. PES membrane showed its sufficient sorption strength and fast desorption of BACs to maintain a consistent exposure concentration. This passive dosing format with PES does not need frequent exchange of exposure media and is inexpensive, biocompatible, and adaptable to a large number of samples. Testing this new method for prolonged exposure in toxicity tests is a next important step of research, which may be useful in examining the cutoff effects observed for BACs with long alkyl chains.

316 Sorption onto glass equipment is crucial in the toxicity test of long-chain BACs and probably other
317 cationic surfactants, as it decreases the actual exposure concentrations and apparent toxicity, particularly at
318 low exposure levels. Hence, relying solely on the conventional nominal concentrations does not accurately
319 represent toxicity. Therefore, to gain reliable data in aquatic toxicity, accessible analytical methods for
320 measuring exposure concentrations are essential. Moreover, long-chain cationic surfactants may have the
321 problem of complete dissolution into the free state, which complicates the interpretation of toxicity test
322 results even when the total aqueous concentration is measured. The presence of solvent (i.e., methanol) in
323 the spiking test might increase the tendency of hydrophobic cationic surfactants to micellize in exposure
324 medium, thereby lowering the bioavailable concentration.

325 A drawback of the PES membrane as a passive dosing phase may be the strong nonlinear sorption
326 of C14–18-BACs (Figure S9, Freundlich exponent: 0.33–0.82) observed over the fully investigated
327 concentration range in the acute toxicity test. Strong nonlinear sorption means that a small change in the
328 concentration in PES leads to a large change in the equilibrium aqueous phase concentration, which makes
329 it difficult to achieve the aimed aqueous phase concentrations for toxicity tests. Therefore, the search for
330 even better materials for passive dosing methods in ecotoxicity testing of cationic surfactants is worthy of
331 further research.

332 The results of the passive dosing tests indicated that the acute EC_{50} of long-chain BACs to *D. magna*
333 was as low as single μg/L and that toxicity increased as the alkyl chain length increased, as opposed to what
334 was indicated by the conventional solvent spiking tests. Therefore, the lowered toxicity of long-chain
335 cationic surfactants may, in part, be due to experimental artifacts of solvent spiking tests and needs further
336 investigation. Furthermore, while this study implemented the newly developed passive dosing method to
337 the acute toxicity test on *D. magna*, this approach may be extended to other aquatic organisms in both acute
338 and chronic toxicity tests of BACs or other cationic surfactants under various exposure conditions (e.g.,
339 suspension with sediment, organic matter, food), which should provide useful information for a
340 comprehensive risk assessment of cationic surfactants in aquatic ecosystems.

ASSOCIATED CONTENT

Supporting Information. Additional information of chemicals, materials, experimental details and methods for instrument analysis and detailed experimental data are provided in the Supporting Information.

AUTHOR INFORMATION

Corresponding Author

- Anh T. Ngoc Do – Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan orcid.org/0000-0001-9650-1579

Authors

- Kyoshiro Hiki – Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan orcid.org/0000-0001-7898-9486
- Haruna Watanabe – Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan orcid.org/0000-0001-5025-1957
- Hiroshi Yamamoto – Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan orcid.org/0000-0003-3865-8708
- Satoshi Endo – Health and Environmental Risk Division, National Institute for Environmental Studies (NIES), Onogawa 16-2, 305-8506 Tsukuba, Ibaraki, Japan orcid.org/0000-0001-8702-1602

Author Contributions

Conceptualization: S.E., K.H., ATN.D. Methodology: S.E., K.H., ATN.D. Visualization: ATN.D., H.K., Investigation: ATN.D., H.K., Formal analysis: ATN.D, S.E., K.H. Validation: ATN.D, S.E., K.H. Writing

– original draft: ATN.D. Writing – review & editing: H.Y, H.W., S.E., K.H. Project administration: H.Y, S.E., K.H.

Notes The authors declare no competing financial interest.

ACKNOWLEDGMENT

The authors would like to thank Sakura Yoshii for her help in LC-MS/MS measurement, Yoko Katakura for her technical assistance, and Ryoko Abe and Mie Suzuki for their help in culturing the test organisms. This study was supported by NIES Research Funding (Type B).

REFERENCES

(1) Arnold, W. A.; Blum, A.; Branyan, J.; Bruton, T. A.; Carignan, C. C.; Cortopassi, G.; Datta, S.; DeWitt, J.; Doherty, A.-C.; Halden, R. U. Quaternary Ammonium Compounds: A Chemical Class of Emerging Concern. *Environ. Sci. Tech.***2023**. [DOI]

(2) Barber, O. W.; Hartmann, E. M. Benzalkonium chloride: A systematic review of its environmental entry through wastewater treatment, potential impact, and mitigation strategies. *Crit. Rev. Environ. Sci. Technol.* **2022**, *52* (15), 2691-2719. [DOI]

(3) USEPA. *Reregistration eligibility decision for alkyl dimethyl benzyl ammonium chloride (ADBAC). EPA739-R-06-009.*;2006.[LINK]

(4) DeLeo, P. C.; Huynh, C.; Pattanayek, M.; Schmid, K. C.; Pechacek, N. Assessment of ecological hazards and environmental fate of disinfectant quaternary ammonium compounds. *Ecotoxicol. Environ. Saf.* **2020**, *206*, 111116. [DOI]

(5) Merchel Piovesan Pereira, B.; Tagkopoulos, I. Benzalkonium chlorides: uses, regulatory status, and microbial resistance. *Appl. Environ. Microbiol.* **2019**, *85* (13), e00377-00319.[DOI]

(6) Mohapatra, S.; Yutao, L.; Goh, S. G.; Ng, C.; Luhua, Y.; Tran, N. H.; Gin, K. Y.-H. Quaternary ammonium compounds of emerging concern: Classification, occurrence, fate, toxicity and antimicrobial resistance. *J. Hazard. Mater.* **2023**, *445*, 130393. [DOI]

(7) Clara, M.; Scharf, S.; Scheffknecht, C.; Gans, O. Occurrence of selected surfactants in untreated and treated sewage. *Water Res.* **2007**, *41* (19), 4339-4348. [DOI]

(8) Chen, Y.; Geurts, M.; Sjollema, S. B.; Kramer, N. I.; Hermens, J. L. M.; Droge, S. T. J. Acute toxicity of the cationic surfactant C12-benzalkonium in different bioassays: How test design affects bioavailability and effect concentrations. *Environ. Toxicol. Chem.* **2014**, *33* (3), 606-615. [DOI]

(9) Ding, W.-H.; Liao, Y.-H. Determination of alkylbenzyldimethylammonium chlorides in river water and sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *Anal. Chem.* **2001**, *73* (1), 36-40. [DOI]

(10) Ferrer, I.; Furlong, E. T. Identification of alkyl dimethylbenzylammonium surfactants in water samples by solid-phase extraction followed by ion trap LC/MS and LC/MS/MS. *Environ. Sci. Tech.* **2001**, *35* (12), 2583-2588. [DOI]

(11) Merino, F.; Rubio, S.; Pérez-Bendito, D. Solid-phase extraction of amphiphiles based on mixed hemimicelle/admicelle formation: Application to the concentration of benzalkonium surfactants in sewage and river water. *Anal. Chem.* **2003**, *75* (24), 6799-6806. [DOI]

(12) Olkowska, E.; Polkowska, Ż.; Namieśnik, J. A solid phase extraction–ion chromatography with conductivity detection procedure for determining cationic surfactants in surface water samples. *Talanta* **2013**, *116*, 210-216. [DOI]

(13) Zhang, C.; Cui, F.; Zeng, G.-m.; Jiang, M.; Yang, Z.-z.; Yu, Z.-g.; Zhu, M.-y.; Shen, L.-q. Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. *Sci. Total Environ.* **2015**, *518*, 352-362. [DOI]

(14) Antunes, S.; Nunes, B.; Rodrigues, S.; Nunes, R.; Fernandes, J.; Correia, A. Effects of chronic exposure to benzalkonium chloride in Oncorhynchus mykiss: cholinergic neurotoxicity, oxidative stress, peroxidative damage and genotoxicity. *Environ. Toxicol. Pharmacol.* **2016**, *45*, 115-122. [DOI]

(15) Kim, S.; Ji, K.; Shin, H.; Park, S.; Kho, Y.; Park, K.; Kim, K.; Choi, K. Occurrences of benzalkonium chloride in streams near a pharmaceutical manufacturing complex in Korea and associated ecological risk. *Chemosphere* **2020**, *256*, 127084. [DOI]

(16) Sánchez-Fortún, S.; Llorente, M.; Castano, A. Genotoxic effects of selected biocides on RTG-2 fish cells by means of a modified Fast Micromethod Assay. *Aquat. Toxicol.* **2005**, *73* (1), 55-64. [DOI]

(17) Garcıa, M.; Ribosa, I.; Guindulain, T.; Sanchez-Leal, J.; Vives-Rego, J. Fate and effect of monoalkyl quaternary ammonium surfactants in the aquatic environment. *Environ. Pollut.* **2001**, *111* (1), 169-175. [DOI]

(18) Lavorgna, M.; Russo, C.; D'Abrosca, B.; Parrella, A.; Isidori, M. Toxicity and genotoxicity of the quaternary ammonium compound benzalkonium chloride (BAC) using Daphnia magna and Ceriodaphnia dubia as model systems. *Environ. Pollut.* **2016**, *210*, 34-39. [DOI]

(19) Li, B.; Li, H.; Pang, X.; Cui, K.; Lin, J.; Liu, F.; Mu, W. Quaternary ammonium cationic surfactants increase bioactivity of indoxacarb on pests and toxicological risk to Daphnia magna. *Ecotoxicol. Environ. Saf.* **2018**, *149*, 190-196. [DOI]

(20) Ndabambi, M.; Kwon, J.-H. Application of an algal growth inhibition assay to determine distribution coefficients of benzalkonium ions between kaolinite and water. *Ecotoxicol. Environ. Saf.* **2019**, *174*, 491-497. [DOI]

(21) Zhu, M.; Ge, F.; Zhu, R.; Wang, X.; Zheng, X. A DFT-based QSAR study of the toxicity of quaternary ammonium compounds on Chlorella vulgaris. *Chemosphere* **2010**, *80* (1), 46-52. [DOI]

(22) Qian, Y.; He, Y.; Li, H.; Yi, M.; Zhang, L.; Zhang, L.; Liu, L.; Lu, Z. Benzalkonium chlorides (C12) inhibits growth but motivates microcystins release of Microcystis aeruginosa revealed by morphological, physiological, and iTRAQ investigation. *Environ. Pollut.* **2022**, *292*, 118305. [DOI]

(23) Sütterlin, H.; Alexy, R.; Kümmerer, K. The toxicity of the quaternary ammonium compound benzalkonium chloride alone and in mixtures with other anionic compounds to bacteria in test systems with Vibrio fischeri and Pseudomonas putida. *Ecotoxicol. Environ. Saf.* **2008**, *71* (2), 498-505. [DOI]

(24) Smith, K. E.; Schäfer, S. Defining and controlling exposure during in vitro toxicity testing and the potential of passive dosing. *In vitro Environmental Toxicology-Concepts, Application and Assessment* **2017**, 263-292. [DOI]

(25) OECD. Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals. *OECD Environ. Health Saf. Publ.* **2019**, *23*, 3-81. [DOI]

(26) Groothuis, F. A.; Timmer, N.; Opsahl, E.; Nicol, B.; Droge, S. T.; Blaauboer, B. J.; Kramer, N. I. Influence of in vitro assay setup on the apparent cytotoxic potency of benzalkonium chlorides. *Chem. Res. Toxicol.* **2019**, *32* (6), 1103-1114. [DOI]

(27) Brown, R. S.; Akhtar, P.; Åkerman, J.; Hampel, L.; Kozin, I. S.; Villerius, L. A.; Klamer, H. J. Partition controlled delivery of hydrophobic substances in toxicity tests using poly (dimethylsiloxane)(PDMS) films. *Environ. Sci. Tech.* **2001**, *35* (20), 4097-4102. [DOI]

(28) Fischer, F. C.; Hiki, K.; Endo, S. Free versus Bound Concentration: Passive Dosing from Polymer Meshes Elucidates Drivers of Toxicity in Aquatic Tests with Benthic Invertebrates. *Environ. Toxicol. Chem.* **2022**. [DOI]

(29) Smith, K. E.; Jeong, Y.; Kim, J. Passive dosing versus solvent spiking for controlling and maintaining hydrophobic organic compound exposure in the Microtox® assay. *Chemosphere* **2015**, *139*, 174-180. [DOI]

(30) Fischer, F.; Böhm, L.; Höss, S.; Möhlenkamp, C.; Claus, E.; Düring, R.-A.; Schäfer, S. Passive dosing in chronic toxicity tests with the nematode Caenorhabditis elegans. *Environ. Sci. Tech.* **2016**, *50* (17), 9708-9716. [DOI]

(31) Kwon, J.-H.; Wuethrich, T.; Mayer, P.; Escher, B. I. Development of a dynamic delivery method for in vitro bioassays. *Chemosphere* **2009**, *76* (1), 83-90. [DOI]

(32) Mayer, P.; Wernsing, J.; Tolls, J.; de Maagd, P. G.-J.; Sijm, D. T. Establishing and controlling dissolved concentrations of hydrophobic organics by partitioning from a solid phase. *Environ. Sci. Tech.* **1999**, *33* (13), 2284-2290. [DOI]

(33) Smith, K. E.; Heringa, M. B.; Uytewaal, M.; Mayer, P. The dosing determines mutagenicity of hydrophobic compounds in the Ames II assay with metabolic transformation: Passive dosing versus solvent spiking. *MRGTEM* **2013**, *750* (1-2), 12-18. [DOI]

(34) Kramer, N. I.; Busser, F. J.; Oosterwijk, M. T.; Schirmer, K.; Escher, B. I.; Hermens, J. L. Development of a partition-controlled dosing system for cell assays. *Chem. Res. Toxicol.* **2010**, *23* (11), 1806-1814. [DOI]

(35) Butler, J. D.; Parkerton, T. F.; Letinski, D. J.; Bragin, G. E.; Lampi, M. A.; Cooper, K. R. A novel passive dosing system for determining the toxicity of phenanthrene to early life stages of zebrafish. *Sci. Total Environ.* **2013**, *463*, 952-958. [DOI]

(36) Kwon, H.-a.; Jeong, Y.; Jeon, H. P.; Kim, S. Comparing passive dosing and solvent spiking methods to determine the acute toxic effect of pentachlorophenol on Daphnia magna. *Ecotoxicology* **2020**, *29*, 286-294. [DOI]

(37) Kwon, H.-C.; Roh, J.-Y.; Lim, D.; Choi, J.; Kwon, J.-H. Maintaining the constant exposure condition for an acute Caenorhabditis elegans mortality test using passive dosing. *Environ. Health. Toxicol.* **2011**, *26*. [DOI]

(38) Vergauwen, L.; Schmidt, S. N.; Stinckens, E.; Maho, W.; Blust, R.; Mayer, P.; Covaci, A.; Knapen, D. A high throughput passive dosing format for the Fish Embryo Acute Toxicity test. *Chemosphere* **2015**, *139*, 9-17. [DOI]

(39) Sigmund, G.; Arp, H. P. H.; Aumeier, B. M.; Bucheli, T. D.; Chefetz, B.; Chen, W.; Droge, S. T.; Endo, S.; Escher, B. I.; Hale, S. E. Sorption and mobility of charged organic compounds: how to confront and overcome limitations in their assessment. *Environ. Sci. Tech.* **2022**, *56* (8), 4702-4710. [DOI]

(40) Yoshii, S.; Hiki, K.; Watanabe, H.; Yamamoto, H.; Endo, S. Freely dissolved concentration profile and Hyalella azteca toxicity of cationic surfactant C12-benzalkonium in spiked-sediment toxicity test. *Sci. Total Environ.* **2023**, *866*, 161226. [DOI]

(41) OECD. *Test No. 202: Daphnia sp. Acute Immobilisation Test*; 2004. [DOI]

(42) Ritz, C.; Baty, F.; Streibig, J. C.; Gerhard, D. Dose-response analysis using R. *PloS one* **2015**, *10* (12), e0146021. [DOI]

(43) Timmer, N.; Scherpenisse, P.; Hermens, J. L.; Droge, S. T. Evaluating solid phase (micro-) extraction tools to analyze freely ionizable and permanently charged cationic surfactants. *Anal. Chim. Acta* **2018**, *1002*, 26-38. [DOI]

(44) Leal, J. S.; Gonzalez, J.; Kaiser, K.; Palabrica, V.; Comelles, F.; Garcia, M. On the toxicity and biodegradation of cationic surfactants Über die toxizität und den biologischen abbau kationischer tenside. *Acta Hydrochim. Hydrobiol.* **1994**, *22* (1), 13-18. [DOI]

(45) Tomlinson, E.; Brown, M.; Davis, S. Effect of colloidal association on the measured activity of alkylbenzyldimethylammonium chlorides against Pseudomonas aeruginosa. *J. Med. Chem.* **1977**, *20* (10), 1277-1282. [DOI]

(46) Lee, J.; Braun, G.; Henneberger, L.; König, M.; Schlichting, R.; Scholz, S.; Escher, B. I. Critical membrane concentration and mass-balance model to identify baseline cytotoxicity of hydrophobic and ionizable organic chemicals in mammalian cell lines. *Chem. Res. Toxicol.* **2021**, *34* (9), 2100-2109. [DOI]

(47) Balgavý, P.; Devínsky, F. Cut-off effects in biological activities of surfactants. *Adv. Colloid Interface Sci.* **1996**, *66*, 23-63. [DOI]

(48) Sandbacka, M.; Christianson, I.; Isomaa, B. The acute toxicity of surfactants on fish cells, Daphnia magna and fish—a comparative study. *Toxicol. In Vitro* **2000**, *14* (1), 61-68. [DOI]

(49) van Wijk, D.; Gyimesi-van den Bos, M.; Garttener-Arends, I.; Geurts, M.; Kamstra, J.; Thomas, P. Bioavailability and detoxification of cationics: I. Algal toxicity of alkyltrimethyl ammonium salts in the presence of suspended sediment and humic acid. *Chemosphere* **2009**, *75* (3), 303-309. [DOI]

(50) Ventura, S. P.; Marques, C. S.; Rosatella, A. A.; Afonso, C. A.; Goncalves, F.; Coutinho, J. A. Toxicity assessment of various ionic liquid families towards Vibrio fischeri marine bacteria. *Ecotoxicol. Environ. Saf.* **2012**, *76*, 162-168. [DOI]

(51) Kusumahastuti, D. K.; Sihtmäe, M.; Kapitanov, I. V.; Karpichev, Y.; Gathergood, N.; Kahru, A. Toxicity profiling of 24 l-phenylalanine derived ionic liquids based on pyridinium, imidazolium and cholinium cations and varying alkyl chains using rapid screening Vibrio fischeri bioassay. *Ecotoxicol. Environ. Saf.* **2019**, *172*, 556-565. [DOI]

(52) Megaw, J.; Thompson, T. P.; Lafferty, R. A.; Gilmore, B. F. Galleria mellonella as a novel in vivo model for assessment of the toxicity of 1-alkyl-3-methylimidazolium chloride ionic liquids. *Chemosphere* **2015**, *139*, 197-201. [DOI]

(53) Jono, K.; Takayama, T.; KUNo, M.; Higashide, E. Effect of alkyl chain length of benzalkonium chloride on the bactericidal activity and binding to organic materials. *Chem. Pharm. Bull.* **1986**, *34* (10), 4215-4224. [DOI]

(54) Garcia, M.; Campos, E.; Sanchez-Leal, J.; Ribosa, I. Effect of the alkyl chain length on the anaerobic biodegradability and toxicity of quaternary ammonium based surfactants. *Chemosphere* **1999**, *38* (15), 3473-3483. [DOI]