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Original Article

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Replacement per- and polyfluoroalkyl substance (PFAS)-free aqueous film-forming foams impact growth more than a PFAS-containing product in the hard clam, *Mercenaria mercenaria*

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Abstract

Aqueous film-forming foams (AFFFs) are widely used fire suppression products that have been identified as a direct source of environmental per- and polyfluoroalkyl substances (PFAS). Per- and polyfluoroalkyl substance exposure has demonstrated chronic and sublethal effects on biota. Ongoing efforts aim to reduce and, ideally, eliminate PFAS use in AFFF products. However, there is little known about the potential toxic effects of the new PFAS-free AFFFs, specifically on benthic organisms. The objective of this study is to quantify the effects of seven AFFFs on growth in the hard clam, *Mercenaria mercenaria*, over a 21-day exposure period with juvenile animals. Additionally, AFFF effects are reported from algal toxicity assays and a feeding study. Five of the PFAS-free AFFFs negatively impacted growth over the exposure period, while one PFAS-free AFFF and the reference PFAS-containing AFFF had no observable effect. Median effect concentrations (EC50) for shell growth ranged from 5.81 mg/L to >100 mg/L. Clam dry and wet weights also decreased with increasing exposure concentration ($p < 0.05$). Algal growth was impacted over a 96-hr exposure. Impacts were observed to final standing biomass and overall growth rates at the highest exposure concentrations. However, complete lethality was only observed for one PFAS-free product, suggesting lack of food availability was likely not the primary driver of growth inhibition for all products. Net particle clearance rates in AFFF-exposed clams were not found to be impacted, suggesting there was no obvious AFFF influence on organismal feeding ability. The presented results identify chronic effects of exposure to these AFFFs in this economically and ecologically important bivalve species and are expected to inform decisions regarding PFAS replacement AFFF products.

Keywords: aqueous film-forming foam, per- and polyfluoroalkyl substances alternatives, estuarine toxicology, chronic exposure

Introduction

Per- and polyfluoroalkyl substances (PFAS) are a large class of ubiquitous environmental contaminants that have raised concerns for environmental and human health. These compounds generally contain a fluorinated C_nF_{2n+1} moiety and are used in multiple industries for their desirable chemical properties, including hydro- and lipophobicity (Buck et al., 2011; Smart, 1994). The strong carbon-fluorine bonds result in compounds that do not readily degrade, leading them to be termed "forever chemicals" (Wang et al., 2017). These characteristics and unique chemical behaviors have led to a large number of compounds being synthesized, with definitions and classification schemes differing between regulatory bodies (U.S. Environmental Protection Agency, 2022). Since their discovery, PFAS have become pervasive contaminants and these compounds are now measured in essentially every global ecosystem (Cousins et al., 2022; Giesy & Kannan, 2001).

The use of traditional aqueous film-forming foams (AFFFs) has been identified as a significant driver of environmental PFAS distribution globally (Moody & Field, 2000; Prevedouros et al., 2006). These products are utilized as Class B fire suppressants to mitigate liquid-based fires, such as oil or fuel fires, and exploit PFAS as ingredients for their heat resistance and film formation properties (Leeson et al., 2021). Aqueous film-forming foams are heavily utilized by the aviation and defense industries and, as such, military bases have been noted as hot spots for PFAS contamination (Prevedouros et al., 2006).

Traditional PFAS-containing AFFFs used by the United States military are governed by specifications detailed in the "MILSPEC" document, MIL-PRF-24385 (Naval Sea Systems Command, 2020). These products are primarily composed of surfactants (both hydrocarbon and fluorosurfactant) and a solvent (Moody & Field, 2000). Initially, AFFFs included longer chain PFAS but as adverse health effects associated with long chain PFAS became more

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apparent, industry substituted shorter chain PFAS as a safety precaution (Brendel et al., 2018). As the persistence and potential toxicity of short-chain PFAS became clearer, a need for a PFAS-free AFFF (also termed F3, FF-AFFF, or PFF) replacement was identified and deemed attainable (Ateia et al., 2019; Brendel et al., 2018; Cousins et al., 2019; Houtz et al., 2013). These new PFAS-free AFFFs must also satisfy performance and safety guidance for fire suppression and environmental risk found in the updated "MILSPEC" document, MIL-PRF-32725 (Naval Sea Systems Command, 2023). As such, the target is for PFAS-Free AFFF products to be overall less hazardous to both human and environmental health than traditional AFFFs while still meeting the fire-suppression performance criteria.

Initially, there was a noted lack of toxicity data associated with PFAS-Free AFFF products, and the Strategic Environmental Research and Development Program (SERDP) funded a cohort of projects to study the toxicity and chemistry of six of these PFAS-free products (SERDP, 2020). To date, these studies have documented biodegradation, human health hazard, and acute and chronic toxicity to many species representing multiple ecosystems (East et al., 2023; Fuller et al., 2024; Gharehveran et al., 2022; Holden et al., 2023; Jones et al., 2022). These products are generally a mixture of five classes of compounds (i.e., carbohydrates and four classes of surfactant, each with different headgroup charges) and have been documented to share a number of individual constituent compounds (Gharehveran et al., 2022; Holden et al., 2023). The precise chemical composition of each of these products is considered a trade secret and is not discussed, however, general product information has been included (see online supplementary material, Supplemental 1A).

The goal of this study is to expand scientific knowledge of the effects of PFAS-free AFFFs on growth at chronic, sublethal concentrations on the hard clam, *Mercenaria mercenaria*. This bivalve is ecologically and economically important, with $46 million USD in reported landings in 2022 (National Oceanic and Atmospheric Administration, 2023). There is little-to-no literature documentation of AFFF toxicity in this organism but toxicity has been measured in other marine species, including vertebrate, invertebrate, and algal species (Fuller et al., 2024; Jones et al., 2022). The impacts of surfactant exposure in general have been reported in other bivalves, with impacts including decreases in feeding ability, immunosuppression, and alterations to oxidative stress mechanisms. However, most of this research has focused on exposure to Sodium Lauryl Sulfate, an ingredient used in many personal care products (Freitas et al., 2020; Ostroumov & Widdows, 2006; Paciello et al., 2023).

Exposure to AFFF products could conceivably have any of these direct impacts in *M. mercenaria*, as well as indirect effects due to AFFF toxicity to their algal food source. In fact, there have been documented incidents of AFFFs entering the coastal environments both intentionally and unintentionally during fire-prevention events and spills, respectively (Katz et al., 2022; Miranda et al., 2024). As industries begin to transition to the usage of PFAS-free AFFFs, it is highly likely that estuarine organisms will be exposed to either whole products or individual components as they migrate within the environment. Both direct and indirect exposure-related effects on *M. mercenaria* could lead to chronic alterations to growth, and by extension, declines in population health. Characterizing how exposure to replacement AFFFs could affect this ecologically important species will help to increase understanding of the environmental hazards of these products. This study serves to document the impacts of exposure to PFAS-free AFFFs on clam growth, algal population growth, and clam feeding ability.

Materials and methods

AFFFs

Seven AFFFs were received from the SERDP for testing at the Hollings Marine Laboratory in Charleston, South Carolina, United States. Six of the seven chosen by SERDP for this project were PFAS-free AFFFs, some of which were commercially available for purchase at the time of testing but were not approved for use by the U.S. Department of Defense. All PFAS-free products have been stripped of identifiable naming at the request of SERDP to avoid association with any current commercial product or products approved for use under the "MILSPEC." As such, the PFAS-free AFFFs will be discussed here as AF1, AF2, AF3, AF4, AF5, and AF6 along with a corresponding "Reference" PFAS-containing AFFF, Buckeye Platinum 3% AFFF (referred to here as Buckeye). These products are consistent with other studies on this subject despite changes in naming convention (e.g., East et al., 2023; Fuller et al., 2024; Gharehveran et al., 2022; Holden et al., 2023; Jones et al., 2022; Leeson et al., 2021).

Each of these PFAS-free products share several individual constituents and can be categorized roughly based on their primary surfactant type. AF5 is the lone product containing siloxane surfactants, while the remaining five products are based on hydrocarbon surfactant mixtures (see online supplementary material, Supplemental 1A). Analytical information on whole, undiluted PFAS-free concentrates received from SERDP are available in prior studies with these products (Gharehveran et al., 2022; Jones et al., 2022). The Reference AFFF, Buckeye, contains three PFAS, a 6:2 fluorotelomer sulfonate (6:2 FTS) zwitterion ($C_{16}H_{23}F_{13}N_2O_6S_2$), 6:2 FTS, and perfluorohexanoic acid as discussed in Jones et al. (2022). This product has been widely used for fire suppression and is well accepted to meet the PFAS-containing AFFF MILSPEC, and as such serves as an ideal positive control "Reference" formulation.

Full quantitative chemical concentrations and product breakdowns of the PFAS-free AFFFs are not discussed in the present study due to the proprietary nature of these products, however, information disclosed in the product's Safety Data Sheets provided by SERDP is summarized in Supplemental 1A (see online supplementary material). Additionally, due to the inherent difficulty in confirming exposure concentrations for these complex proprietary mixtures, all exposure concentrations are reported here on a nominal basis. However, the present study's testing methodology including stock preparation, dilution, and exposure concentration ranges were performed as in previous studies on these products using marine organisms (Fuller et al., 2024; Jones et al., 2022).

Manufacturers typically recommend that these products are generated and stored as pre-prepared concentrates that are then diluted as directed typically to 3% or 6% prior to use in fire control. As such, working stocks were prepared gravimetrically for each product at a 3% (30,000 mg/L) dilution of the supplied concentrate by dissolving them in deionized water, mimicking each manufacturer's directions. As necessary, secondary stocks (e.g., dilution to 300 mg/L) were created gravimetrically. All exposure media were made by volumetric dilutions of each 30,000 or 300 mg/L working stock. Preliminary nontarget analysis on the stability of these stocks indicated six of the tested products were stable in deionized water for up to 14 days, whereas AF5 was less stable (up to 2 days; Wirth et al. in press). Stocks were remade as

needed such that all treatment exposure media were made from a less than 14-day-old stock, or in the case of AF5, less than 2 days old, thus not exceeding the stability limit for each product.

Experimental design—growth assay

All clam growth methods were based on those documented by Chung et al. (2007). Hard clams (*Mercenaria mercenaria*; 1–2 mm dorsal-ventral length) were received from Bay Shellfish, Inc. (Terra Ceia Island, FL, United States) in three batches in November 2023, January 2024, and February 2024. Upon receipt, clams were sieved and those retained on a 1-mm sieve (ASTM E-11 No. 18) were used for testing. Clams were held for a 4-day acclimation period prior to exposure and were fed cultured *Isochrysis galbana* daily (AlgaGen, Vero Beach, FL, United States). All clams used for testing were 1 to 2 mm in Posterior-Anterior shell axis length (termed here, shell length).

Individuals were exposed to a range of concentrations chosen based on initial range-finding tests (see online supplementary material, Supplemental 1B) and a no-treatment control for each formulation in 475-mL glass jars containing 180 mL of exposure media. Each exposure concentration and the controls included five replicates with 30 clams each (Figure 1A). Exposures took place in controlled environmental chambers (Percival Scientific IntellusUltra C8) with a 16:8-hr light: dark photoperiod under standard fluorescent lighting. All replicates were gently aerated taking care not to induce foaming and fed 5 mL ($5–6 \times 10^6$ cells/mL) of cultured *I. galbana* per day. All tests were run static with daily 100% renewal of the test solution for 21 days. Water quality was confirmed by daily measurements from one replicate per exposure concentration for temperature (23.9°C ± 0.0224; 21 day mean ± SE), salinity (21.5 ppt ± 0.0342), dissolved oxygen (7.30 mg/L ± 0.103), and pH (7.87 ± 0.00351) using a YSI ProQuatro Multiparameter Meter (Xylem, Inc.). Clams ($n = 5$ reps, 30 individuals each) were also retained at the start of each exposure for baseline size and weight measurements.

Each day, organisms were removed from exposure containers and placed in 60-mm Petri plates (Falcon 351007). All organisms were observed under a dissection microscope and those that did not exhibit a locomotory response within 2 min when exposed to bright white light were deemed dead and were removed from further testing (Chung et al., 2007). On test Days 7 and 14, one replicate per treatment of each product was randomly selected after mortality assessment and individuals were imaged for organismal size measurements and then returned to the exposure chamber. The exposures ended on Day 21 when all clams were removed from jars and observed for mortality. Surviving individuals from each replicate of a treatment were pooled into aluminum weight boats and the wet weight was obtained on a 6-point balance (Sartorius ME36S). Clams were then dried at 70°C for 24 hr after which a dry weight was obtained. The dried clams were then gently transferred to 60-mm Petri plates and imaged under a dissecting scope for size analysis (Olympus ZSH10; Olympus DP73). Measurements of clam Posterior-Anterior (shell length) and Dorsal-Ventral (shell width) shell axes were obtained using FIJI (Schindelin et al., 2012). The lengths for both axes and both dry and wet weights were then pooled at a replicate level for statistical analysis.

All statistical analyses estimating exposure impacts were performed using the R statistical language version 4.3.0 (R Core Team, 2021). Data were visualized using the *ggplot2* package (Wickham, 2016). For products where growth estimates demonstrated a dose–response relationship over the tested exposure concentrations, four-parameter log-logistic models were fit to the final 21-day growth data using the *drc* package (Ritz, 2010; Ritz et al., 2015). In situations where a four-parameter model yielded a statistically significant negative lower asymptote estimate, a three-parameter log-logistic model with the lower

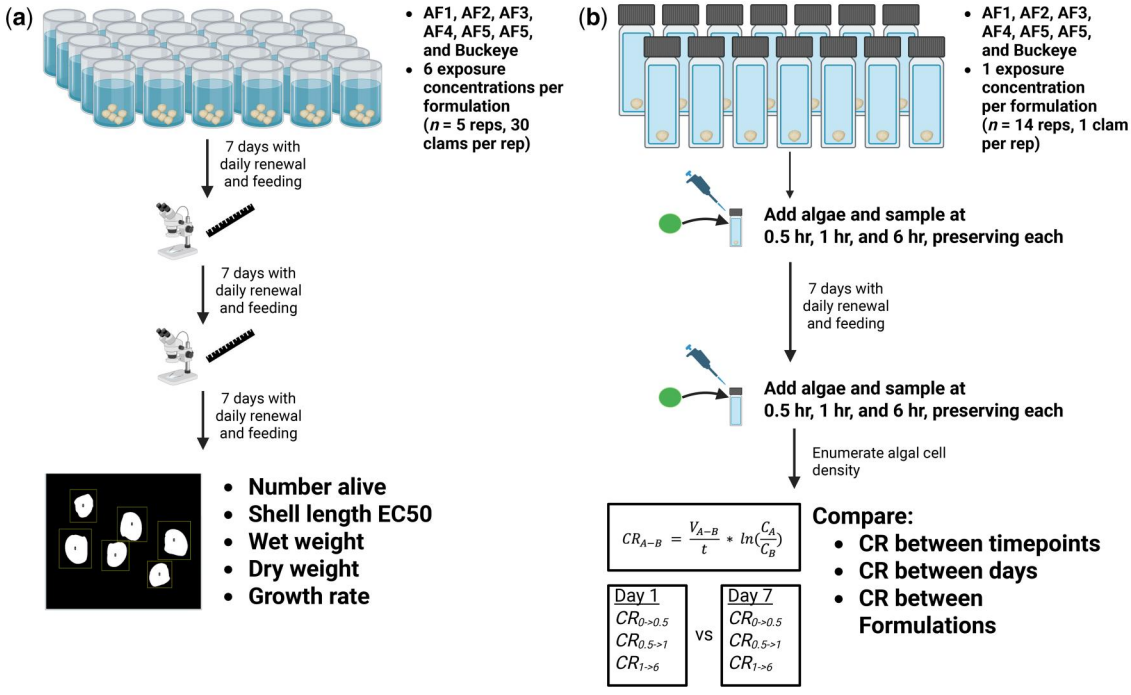

Figure 1. (A) Experimental diagram representing the clam growth assay. Clams were exposed to each product for 21 days statically with daily renewal and feeding. On Days 7 and 14, clams were imaged, and the shell posterior-anterior and dorsal-ventral axes were measured. On Day 21, clams were removed from jars, wet weight was measured, dried, dry weight determined and imaged for size analysis. (B) Experimental design investigating the impact of each product on clam feeding ability. Individual clams were exposed to each product, fed *Isochrysis galbana*, and the rate of algal clearance was measured 0.5 hr postfeeding, 1 hr postfeeding, and 6 hr postfeeding. EC50, median effect concentration; CR, clearance rate.

asymptote fixed at 0 was used. All effect concentrations, including median (EC50) as well as at a 10% and 90% response level (EC10 and EC90) and their respective 95% confidence intervals, were derived from the fitted –log-logistic models. No observable effect concentrations (NOECs) and lowest observable effect concentrations (LOECs) were determined using Dunnett's post hoc test.

Comparisons between AFFF growth dose–response models and thresholds were performed using relative potency (*r*), defined as:

$$r\ (x) = \frac{EC_{xA}}{EC_{xB}}$$

where EC_{xA} is the effective concentration at response level *x* for AFFF *A* (e.g., $EC50_A$) and EC_{xB} is the corresponding effective concentration for AFFF *B* (e.g., $EC50_B$; Ritz et al., 2006).

Average wet weight per clam and average dry weight per clam were compared among treatments and formulations via two-way analysis of variance (ANOVA). The NOEC and LOEC values were determined with Dunnett's post hoc test. Growth over the 21-day exposure period was found to be approximately linear, albeit highly variable. As such, for each product, growth rates per treatment were estimated and compared via hierarchical linear regression (see online supplementary material, Supplemental 1D).

Experimental design—algal toxicity assay

A monoculture of *I. galbana* was received from AlgaGen, LLC (Vero Beach, FL, United States) in September 2024. Initial density was determined via hemocytometer and a stock of 0.22 μm filtered Guillard's F/2 marine algal media (20 ppt salinity) was inoculated using axenic techniques. An algal monoculture was then maintained at log-phase growth on an orbital shaker (150 rpm) in an environmental chamber at 25°C under white light (16:8-hr light: dark photoperiod) with daily density enumeration and weekly transfers.

Algal toxicity testing followed ASTM E1218-21 modified for use with *I. galbana* using 20 ppt salinity F/2 marine algal media, autoclaved and sterile filtered (ASTM International, 2021). Exposure concentrations were chosen to exactly follow those used to evaluate AFFF impacts on clam growth (see online supplementary material, Supplemental 1B). On test start days, three replicate glass flat-bottomed algal culture tubes containing sterile filtered F/2 media and AFFF were inoculated with a volume of algal culture to yield 25 mL at an initial target density of 20,000 cells/mL for each targeted exposure concentration. Density was estimated daily over the testing duration using a Beckman-Coulter Multisizer 3 or by measuring absorbance at 680 nm (Agilent BioTek Epoch 2). Both methods were validated by direct hemocytometer counts at 0, 48, and 96 hr.

Threshold toxicity values were estimated for standing biomass and population growth rate using Log-Logistic Four Parameter models in the *drc* package in the R statistical language (Ritz et al., 2015). Threshold median inhibitory concentrations (IC50s) were estimated for both 48 and 96 hr standing biomass. The population growth rate over the test period was calculated according to Sorokin by fitting a linear regression to the algal density over the length of the test and multiplying the slope by the logarithm conversion factor, 3.32 (Sorokin, 1973).

Experimental design—feeding assay

A feeding study was designed to isolate the effects of AFFF exposure on clam net clearance rate (CR). Clams (1–2 mm) were received from Bay Shellfish and held for at least 4 days prior to exposure. Individual clams were placed in glass scintillation vials (Wheaton 986540) containing 20 mL of 0.22 μm sterile filtered exposure media for 7 days (Figure 1B). Treatments included one concentration from each of the seven AFFFs and a control ($n = 14$ replicates per treatment) with nonaerated conditions and daily renewal. Exposure concentrations were determined based on the 21-day EC50 for growth established in this study for each product. In situations where no EC50 was estimated, the NOEC for growth was determined via Dunnett's test and was used. Clams were fed an aliquot of cultured *I. galbana* daily to yield a target density of 20,000 cells/mL in each exposure chamber. Measurements of temperature (23.9°C ± 0.0614; 7 day mean ± SE), salinity (20.2 ppt ± 0.0507), dissolved oxygen (7.04 mg/L ± 0.355), and pH (7.82 ± 0.0227) were obtained daily by pooling replicates for each product and measuring with a YSI ProQuatro Multiparameter Meter.

On experimental Days 1 and 7, exposure media were renewed and *I. galbana* was added to each vial with a target density of 20,000 cells/mL in 20 mL exposure media; the water column was mixed via gentle pipetting, and a 1-mL aliquot was retained and preserved in 1% Lugol's iodine. Additional aliquots of the mixed exposure media were also obtained at 0.5 hr postrenewal, 1 hr postrenewal, and 6 hr postrenewal and each preserved in 1% Lugol's iodine. Each sample was then enumerated using a Beckman-Coulter Multisizer 3.

Individual CRs were then calculated between each sampling timepoints using the following formula (Coughlan, 1969; Rosa et al., 2020):

$$CR_{A-B} = \frac{V_{A-B}}{t} * \ln\left(\frac{C_A}{C_B}\right)$$

where CR_{A-B} is the net CR between sampling timepoints *A* and *B*, *t* is the duration of exposure and *V* is the volume of the experimental container between *A* and *B*, C_A is the particle concentration at timepoint *A*, and C_B is the particle concentration at timepoint *B*. Each CR was then compared via three-way repeated measures ANOVA comparing CR between each timepoint, the effects of individual AFFFs, and differences between sampling days.

Results

Clam growth assay

Across all exposure concentrations clam Posterior-Anterior (shell length) and Dorsal-Ventral (shell width) axes were found to be allometrically related and did not vary across treatments (see online supplementary material, Supplemental 1C). As such, all shell measurement data and comparisons are presented in terms of shell length for brevity. After 21 day exposure, no-treatment control shell length averaged 2.57 mm ± 0.036 (mean ± SE) across all tested products. Of the seven tested AFFFs, all but AF5 and Buckeye demonstrated negative impacts to shell length over the tested exposure concentrations (ANOVA, $p < 0.05$; Figure 2). Threshold EC50 values for shell length varied from 5.81 mg/L ± 2.13 (est. ± SE) for AF1, 36.4 mg/L ± 27.2 for AF2, 94.0 mg/L ± 13.7 for AF3, 13.0 ± 1.13 for AF4, 22.7 ± 3.03 for AF6, and >100 mg/L for Buckeye and AF5. The LOEC where growth was slowed varied between formulations from 1.563 mg/L for AF1 to >100 mg/L for Buckeye and AF5 (Table 1). Average clam growth rate was also impacted by exposure, decreasing from a maximum of 0.0424 mm/day ± 1.18×10^{-3} (mean ± SE) in control replicates to a minimum of −0.00413 mm/day ± 1.670×10^{-3} mm/day at 50 mg/L for AF4 (see online supplementary material, Supplemental 1D).

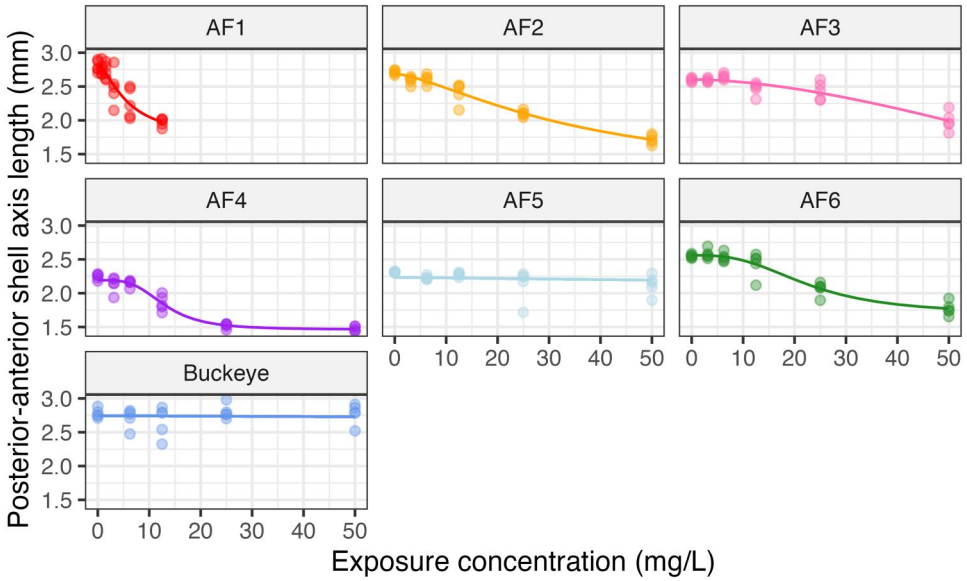

Figure 2. Posterior-anterior axis length (mm) of *Mercenaria mercenaria* hard clams after a 21-day exposure to aqueous film-forming foam products. Curves represent fitted log-logistic dose–response models.

Table 1. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for growth (measured in mm), wet weight (measured in mg), and dry weight (measured in mg) and the median effect concentration (EC50) for growth (± SE; measured in mm) in *Mercenaria mercenaria* following a 21-day exposure to aqueous film-forming foam products.

	Growth			Wet weight per clam		Dry weight per clam	
Formulation	**EC50 (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**
AF1	5.81 ± 2.13	1.563	3.125	1.563	3.125	1.563	3.125
AF2	36.4 ± 27.2	6.25	12.5	6.25	12.5	0	3.125
AF3	94.0 ± 13.7	12.5	25	25	50	25	50
AF4	13.0 ± 1.13	6.25	12.5	0	3.125	0	3.125
AF5	>100	100	ND	>100	ND	>100	ND
AF6	22.7 ± 3.03	12.5	25	12.5	25	6.25	12.5
Buckeye	>100	100	ND	100	ND	100	ND

Note. ND = value not determined.

Table 2. Relative potency estimates (mean and 95% confidence intervals) at three effect concentrations (r10, r50, and r90) comparing the toxicity of each aqueous film-forming foam (AFFF) product to growth in *Mercenaria mercenaria* over a 21-day exposure period.

AFFF A	**AFFF B**	**r10**	**r50**	**r90**
AF1	AF2	0.183 (0.0575, 0.309)	0.159 (–0.103, 0.422)	0.139 (–0.289, 0.566)
AF1	AF3	0.0502 (0.0147, 0.0857)	0.062 (–0.00425, 0.128)	0.0765 (–0.0955, 0.248)
AF1	AF4	0.206 (0.0639, 0.349)	0.448 (0.113, 0.783)	0.973 (–0.587, 2.53)
AF1	AF6	0.14 (0.0527, 0.225)	0.256 (0.0578, 0.453)	0.47 (–0.301, 1.24)
AF2	AF3	0.274 (0.151, 0.396)	0.384 (–0.0526, 0.821)	0.54 (–0.619, 1.7)
AF2	AF4	1.13 (0.333, 1.92)	2.81 (–1.37, 6.99)	7.00 (–12.3, 26.3)
AF2	AF6	0.759 (0.275, 1.24)	1.60 (–0.804, 4.01)	3.39 (–6.00, 12.8)
AF3	AF4	4.12 (2.32, 5.93)	7.26 (4.99, 9.52)	12.8 (1.68, 23.8)
AF3	AF6	2.77 (1.56, 3.98)	4.14 (2.59, 5.68)	6.18 (0.158, 12.2)
AF4	AF6	0.673 (0.241, 1.11)	0.571 (0.390, 0.751)	0.483 (0.0568, 0.91)

Shell malformations of a notch along anterior side of the ventral edge were noted for only some individuals in some replicates of the 50-mg/L exposure concentration of AF5 and as such, are not presently attributed to any exposure-related impact.

Relative potency for shell length at each effect concentration (i.e., r10, r50, r90) indicates that products vary in their toxicity to growth and these differences are consistent across most effective concentrations, indicating largely parallel shifts in dose-responses (Table 2). The lowest observed r50, indicating the largest difference in median growth inhibition, was between AF1 and AF3 at 0.062 ± 0.034 (estimate ± SE). At the lower r10, some products were more impactful to growth than others. Differences were observed at an r10 for comparisons between AF1/AF2, AF1/AF3, AF1/AF4, AF1/AF6, and AF2/AF3 with the remaining being nonsignificant. A similar pattern of differences is observed at an r50, with the addition of AF4/AF6. The upper r90 values are characterized by higher uncertainty in the underlying effective concentration values and do not significantly differ among products with the exception of AF1/AF2 and AF4/AF6.

Wet weight and dry weight decreased significantly with increasing exposure concentrations for AF1, AF2, AF3, AF4, and AF6 (ANOVA, $p < 0.05$). Across all seven no-treatment controls,

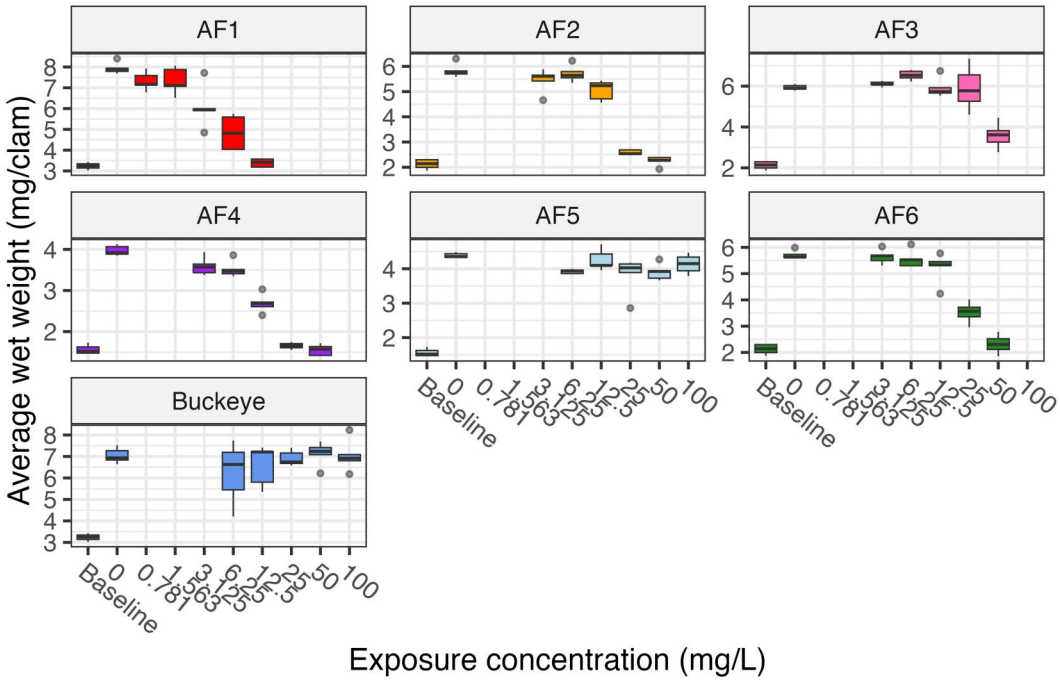

Figure 3. Average wet weight (mg/clam; ± SD) of *Mercenaria mercenaria* hard clams after a 21-day exposure to aqueous film-forming foam products.

wet weight averaged 5.83 mg ± 0.222 mg/clam (Figure 3) and dry weight averaged 2.84 mg ± 0.103 mg/clam. Exposure to Buckeye did not affect these endpoints ($p > 0.05$). The NOEC and LOEC values for weight differ from those observed for shell length for AF2 dry weight per clam, AF3 dry and wet weights, AF4 dry and wet weights, and AF5 dry and wet weights (Table 1). The AF5 had no dose-dependent effect, with only the 25-mg/L concentrations differing from the control (Dunnett's test, $p < 0.05$). Clams at the highest tested exposure concentration for AF1, AF2, AF4, and AF6 did not significantly differ in average wet weight from the baseline starting clams (two-tailed *t* test, $p > 0.05$, Figure 3). Exposure to the highest tested concentrations for AF1, AF2, AF4, and AF6 resulted in a lower average dry weight than recorded in the baseline clams (one-tailed *t* test, $p < 0.05$).

Effects on algal population growth

Each of the tested PFAS-free AFFFs impacted algal growth and survival over the 96-hr exposure period. Buckeye had no observed impact (Dunnett's test, $p > 0.05$). Final standing biomass IC50 values ranged from 0.951 mg/L (± 0.636; ± SE) for AF2 to 32.9 mg/L (± 5.55) for AF5 (Table 3). Algal growth rates consistently remained positive across exposure concentrations except for the highest exposure concentrations in AF1, AF2, AF3, AF4, and AF5 (Figure 4). Overall growth rate IC50s for the PFAS-free AFFFs ranged from 3.76 mg/L (± 0.357) for AF2 to 55.0 mg/L (± 30.4) for AF5. Highest exposure concentrations for AF1, AF2, and AF5 resulted in no observable algae after the initial inoculation. No cellular abnormalities were observed in any exposure concentration during microscope enumeration.

Effects on feeding ability

Overall net CRs were observed to be variable, with no significant effect of repeated measures within individuals or between measurement test days (likelihood ratio test, $p > 0.05$). Product AF2 was observed to be potentially algicidal and had rapid onset of toxicity, resulting in an artificially inflated CR. As such, no CR was calculated and AF2 is thus excluded from any further analysis for this endpoint. No statistical differences were observed between the control group and any of the formulations; however, the difference between AF1 and the control group approached the traditional cutoff for significance (Tukey's honest significant difference, $p = 0.06$; Figure 5). The interaction term between AF1, the Control group, and the earliest sampling timepoint was statistically significant ($p < 0.05$). Differences in CR were observed between AF1 and all AFFFs but AF6, with the interaction terms for the earliest sampling timepoint also being significant ($p < 0.05$).

Table 3. Threshold median inhibition concentrations (IC50; mean and 95% confidence intervals) for decreases in final standing biomass for *Isochrysis galbana* after 48 and 96 hr of exposure to aqueous film-forming foam formulations.

	Standing biomass		Growth rate
Formulation	IC50 48 hr (mg/L)	IC50 96 hr (mg/L)	IC50 (mg/L)
AF1	3.52 (1.92, 5.12)	3.59 (0.742, 6.43)	>12.5
AF2	2.07 (1.03, 3.11)	0.951 (–0.313, 2.21)	3.76 (3.00, 4.52)
AF3	5.43 (3.53, 7.34)	6.29 (4.76, 7.83)	44.8 (–35.0, 125)
AF4	17.4 (–9.97, 44.9)	18.2 (–10.3, 46.7)	>50
AF5	33.1 (26.6, 39.5)	32.9 (22.0, 43.7)	55.0 (–10.2, 120)
AF6	16.9 (11.2, 22.6)	12.8 (9.46, 16.2)	26.5 (20.0, 32.9)
Buckeye	>100	>100	>100

Discussion

Previously documented studies investigating these PFAS-free AFFFs have suggested that toxic thresholds generally fall within similar orders of magnitude for all products and that they had differing levels of toxicity from the reference foam, Buckeye (East et al., 2023; Fuller et al., 2024; Holden et al., 2023; Jones et al., 2022). This trend holds in the present study as growth of the hard clam was directly impacted by exposure to all but one of the tested PFAS-free AFFFs (AF5). The PFAS-free AFFFs have been previously shown to impact growth and weight in multiple

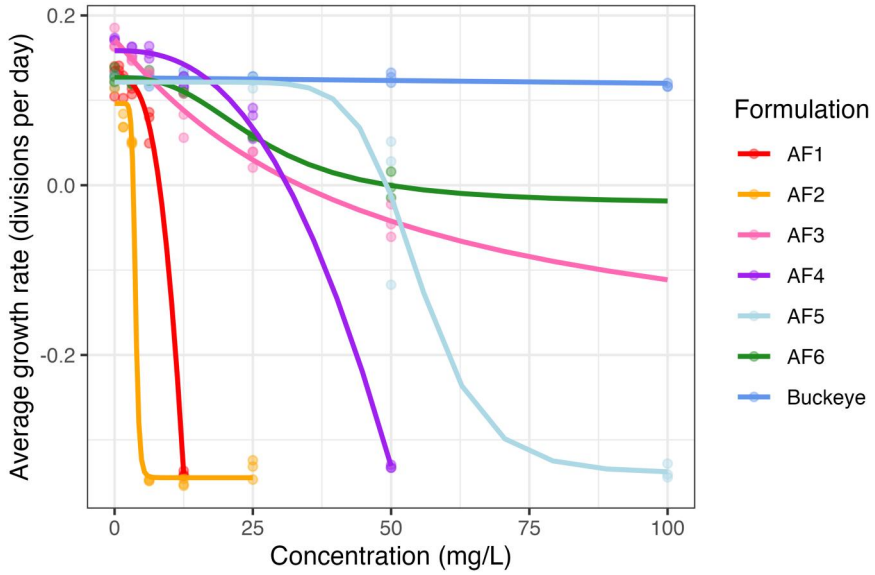

Figure 4. Average growth rate for *Isochrysis galbana* over 96 hr during exposure to aqueous film-forming foam products. Mean control growth rate was 3.16 division/day ± 0.653 (± SD). Growth rate calculated according to Sorokin (1973).

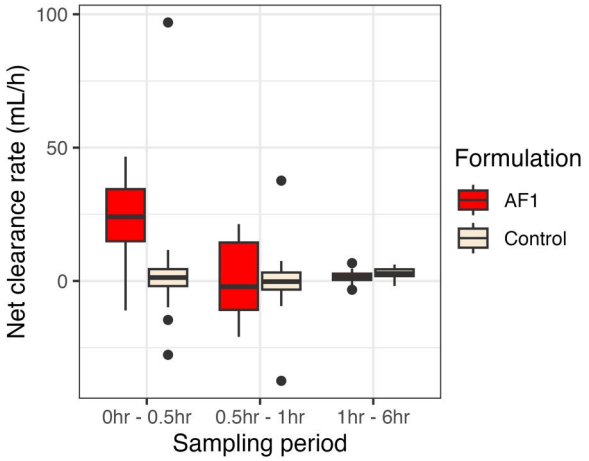

Figure 5. Clam net clearance rate (mL/hr) for one of the tested aqueous film-forming foam (AFFF) products over multiple sampling periods. No significant differences were observed between any of the tested AFFFs concentrations and their corresponding controls.

species spanning different taxa, with exposure being potentially stimulatory to growth in *Daphnia magna* for a number of the tested PFAS-free AFFFs (Fuller et al., 2024). Similar to these other studies with these products, Buckeye had no effect on the studied endpoints in *M. mercenaria* (Fuller et al., 2024; Jones et al., 2022).

Comparable to Buckeye, AF5 also did not appear to impact the endpoints in this study. These results set AF5, the only siloxane surfactant-containing product in this study, apart from the other PFAS-free AFFFs. This breaks with previously documented trends as AF5 has been shown to impact growth in other species and was even potentially stimulatory in some cases (Fuller et al., 2024; Jones et al., 2022). Product AF5 has also been suggested to be among the least hazardous of the tested PFAS-free AFFFs due to its relatively low aquatic toxicity, however, it did have higher oral toxicity than other products in mice (East et al., 2023; Holden et al., 2023). The present study supports this as little-to-no impact was documented for exposure to AF5. Siloxanes as a class of chemicals are known to readily hydrolyze at lower concentrations and at the pHs used in the present study (Ananth et al., 2020; Cypryk & Apeloig, 2002). While AF5 is not entirely comprised of siloxane surfactants, it is conceivable that the lack of toxicity observed here is attributable to the rapid degradation of an individual class of constituents, however, more experimentation is needed.

Of the remaining PFAS-free AFFFs, AF1 displayed the lowest growth EC50 (i.e., most potent to this endpoint) and the only potential impact to clam feeding ability, measured here using net CR. While statistically insignificant, the increase in CR could potentially be due to a "low dose stimulation" or hormesis phenomena, which has been documented in chronic endpoints for other species with this product (Fuller et al., 2024). However, it is difficult to make this determination with the given dataset as only one treatment concentration was used to examine feeding ability. Product AF1 has frequently been noted among other studies of these same products to be the most toxic PFAS-free AFFF (Fuller et al., 2024). This trend holds in this study and the relative potency of AF1's growth impacts compared to other products suggests that the magnitude of any differences in toxicity is consistent across the range of observed responses.

The similarities in relative potency between each formulation across all effect levels suggest that they are horizontally shifted parallel curves over most growth ECs (i.e., EC10, EC50, EC90). This is consistent with the observations that these products share some primary components at differing concentrations and fractions (Gharehveran et al., 2022). This trend of parallel responses, however, does not hold true for all formulations. At some lower effect levels, relative potency does differ (e.g., AF2/AF6) which suggests lower onset of toxicity for some products. Gharehveran et al. (2022) studied the biodegradation and chemical oxygen demand of each of these AFFF product at varying concentrations up to 28 days and noted different degradation profiles for each product. While the exposure concentrations used in this study are an order of magnitude lower than those used by Gharehveran et al., it is possible that differences in toxicity at lower effective concentrations (e.g., EC10) are due to changes in these complex mixtures over time. It is also possible that this phenomenon is attributable to an increase in uncertainty at higher effect concentrations (e.g., EC90) and that the responses are not truly parallel; however, it is also possible that

this is an inherent feature due to the variability of growth as an endpoint. Both organismal wet and dry weights followed the same general trend as shell length, decreasing monotonically with increasing exposure concentration, with the exception of AF5 and Buckeye which showed no clear dose–response impacts.

Clam growth was likely not inhibited by a lack of an available or degraded food source. The measured 96-hr *I. galbana* density IC50 values are generally observed to be higher than the measured 21-day EC50 clam growth values. These inhibition thresholds generally fall in the same order of magnitude or higher than the previously reported impacts of these products to the freshwater alga *Raphidocelis subcapitata* and the marine diatom *Phaeodactylum tricornutum* (Jones et al., 2022; Wirth et al., in press). Additionally, algal growth rates remained positive over the test duration in all but the highest exposure concentrations for a majority of the PFAS-free products (Figure 5).

Results from the AF2 exposure were different from the other tested products in the present study and displayed a high disparity between algal IC50s and clam growth IC50s. Additionally, the acute algacidal nature observed in both the feeding study and the algal test could have contributed to clam growth inhibition. However, given the design of the clam growth study with daily renewals, it seems much less likely that food availability was a driver of decreased growth. The observed clam AF2 EC50 is also above the measured algal thresholds. Hard clams have been observed to derive free amino acids from ambient seawater and incorporate them into tissues (Rice & Stephens, 1988). It is unlikely, however, that this alone explains the disparity between clam growth EC50 and the observed algal thresholds given the energy demands of juvenile *M. mercenaria*.

There is also not enough evidence to attribute decreased organismal weight and growth rates to acute inhibition of organismal feeding. A previous study on Sodium Lauryl Sulfate, a common anionic surfactant, documented impacts on feeding ability in the Mediterranean mussel, *Mytilus galloprovincialis* (Ostroumov & Widdows, 2006). Another study investigating lower concentrations of multiple surfactants in *M. edulis* documented impacts to capture efficiency for Triton-X, a nonionic surfactant, on 3 μm polystyrene microspheres but found no observable impact of exposure on CR (Rosa et al., 2020). Net CR values measured here are more variable than those documented in previous studies, such as those observed by Rosa et al. (2020); however, the experimental designs are not directly comparable. Given the important role surfactant mixtures play in these PFAS-free AFFFs and Buckeye, it was hypothesized that feeding inhibition could have served as a potential mechanism of growth inhibition in *M. mercenaria* for these formulations. Alterations to organismal feeding ability likely would also indirectly impact organismal energy budgets, which may then drive changes in growth through alterations of energy allocation. These results observed here suggest that growth inhibition occurred through another mechanism and was not related to CR, though it could be related to another aspect of feeding, such as capture efficiency, or an aspect of metabolism. For example, low concentrations of Sodium Lauryl Sulfate have been shown to impact respiration and metabolic capacity in chronic exposure with *M. galloprovincialis* (Freitas et al., 2020). However, evaluating the PFAS-free AFFF toxicity from a mixture perspective (i.e., testing the whole product) obfuscates the impacts of any individual class of ingredient on feeding in the absence of all other classes, making it difficult to identify any individual components that may drive toxicity.

There have been recorded applications of PFAS-containing AFFFs in estuaries known to be inhabited by *M. mercenaria*; however, one such study noted a rapid spatial and temporal drop in individual PFAS ingredients following application (Katz et al., 2022). The tested exposure concentrations used in this study are orders of magnitude lower than the manufacturer's recommended usage concentration, which is typically 3%. It is unlikely that direct estuarine application of any of the PFAS-free products used in this study would result in sustained environmental concentrations that inhibited growth, particularly given the chronic timescale used in this study and the expected short-lived nature of these products. However, given the unknown mechanism of growth inhibition, it is possible that other effects may be seen in more dilute or in more acute exposures. No studies have been conducted to date on the fate of these PFAS-free AFFFs in the natural environment given their relatively novel nature and the ongoing development of PFAS-alternatives in general. Such studies would help further elucidate any potential risks posed to estuarine ecosystems.

Conclusion

The present study evaluated the effects of chronic exposure to PFAS-free AFFFs on the hard clam, *Mercenaria mercenaria*, and examined energy availability and feeding inhibition as potential mechanisms of toxicity. All but one PFAS-free AFFF impacted growth over a 21-day exposure period. Growth of *Isochrysis galbana* was inhibited by exposure to these products, but not at levels to impact clam growth for all products. One PFAS-free AFFF was found to be potentially stimulatory to organismal feeding, with the rest having no observable impact. As such, there was not enough evidence to identify feeding inhibition as a primary driver of growth inhibition. These results align with the existing research that suggest the aquatic toxicity of these potential replacement PFAS-free AFFFs is higher than a PFAS-containing AFFF and also points to an unidentified mechanism for chronic toxicity in this benthic species. Ultimately, this study is expected to inform the use and development of less hazardous AFFFs for use in fire suppression in multiple industries.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

The data are publicly available upon email request to the corresponding author and have been made available to the reviews and editors.

Author contributions

Jonathan A. Stewart (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing—original draft, Writing—review & editing), Katy W. Chung (Conceptualization, Investigation, Methodology, Resources, Writing—review & editing), Peter Key (Conceptualization, Investigation, Methodology, Resources, Writing—review & editing), Edward F. Wirth (Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing—review & editing), and Marie E. DeLorenzo (Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing—review & editing)

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Conflicts of interest

The authors declare no competing financial interest or other conflicts of interest.

Disclaimer

The scientific results and conclusions, as well as any opinions expressed herein, are those of the author(s) and do not necessarily reflect the views of the National Oceanic and Atmospheric Administration or the U.S. Department of Commerce. The mention of any commercial products is not meant as an endorsement by the Agency or Departments.

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