The silence of the clams: Forestry registered pesticides as multiple stressors on soft-shell clams

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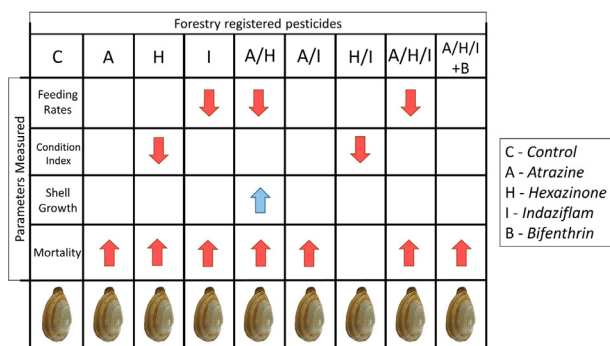[c] *U.S. Geological Survey, California Water Science Center, 6000 J St, Placer Hall, Sacramento, CA 95819, United States*
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HIGHLIGHTS

- Forestry herbicide mixtures pose an environmental risk to non-target shellfish.
- Clam fitness declines after exposure to environmental concentrations of herbicides.
- Clam mortality increases with chronic exposure to herbicide concentrations.
- Low concentrations of indaziflam are toxic to clams and accumulate in tissue.
- Herbicide mixtures produce distinct, complex sub-lethal effects.

GRAPHICAL ABSTRACT

ABSTRACT

Contaminants are ubiquitous in the environment, often reaching aquatic systems. Combinations of forestry use pesticides have been detected in both water and aquatic organism tissue samples in coastal systems. Yet, most toxicological studies focus on the effects of these pesticides individually, at high doses, and over acute time periods, which, while key for establishing toxicity and safe limits, are rarely environmentally realistic. We examined chronic (90 days) exposure by the soft-shell clam, *Mya arenaria*, to environmentally relevant concentrations of four pesticides registered for use in forestry (atrazine, 5 μg/L; hexazinone, 0.3 μg/L; indaziflam, 5 μg/L; and bifenthrin, 1.5 μg/g organic carbon (OC)). Pesticides were tested individually and in combination, except bifenthrin, which was tested only in combination with the other three. We measured shell growth and condition index every 30 days, as well as feeding rates, mortality, and chemical concentrations in tissue from a subset of clams at the end of the experiment to measure contaminant uptake. Indaziflam caused a high mortality rate (max. 36%), followed by atrazine (max. 27%), both individually as well as in combination with other pesticides. Additionally, indaziflam concentrations in tissue (61.70–152.56 ng/g) were higher than those of atrazine (26.48–48.56 ng/g), despite equal dosing concentrations, indicating higher tissue accumulation. Furthermore, clams exposed to indaziflam and hexazinone experienced reduced condition index and clearance rates individually and in combination with other compounds; however, the two combined did not result in significant mortality. These two compounds, even at environmentally relevant concentrations, affected a non-target organism and, in the case of the herbicide indaziflam, accumulated in

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clam tissue and appeared more toxic than other tested pesticides. These findings underscore the need for more comprehensive studies combining multiple compounds at relevant concentrations to understand their impacts on aquatic ecosystems.

1. Introduction

Pesticide use spans multiple industries ranging from its more commonly known application on agricultural sites, to forestry, and personal home applications. Each of these uses have similar objectives—to remove unwanted threats, such as pests or competition from other plants, and to maximize crop yield in agricultural and forestry use (Shepard et al., 2004; Zhang et al., 2011; Tatum et al., 2017). Post application, many of these compounds are transported away from the application area either aerially or via runoff (soil, surface water/groundwater), then deposited into streams/rivers and coastal watersheds (Oregon concentrations available in Table A1; Gilliom, 2007; Arias-Estevez et al., 2008; Greco et al., 2011; Scholz et al., 2012; Caldwell and Courter, 2019; USGS, 2020; Scully-Engelmeyer et al., 2021). While the transport and fate of these compounds vary based on numerous physical factors (Arias-Estevez et al., 2008) and chemical attributes, their presence in aquatic ecosystems and resident species has been reported, confirming their transport from application sites to waterways and subsequent uptake by organisms (Katagi, 2010; Scully-Engelmeyer et al., 2021).

In the Pacific Northwest, commonly applied forestry pesticides include but are not limited to: 2,4-D, aminopyralid, atrazine, clopyralid, glyphosate, hexazinone, imazapyr, indaziflam, metsulfuron methyl, oxyfluorfen, penoxsulam, and sulfometuron methyl (Oregon Water Quality Management Team, 2019; FERNS, 2021). A recent study by Scully-Engelmeyer et al. (2021) focused on forestry management in Oregon coastal watersheds detected combinations of these commonly used compounds, including the herbicides atrazine, hexazinone, and indaziflam, and the insecticide bifenthrin in water and tissue samples across different management regimes. Each of these forestry registered compounds have been detected in aquatic systems globally, except indaziflam, which is not yet widely monitored (Michael et al., 1999; Clark et al., 2009; Weston et al., 2015; Rogers et al., 2016; U.S. Geological Survey, 2020). Atrazine, hexazinone, and indaziflam were notified for 539 (19%), 1070 (38%), and 30 (12%) forestry application spray events in the Spring of 2020 (FERNS, 2021). While use is declining in the Pacific Northwest, atrazine is still applied and subsequently detected in field studies (Oregon Water Quality Management Team, 2019; Scully-Engelmeyer et al., 2021). As one of the most widely applied herbicides globally, with uses ranging from controlling grasses and broad leaf plants via photosynthesis inhibition, to eliminating competition plants in forestry and agriculture (Graymore et al., 2001), atrazine is readily transported to aquatic systems due to its moderately hydrophilic nature (Graymore et al., 2001). Hexazinone, a globally registered triazine herbicide and a photosynthesis inhibitor like atrazine, is predominantly used by the forestry industry (Michael et al., 1999; Shepard et al., 2004). Indaziflam is a relatively new herbicide and effective weed killer through inhibition of cellulose binding (Kaapro and Hall, 2012; Brabham et al., 2014); it is increasingly used to control invasive weeds (Sebastian et al., 2017), but its effects on aquatic invertebrates have yet to be adequately studied (Tompkins, 2010). Bifenthrin, a pyrethroid insecticide that targets the nervous system of insects, is frequently used in agricultural and urban settings due to its high effectiveness (Weston et al., 2011, 2015). While registered for use in the forestry industry, its forestry use has not been notified since 2014 (FERNS, 2021), yet its remains the most frequently detected pyrethroid in numerous stream studies along the United States West Coast (Kuivila et al., 2012; Weston et al., 2011, 2015; Scully-Engelmeyer et al., 2021).

Research on these compounds and their toxicity has historically focused on individual compound effects at high concentrations to determine lethal endpoints, which establish important toxicity limits for a small number of species (Lawton et al., 2006; Lindsay et al., 2010; Rogers et al., 2016). These studies are key in understanding the toxicity of these compounds and determining environmental and human health hazards to properly restrict their use and inform regulation decisions (EPA, 2021). However, these high concentrations of individual compounds under acute time frames are not environmentally realistic, nor do acute studies consider long-term effects on non-target aquatic organisms and populations. Multiple land management industries apply these compounds for pest management (Spies et al., 2007; Scully-Engelmeyer et al., 2021), after which, they can be transported within watersheds and expose organisms to a mixture of contaminants. The focus on single compounds ignores potential additive, synergistic, or antagonistic organismal effects of multiple compounds.

To address these major data gaps, we examined individual and mixture effects from chronic (3-month) exposure to environmentally relevant concentrations of compounds registered for use in the forestry industry and previously detected in field sampling of water and aquatic bivalves (Bouchard et al., 1985; Graymore et al., 2001; Shepard et al., 2004; Weston et al., 2015; U.S. Geological Survey, 2020; Scully-Engelmeyer et al., 2021). We exposed adult soft-shell clams, *Mya arenaria*, to four previously detected forestry-use compounds: atrazine, hexazinone, indaziflam, and bifenthrin during a 90-day tank experiment with monthly subsampling to test the following hypotheses:

1. Pesticides in combination will show an additive or synergistic relationship, generating negative and more impactful effects on the biological performance and fitness of clams in comparison to singular compounds and relative to controls.
2. Long-term exposures will significantly affect clam growth and condition.
3. Atrazine, hexazinone, and indaziflam doses will be detected in water samples post-dosing due to their hydrophilic (hexazinone) or moderately hydrophobic (atrazine, indaziflam) properties.
4. Moderately hydrophobic compounds (atrazine, indaziflam) and hydrophobic bifenthrin will be detected in tissue samples at concentrations found in natural systems.

2. Methods

2.1. Study species

Mya arenaria, the soft-shell clam, is an estuarine bivalve commonly found on the West Coast of the United States, frequently in high densities, in fine-grain mud. As estuarine organisms that require 2–5 years to reach their adult stage, they can be exposed to contaminants from diverse upland/upstream sources (Lindsay et al., 2010). As filter feeding organisms, bivalves are a popular organism choice in toxicological studies (Frouin et al., 2007; Greco et al., 2011). We chose this particular species due to its widespread occurrence on the Oregon coast, and results of a recent study that detected multiple pesticides in the tissue of field collected soft-shell clams (Scully-Engelmeyer et al., 2021).

2.2. Organism collection and laboratory set-up

Adult *M. arenaria* were collected by hand in July 2020 from a mud flat on the west bank of the North Fork Siuslaw (43°58′37.0″N 124°04′39.6″W) in Florence, Oregon. In coolers chilled indirectly with ice, the clams were transported to the Applied Coastal Ecology laboratory at Portland State University where they were maintained in 120 L acclimation tanks on a recirculating water table with artificial Instant Ocean® sea water (for composition see Dickman and Christy, 2002) for no more than a week before being measured into size classes and randomly distributed into 27 experimental tanks (64 L, plastic) with individual chillers and filters (Aquatic

Enterprises). Wet weight (g) of each clam was measured by first lightly stroking the mantle and siphon to ensure expulsion of most of the water, then placing each on a Sartorius TE313S balance. Shell length (mm) from the anterior to posterior sides of the shell, as well as the width from umbo to the ventral edge, were measured and recorded.

Pesticides were administered across a total of 8 treatments: atrazine, hexazinone, indaziflam, atrazine and hexazinone (At/Hex), atrazine and indaziflam (At/Ind), hexazinone and indaziflam (Hex/Ind), atrazine, hexazinone, and indaziflam (3-Way), and finally a combination of atrazine, hexazinone, and indaziflam with bifenthrin (Bif + 3). Tanks spanned three racks (8–10 tanks/rack) with 11 clams placed in each tank with a mean shell length of 76.6 ± 3.3 SE (mm) per tank; there were 3 tanks per treatment group. Mean shell length and mass of individual clams did not differ among treatments (mean length one-way ANOVA, $p = 0.596$, mean mass one-way ANOVA, $p = 0.512$). Clams were gradually introduced to the experimental tanks, two per day, over a week to avoid nitrogen spikes, to reach a total of 11 clams per tank. Saltwater tanks contained Instant Ocean® mixed with deionized water with bacterially-colonized BioBall filter media to control nitrogen levels.

To mimic estuarine mud flat conditions with enough pressure to maintain clams upright in their natural position, smaller tanks (12 L, glass) were placed within the housing tanks and filled two-thirds full with clean sand collected from Florence, Oregon (Fig. A1). Each housing tank had an independent water chilling and filtration system (Aquatic Enterprises), and salinity and temperature were maintained at 17 practical salinity units (PSU) and 13 °C, respectively, to replicate conditions measured at the collection site. Temperature and salinity were measured before and after each water change (every 10 and 11 days) using a YSI Pro 2030 (YSI Inc. Yellow Springs, OH, USA). Water chemistry (ammonia, nitrate, and nitrite) was measured weekly and suitable levels were confirmed but not recorded (as per Peters and Granek, 2016). Ultraviolet lights above each tank were set on a 12-hour light cycle and blackout curtains closed off the experimental area to eliminate light pollution.

Every other day, organisms were fed Shellfish Diet 1800® (Reed Mariculture) diluted with fresh saltwater to a 1:1 ratio; feed volume was based on total clam biomass per tank as recommended by the supplier. Clam mortality was closely monitored and recorded throughout the one-month acclimation period and dead clams were immediately replaced by individuals of

a similar size. Throughout the experiment, clams were monitored daily for mortality and dead clams were removed immediately to avoid stress to other clams.

2.3. Pesticide exposures

Every 10 days, tanks were dosed with pesticides singularly or in combination (Section 2.3.1), excluding bifenthrin, which was dosed only in combination with all other compounds (Section 2.3.2, Fig. 1). Environmentally relevant dosing concentrations (Table 1) were determined by comparing a thorough literature review with local USGS data as a foundation, then selecting concentrations to mimic measured environmental concentrations post spring spray application and rain events (U.S. Geological Survey, 2020). Pesticides were purchased from Sigma-Aldrich in pure powder form. Before each tank dosing, a 20% water change was completed to manage nitrogen levels and maintain pesticide levels, mimicking industrial applications across upstream watershed parcels and/or flushing from rainfall events (Fig. 1).

2.3.1. Atrazine, hexazinone & indaziflam

Fresh solutions of these water-soluble compounds were prepared the day of each dosing to avoid chemical decomposition between 10-day dosing periods. Although in forestry applications, all compounds are combined into one mixture, each stock solution was prepared separately to avoid cross contamination, thoroughly mixing the powdered compound in Instant Ocean seawater and using serial dilutions to achieve the experimental concentrations: atrazine 5 μg/L, hexazinone 0.3 μg/L, and indaziflam 5 μg/L (Table 1). Solutions were administered in 1 mL quantities using a micropipette, changing tips between solutions.

2.3.2. Bifenthrin

Due to its hydrophobicity (log K_{ow} 5.3; Kuivila et al., 2012), bifenthrin doses were administered using acetone-spiked sediment (Pennington et al., 2014; Boyle et al., 2016; Rogers et al., 2016) collected at the clam collection site and allowed to fully dry before preparation; sediment had a mean organic carbon (OC) content of 1.32% (SE 0.074). Serial dilutions were made by adding bifenthrin to acetone using a glass pipette. A final 500 mL acetone bifenthrin solution was created and applied to the
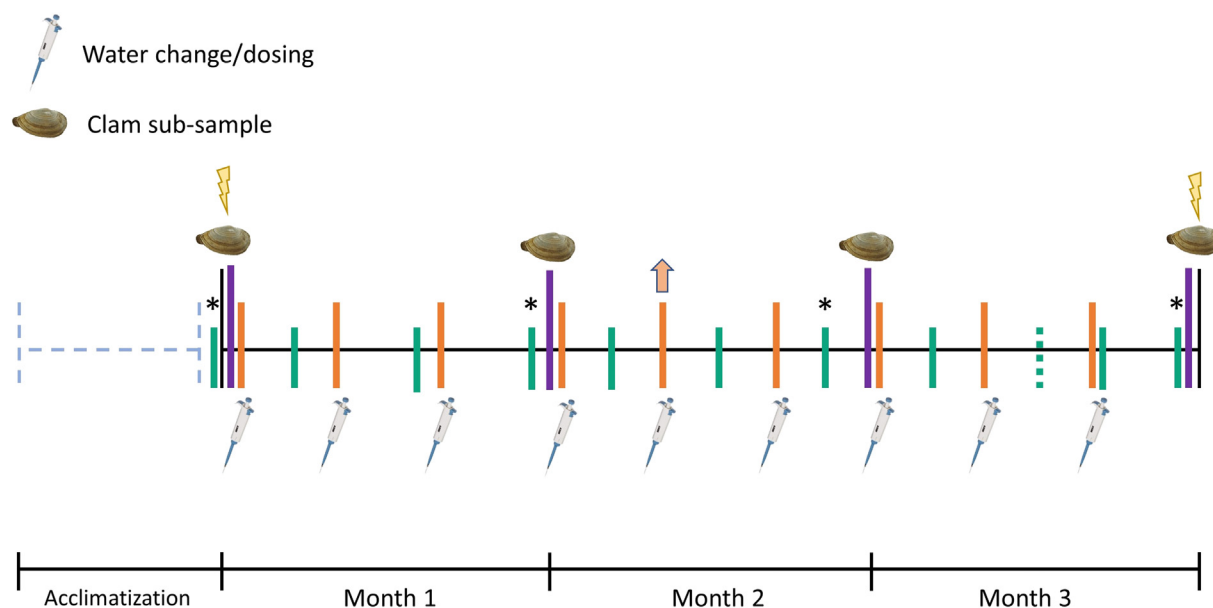

Fig. 1. Experimental timeline including 10-day water changes and subsequent dosing represented by medium orange lines and pipette, with an orange arrow to indicate the increase in bifenthrin dose; clam measurements and water samples represented by long purple line and clam shell; clam tissue samples represented by lightning bolt; feeding rate samples represented by short green lines, dashed line indicates missing feeding rate sample and asterisk indicates samples chosen for analysis. Acclimatization period indicated by dashed, light blue lines.

Compound	Concentration
Atrazine	5 μg/L
Hexazinone	0.3 μg/L
Indaziflam	5 μg/L
Bifenthrin	0.17 μg/g OC

sediment in a metal tray. Once the solution was applied, the sediment was thoroughly mixed until evenly distributed and stored in a fume hood for 24 h to allow the acetone to evaporate. The spiked sediment was then stored in a glass container in a dark refrigerator at 4 °C and mixed every other day during the week leading up to the experiment. Un-spiked sediment (for non-bifenthrin treatments) was prepared, stored, and administered in the same manner. For application, 50 g of spiked sediment was added to a glass jar with seawater from the experimental tank and mixed thoroughly before being released into the tank under the surface to ensure all spiked sediment was administered to the habitat. Sediment was agitated every other day in all tanks to resuspend the particles for full exposure and to model sediment movement in rivers and estuaries. A second spiking was conducted during week 8 (dose 5/9) of the experiment (Fig. 1) to imitate a subsequent runoff event (Weston et al., 2015).

2.4. Sampling

2.4.1. Clams

Seven baseline clams were randomly selected and sacrificed at the start of the experiment, prior to the addition of chemicals. Then, three individuals per tank were collected for growth and weight measurements every 30 days (at 30, 60, 90 days). To avoid disturbance to other organisms, clams at each sample period were chosen based on accessibility, wherein the researcher would randomly select the first three clams found without digging through the sediment. Selected clams were immediately measured, wet weight recorded using previously described methods, dissected, then wet tissue and shells were weighed separately. Tissue was stored in a clean jar following USGS cleaning method Version 2.0 4/2004 for organic compounds (Wilde and Radtke, 1998) and stored at −80 °C until further analysis.

For condition index (CI) as well as chemical composition, tissues were freeze-dried using a Harvest Right freeze dryer (HRFD-P-M-AL) until no further weight loss occurred and then weight was recorded. Shells were baked in a standard drying oven (Fisher Isotemp 625G) for 48 h at 50 °C to measure dry weight. The condition index was calculated using the formula (Lucas and Beninger, 1985; Leavitt et al., 1990):

$$CI = (DW \text{ soft tissue}/DW \text{ shell}) \times 1000.$$

Tissues were individually homogenized using a mortar and pestle and stored in clean jars then shipped overnight on ice to the USGS Organic Chemistry Research Laboratory in Sacramento, California for chemical analysis (see Section 2.5).

2.4.2. Algal clearance rates

Algal clearance rates (CR) were measured weekly, except for week 9 (due to uncontrollable factors). A preliminary analysis of algal cell distribution from their release point into the tank was confirmed visually under a compound microscope by counting total cells in samples collected from the tank after allowing 3 min for circulation. These results confirmed selection of a central point in each tank to pour the algal mixture for thorough mixing. To quantify feeding rates, single 1-mL samples were collected by pipette from each tank 3 min after feeding (baseline algal density post mixing, Table A2), and again after 3 h–to quantify clam feeding rate based on change in algal cell density (Peters and Granek, 2016). Samples were collected in cryovials to which 0.02% glutaraldehyde was added, then

incubated in the dark at room temperature for 10 min. Vials were then flash frozen using liquid nitrogen and stored at −80 °C until further analysis.

Samples were analyzed using a flow cytometer (Becton Dickinson Influx high speed cell sorter equipped with a small particle detector) to characterize prey particles. Particles were interrogated with a 488 nm laser, with data collection triggered on forward scattered light (FSC). Relative red fluorescence of each particle was detected using a 692/40 bandpass filter as a proxy for chlorophyll. Flow rate was determined by measuring the amount of liquid that passed through the instrument during each sample and was used to calculate particles (i.e., cells) per volume for each sample. FSC was calibrated by spiking each sample with 1 μm polystyrene beads as an internal control to allow for assessment of particle size as the size range of algal particles in the mixture was known (Reed Mariculture). Change in diatoms per mL was calculated to determine algal clearance (Coughlan, 1969; Hansen et al., 2011; Peters and Granek, 2016):

$$CR = (v/t * n) \left[\ln \left(C_0/C_t \right) \right]$$

where CR = clearance rate (cells ml^{-1} min^{-1}); v = volume of tank (ml); t = time of measurement (m, $t = 180$); n = total bivalve dry weight of soft parts (g) (as calculated using a linear relationship, see below); C_0 ad C_t = concentration of particles in tank at time 0 and t, respectively.

Dry weight of soft parts was unknown for clams who experienced mortality before sampling dates; therefore, their dry weight was calculated by linear modeling using their initial full wet weights ($R^2 = 0.857$).

2.4.3. Sediment/water

Sediment and water samples were collected to confirm dosing concentrations at each 30-day sampling period. Sediment was sampled from the tank immediately after dosing to measure the bifenthrin dosing concentration following possible loss from mixture with water, using EPA sampling methods (EPA, 2020; Wilde and Radtke, 1998). Control tank sediment samples were consolidated and tested as one aggregated sample. Water samples were collected by submerging 40 mL amber glass bottles in each tank until full and immediately sealing and maintaining bottles below 13.3 °C until shipment in coolers to ANATEK Laboratories (Moscow, ID). Samples were analyzed as described below in Section 2.5.4 Water. Bifenthrin was not analyzed in water samples due its hydrophobic properties.

2.5. Chemical analysis

2.5.1. Organic carbon content

Baseline bed sediment samples were analyzed for organic carbon content using a modified version of USEPA method 440.0 (Zimmermann et al., 1997) at the USGS Organic Chemistry Research Laboratory in Sacramento, California. Sediment samples were freeze-dried, homogenized using a mortar and pestle, then sub-sampled; 5 to 10 mg of sediment were weighed into silver capsules and exposed to concentrated hydrochloric acid fumes in a desiccator for 14 h to remove inorganic carbon. The sediment samples were then dried in an oven at 60 °C to remove any remaining acid or water before being pressed into sealed balls. Samples were analyzed on a Costech ECS 4010 CHNSO analyzer (Costech Analytical Technologies Inc., Valenica, CA) in carbon nitrogen mode. The combustion furnace temperature was 980 °C, the reduction furnace temperature was 650 °C, the gas chromatographic column temperature was 65 °C, and the carrier gas flow rate was 110 mL per min.

2.5.2. Sediment

Freeze-dried samples (5 g) were homogenized with sodium sulfate (Na$_2$SO$_4$) and spiked with ^{13}C$_{12}$-p,p'-DDE (Cambridge Isotope, Cambridge MA) as a recovery surrogate and extracted with dichloromethane (DCM) using a Dionex 200 accelerated solvent extractor (ASE) at 1500 psi and 100 °C. The extract was exchanged into 6 mL of ethyl acetate, the eluent was reduced to 0.2 mL, and an internal standard was added (d$_{10}$-acenaphthene). Samples were analyzed for bifenthrin using ether gas

chromatography mass spectrometry (GC–MS/MS; Agilent 7890 GC coupled to an Agilent 7000 MS/MS operating electron ionization (EI) mode) and data were collected in multiple reaction monitoring (MRM) mode with each compound having one quantifier MRM and at least one qualifier MRM. The limit of detection (LOD), defined as the value greater than three times the signal-to-noise ratio, was 0.2 ng/g. Further instrument details can be found elsewhere (Hladik et al., 2016).

2.5.3. Tissue

Freeze-dried samples (0.2 to 0.3 g) were homogenized with sodium sulfate (Na_2SO_4) and spiked with $^{13}C_{12}$-*p*,*p*′-DDE, d_4-imidacloprid, $^{13}C_6$-*cis* permethrin, and d_{10}-trifluralin (Cambridge Isotope, Cambridge MA) as recovery surrogates and extracted with 50:50 acetone:dichloromethane (DCM) using a Dionex 200 accelerated solvent extractor (ASE) at 1500 psi and 100 °C. The extract was exchanged into 6 mL of acetonitrile, co-extracted matrix interferences were removed with 0.5 g *Z*-sep + (Sigma-Aldrich, St. Louis, MO) and then the eluent was reduced to 0.2 mL and internal standards were added (d_{10}-acenaphthene and d_{10}-phenanthrene and d_3-chlothianidin). Samples were analyzed for a total of 146 pesticides and pesticide degradates using either gas chromatography triple quadrupole mass spectrometry (GC–MS/MS; Agilent 7890 GC coupled to an Agilent 7000 MS/MS operating electron ionization (EI) mode) or liquid chromatography tandem mass spectrometry (LC-MS/MS; Agilent 1260 bio-inert LC coupled to an Agilent 6430 MS/MS). Data for all pesticides were collected in multiple reaction monitoring (MRM) mode with each compound having one quantifier MRM and at least one qualifier MRM. The limits of detection (LOD), defined as the value greater than three times the signal-to-noise ratio, were 5 to 10 ng/g for a 0.2 g sample. Further instrument details can be found elsewhere (Hladik et al., 2016).

2.5.4. Water

Samples were analyzed by a modified version of EPA method 8321B at Anatek Labs, Inc., in Moscow Idaho. A 1.0 mL sample aliquot was fortified with 10 μL of each internal standard (^{13}C-atrazine, 0.1 mg/L; tebuthiuron, 1.0 mg/L). A 10 μL injection of standard-spiked samples was made on a Shimadzu LC equipped with a Waters Xterra C18 column (2.1 × 50 mm, 3.5 μm) and interfaced to an API 4000 MS/MS (AB Sciex). All blanks and controls were treated as samples. A fortified laboratory blank, matrix spike sample, matrix spike sample duplicate, and reagent blank were prepared with each sample batch. The minimum reporting level (MRL) for the method is 0.2 μg/L for indaziflam and atrazine, and 0.005 μg/L hexazinone.

2.6. Statistical analysis

Data for each parameter were analyzed in R Studio version 1.4.1103 (Horton and Kleinman, 2015). Statistical significance was defined as $p < 0.05$, marginal significance defined as $0.08 > p \geq 0.05$, and high statistical significance determined as $p < 0.0001$. Growth data from the final sample period were not normally distributed, so were analyzed using a nonparametric Kruskal-Wallis test with a Dunn's test in the rstatix package (Kassambara, 2020). Condition index data at the final sample period were analyzed using a three-way ANOVA to compare the individual (atrazine, hexazinone, indaziflam) and combination treatments, and a one-way ANOVA to compare bifenthrin to the other treatments. Normality and equal variance of condition index data were tested by a Shapiro-Wilk and Bartlett's test, respectively, and all ANOVA assumptions were met.

While 11 time periods were sampled, clearance rate data did not fit the statistical model for a full time series and were therefore analyzed at four sample points that aligned with organismal sample points for stronger comparison to organism measurements; these included the initial, pre-experimental baseline clearance rates, as well as samples taken before organism sub-sampling (i.e., weeks 0, 3, 6, and 10). Clearance rate data were non-normal and heteroscedastic, therefore the Kruskal-Wallis test with a post-hoc Dunn's test was used to analyze the four sample points (rstatix package).

Mortality data were analyzed using a three-way ANOVA, with a one-way ANOVA to test the bifenthrin combination treatment compared to the others. Mortality data met all ANOVA assumptions. Water and tissue samples were analyzed by calculating mean and standard errors for each compound using the stats package in R Studio (R Core Team, 2013).

3. Results

3.1. Shell growth

At the final sample point, At/Hex treated clams grew significantly more than those in the control, atrazine, hexazinone, At/Ind, and 3-Way combination treatments ($p < 0.05$, Fig. 2A). While not significantly different from the control, clams in the atrazine treatment grew significantly less than those in the indaziflam treatment ($p < 0.05$) and marginally less than those in the Bif + 3 combination treatment ($p = 0.0576$), with the indaziflam treatment showing significantly more growth than the At/Ind treatment ($p < 0.05$, Fig. 2A).

3.2. Condition index

The mean condition index for all treatments at the final sample point was marginally lower than the control (Fig. 3A). However, this difference was only significantly lower for the hexazinone and Hex/Ind treatments ($p < 0.05$, Fig. 3A).

3.3. Clearance rates

At the pre-experiment sample point (0), mean clearance rates in treatment tanks did not significantly differ from those in control tanks (Fig. 4). However, comparing among treatments at sample point 0, Hex/Ind treated clams initially consumed significantly more algae than those in the At/Hex, At/Ind, indaziflam, and 3-Way combination treatments, potentially due to natural variability in organism activity ($p < 0.05$, Fig. 4). At sample point 1, clams treated by At/Hex, indaziflam, and 3-Way combination had significantly lower clearance rates than controls ($p < 0.05$), and those in the atrazine, hexazinone, indaziflam, and 3-Way combination treatments had significantly lower clearance rates than those in the Hex/Ind treatment ($p < 0.05$, Fig. 4). At sample point 2, At/Hex and 3-Way combination treated clams had significantly lower clearance rates than the controls ($p < 0.05$), whereas hexazinone ($p = 0.0506$) and indaziflam ($p = 0.0570$) treated clams had marginally lower clearance rates than those of the controls ($0.08 > p \geq 0.05$; Fig. 4). At sample point 3, the 3-Way combination treated clams were consuming significantly less than the controls ($p < 0.05$), and those in the indaziflam treatment consumed marginally less than the controls ($p = 0.0570$, Fig. 4). Algal consumption increased over time across all treatments (Fig. A3). The Bif + 3 treatment did not differ significantly from the control or any other treatments at any of the analyzed sample points.

3.4. Mortality

Nearly all treatments experienced significantly higher mortality than in the control tanks ($p < 0.05$, Fig. 5A), except the Hex/Ind treatment (Fig. 5A). The indaziflam treatment resulted in the highest mortality of all treatments ($p < 0.001$; Fig. 5A).

3.5. Sediment/water/tissue concentrations

3.5.1. Sediment

Mean initial sediment bifenthrin dose concentrations were 0.06 μg/g OC (SE 0.004). The week 8 spiking resulted in a mean bifenthrin concentration of 1.5 μg/g OC (SE 0.064).
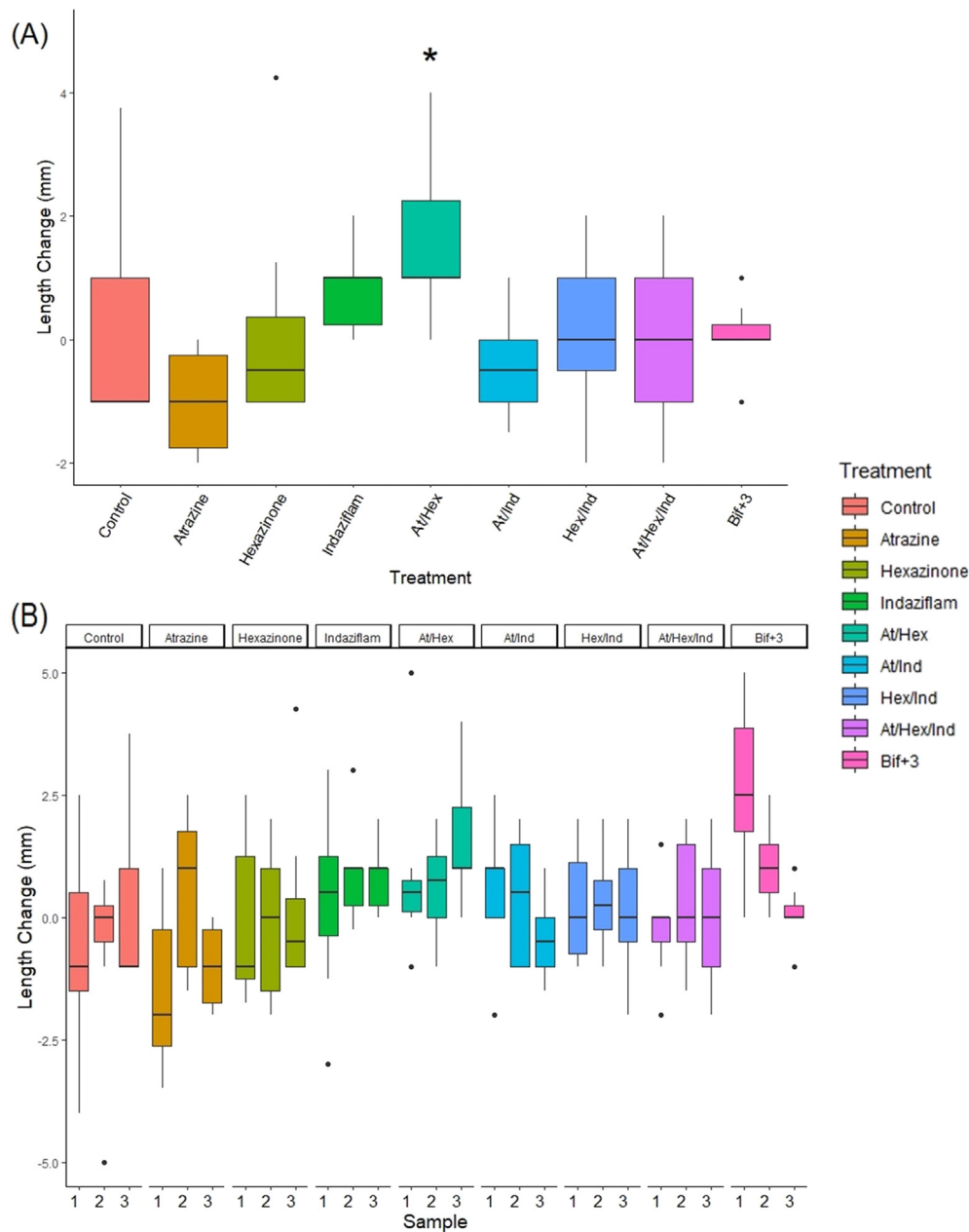
Fig. 2. Change in shell length from beginning of experiment to sample date A) at the final sample point and B) at all 3 sample points. Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values with abnormal outliers represented by black dots. Statistically significant difference ($p < 0.05$) noted in figure solely in comparison to control and noted by asterisk.

3.5.2. Water

Mean water and tissue concentrations are summarized in Table A3 and Fig. 6. Mean atrazine concentrations in water samples post dosing were only 4% lower than the administered dose of 5 μg/L; however, mean hexazinone concentrations were 93% lower, and mean indaziflam concentrations were 84% lower than the administered doses (Tables 1, A3, Fig. 6a). Hexazinone contamination was detected at the second sampling point in which low hexazinone concentrations were detected in all of the atrazine and At/Ind treatment tanks (Table A3, Fig. 6A).

3.5.3. Tissue

In tissue samples, although dosed at the same concentration, mean indaziflam concentrations were 61% higher than those of atrazine (Fig. 6B). Mean detected experimental compound concentrations did not

exceed field detected concentrations in Oregon soft-shell clams (Scully-Engelmeyer et al., 2021), validating that the concentrations utilized in the study are environmentally relevant regionally.

4. Discussion

This study identified effects of forestry associated pesticides on soft-shell clams, including the effects of time and compound combinations on clam performance, resulting in sub-lethal effects as well as unexpected mortality. This study identified interactive effects among pesticides on growth, condition index, mortality and uptake of lipophilic compounds in clam tissues at concentrations higher than previously expected. The results of this study begin to fill an existing literature gap in studies on hexazinone and indaziflam exposure to bivalve organisms at environmentally relevant levels while supported by previous studies on atrazine and bifenthrin.
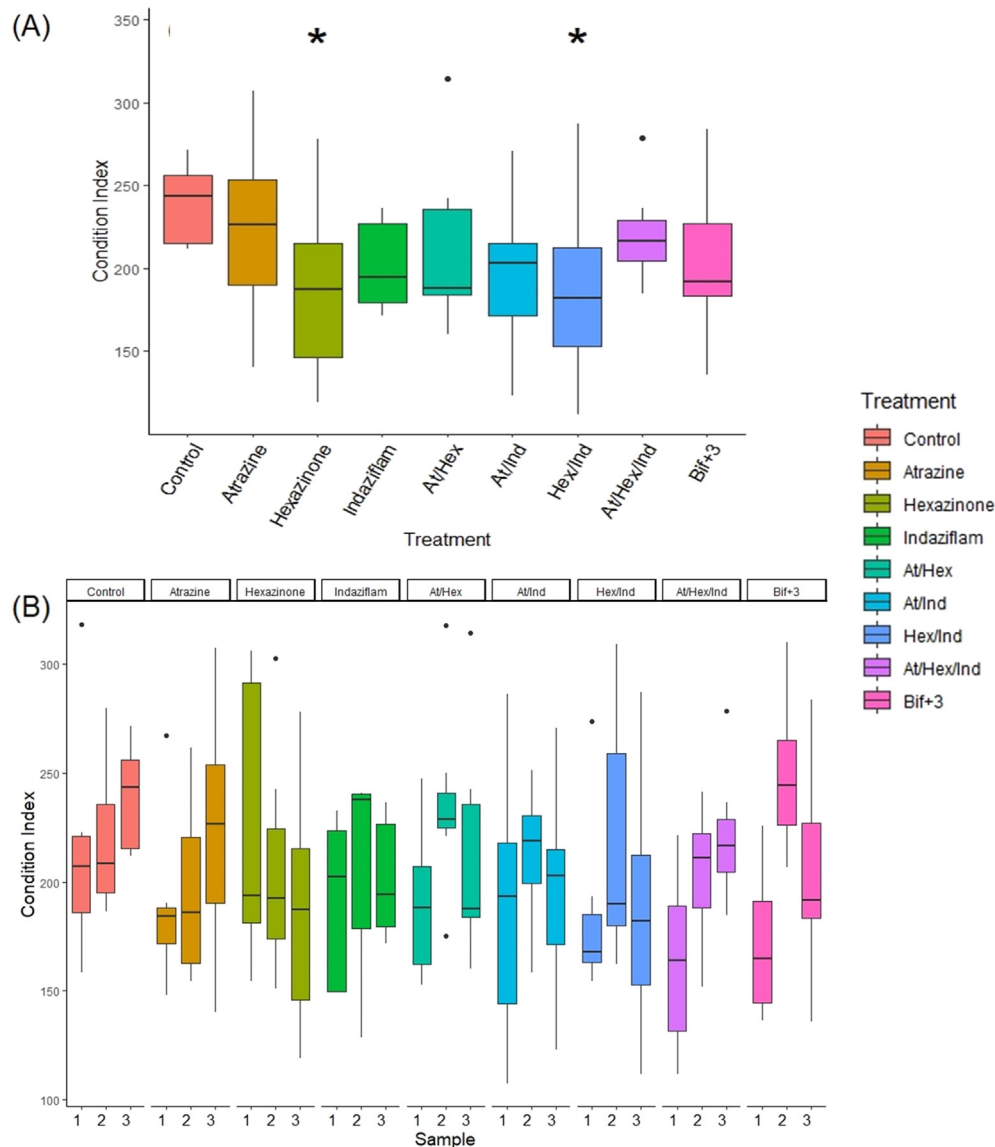
Fig. 3. Differences in condition index among treatments A) at the final sample point and B) at all 3 sample points. Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values with abnormal outliers represented by black dots. Statistically significant difference ($p < 0.05$) noted in figure solely in comparison to control and noted by asterisk.

These findings demonstrate that, in addition to accumulating in tissues, compounds in different combinations result in varying effects on non-target organisms sensitive to these pesticides, even at low concentrations (Table 2).

4.1. Individual and interactive effects of pesticides

Exposure to indaziflam and atrazine led to the highest mortality among all treatments individually and in combination with most other tested compounds; lower mortality in the hexazinone/indaziflam treatment was the exception and suggests potential antagonistic effects when combined. Yet, in terms of sub-lethal effects, hexazinone individually and in combination with indaziflam negatively affected condition index, indicating that both herbicides affect clams when combined with each other. Antagonistic relationships between compounds are currently not well understood, therefore the antagonistic effect of hexazinone with indaziflam on mortality impresses the need for further study in this area (Rizzati et al., 2016).

Changes in parameters such as shell length, condition index, clearance rates, and mortality can be complicated to interpret. For example, if condition index and/or clearance rates are decreasing, an increase in bivalve shell length may indicate a sub-lethal negative effect such as a stress response to low food availability or assimilation (Alunno-Bruscia et al., 2001; Teixeira, 2016). The combination of low clearance rates, elongated shells, and low tissue weight, as observed in the atrazine/hexazinone treatment, may indicate starvation, a potential population-level effect of mixed pesticides exposure. Even when condition index wasn't significantly impacted, lower clearance rates indicate a reduction in consumption and potential decrease in energy and overall physical health (Kesarcodi-Watson et al., 2001). Thus, while shell growth was observed in response to atrazine and hexazinone, the compounded effects of mixtures on clearance rates may indicate long term population-level impacts.

While individual compounds may not have had significant effects on the measured endpoints, these same compounds in combination, as is frequently the case during forestry applications, affected multiple biological parameters. For example, while indaziflam alone did not significantly affect condition index, when in combination with hexazinone, a decrease in condition index was observed. Additionally, decreased clearance rates were observed after exposure to indaziflam, atrazine with hexazinone, and all three
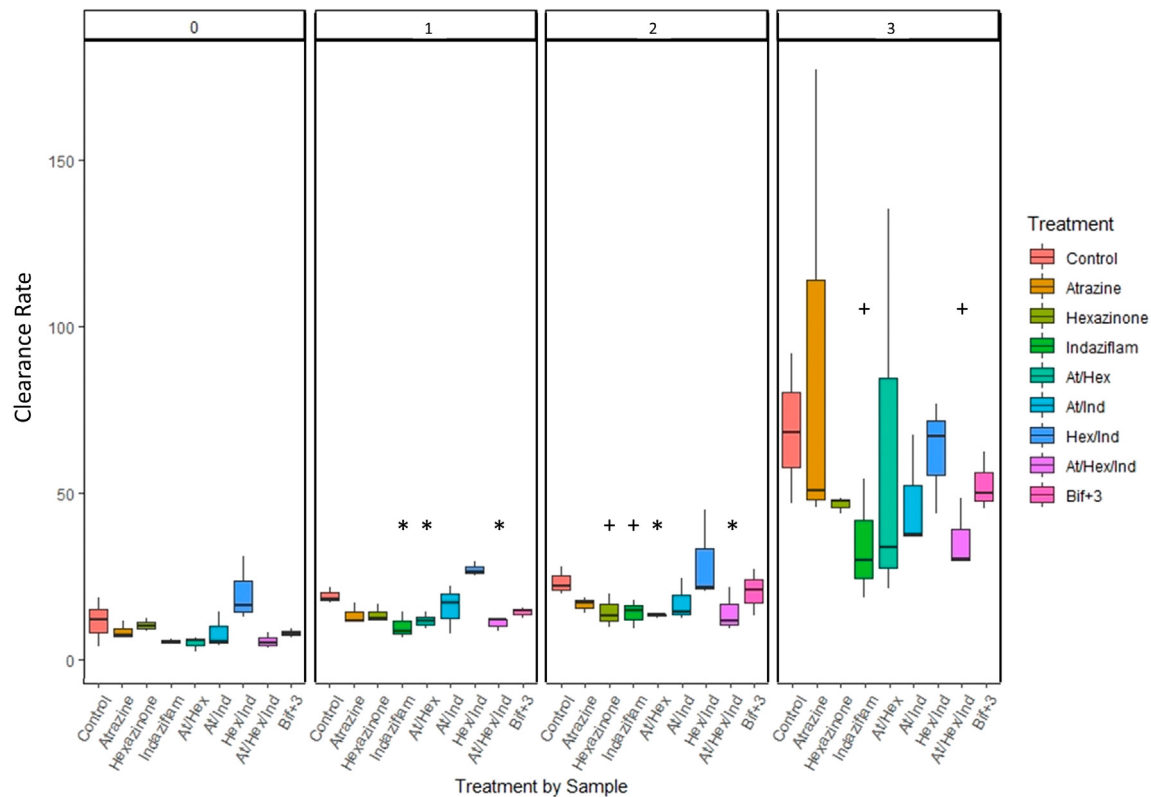
Fig. 4. Algal clearance rates (CR, cells mL^{-1} min^{-1}) by treatment, separated by sample point: 0 (pre-experiment), 1 (week 3), 2 (week 6), and 3 (week 10). Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values with abnormal outliers represented by black dots. Statistically significant difference ($p < 0.05$) noted by asterisk, marginally significant difference ($0.08 > p$ $p \geq 0.05$) noted by +, both noted in figure solely in comparison to control.

combined, suggesting a synergistic relationship between atrazine and hexazinone as well as additive effects from indaziflam. Both sub-lethal and lethal effects observed in treatments with indaziflam provide strong indication of its toxicity to non-target organisms.

4.2. Chemical-organismal interactions

Examining the combination of tissue chemical accumulation rates, modes of action, and biological effects may facilitate predicting future impacts of compounds on non-target organisms. While specific effects may vary among pesticides of a group (i.e., oxidative stress, genotoxicity, etc.), there is a potential for generalized patterns such as sub-lethal versus lethal effects on groups of organisms (Semren et al., 2018).

The chemical mechanism of atrazine is photosynthetic inhibition; therefore, the most at-risk non-target aquatic organisms are algae and phytoplankton (Graymore et al., 2001). However, a study by Britt et al. (2020) found that at environmentally relevant concentrations, atrazine causes a significant loss of beneficial bacteria in oysters, potentially making these organisms susceptible to harmful bacteria or negatively affecting food processing and growth rates. A relative decrease in algal clearance rates as compared to the control, in conjunction with a high mortality rate may suggest such a loss of beneficial gut microbia, causing a decrease in feeding activity and an eventual spike in mortality due to malnutrition or stress. The overall increase in clearance rates throughout the 90 days was observed in a study by Peters and Granek (2016) where they postulated the increase was a result of the removal of individuals at sub-sampling dates causing a reduction in competition pressure. Effects of atrazine exposure on mussel gill structures as well as immune and endocrine systems observed at similar concentrations (Nogarol et al., 2012; Juhel et al., 2017) suggest a range of sub-lethal effects on bivalves that may result in eventual lethality after long-term exposure, supporting our findings for this species.

Hexazinone operates similarly to atrazine, as a triazine herbicide that inhibits photosynthesis. However, it is much more water soluble and does not tend to bioaccumulate in organism tissue (Mayack et al., 1982; Michael et al., 1999; Scully-Engelmeyer et al., 2021; Fig. 6A, B). Hexazinone can inhibit phytoplankton growth (Clark et al., 2009) and reduce larval survival of *Mya arenaria* (Lindsay et al., 2010); however further study of environmentally relevant exposure effects to adult bivalves is needed (EPA, 2021). Study findings of a decrease in condition index and clearance rates and an increase in mortality, may indicate an effect of hexazinone on digestive processes and overall clam performance.

The effects of indaziflam on aquatic organisms are not well studied. As a more recently registered herbicide (registered in the US in 2010), it's mode of action is inhibition of cellulose production in plants (Brabham et al., 2014). Given its detection in both water and tissue samples in this study (Fig. 6A, B), its high concentrations in clam tissue and strong effects on condition index, feeding rates, and mortality, indaziflam appears to be more toxic to bivalves than atrazine, a known toxicant to aquatic organisms (Rohr and McCoy, 2010; Hayes et al., 2011; Abdulelah et al., 2020; National Center for Biotechnology Information, 2021).

Bifenthrin is a pyrethroid insecticide that targets the nervous system (Pennington et al., 2014). While not commonly used in the forestry industry, bifenthrin is commonly detected in the environment (Kuivila et al., 2012; Weston et al., 2011, 2015; Scully-Engelmeyer et al., 2021). While mortality was observed in our bifenthrin treatment, it was not significantly different from the 3-way combination treatment, indicating it may not cause an additive effect on mortality. However, Zhang et al. (2020) observed various sub-lethal genetic and cellular effects of bifenthrin on bivalves at environmentally relevant concentrations. The results of this study may be limited by the lack of an individual bifenthrin treatment to rule out additive effects.
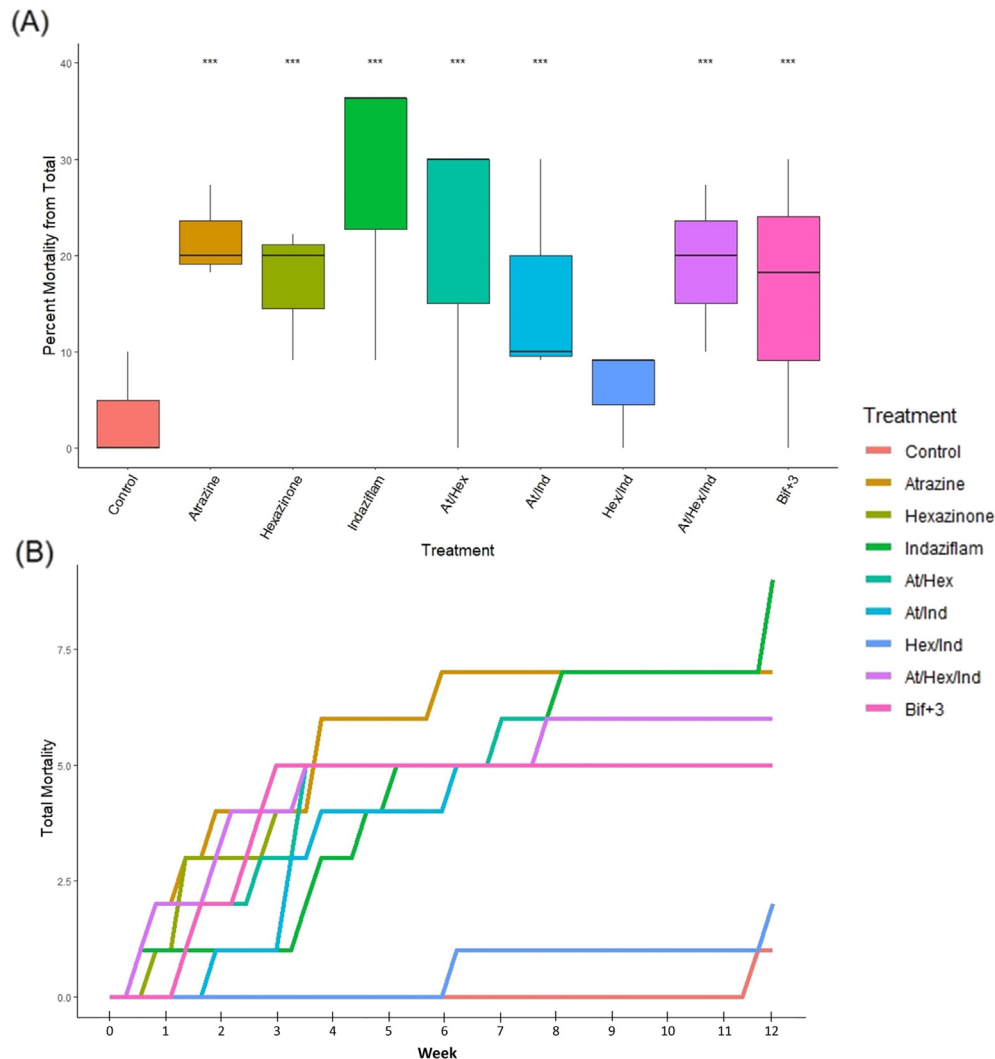
Fig. 5. Average total mortality A) by percent mortality from total starting clam population by treatment and B) throughout the experiment by treatment. Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values with abnormal outliers represented by black dots. High statistically significant difference ($p < 0.0001$) noted in figure solely in comparison to control and noted by triple asterisk.

Our results indicate that triazine compounds negatively affect clam health and lipophilic properties increase toxicity with potential lethal effects. Atrazine and hexazinone both share the same mechanism; however, the increased bioavailability of atrazine by comparison seems to increase its toxicity to clams. Indaziflam appears to be more toxic to clams than atrazine, at the same concentrations. Future work on pesticides' toxicity involving a more comprehensive suite of environmental contaminants aimed at observing effects across chemical classes and physical parameters will facilitate a fuller understanding of their combined, interactive effects.

4.3. Study limitations

Aspects of our study system may limit the breadth of findings. First, the wide variation in clam size among tanks, due to natural limitations in organism field collection, may have driven the lack of significant effects on condition index and shell length (Figs. 2, 3). While all clams were adults and size ranges did not differ among tanks, the range within tanks was wide meaning that younger, smaller adults had more potential for growth than older, larger clams, which produced variability in condition index. Ideally future studies would select individuals from a smaller size range, potentially purchasing them from a grower. As the protocol for aging soft-shell

clams requires sacrificing the animal (MacDonald and Thomas, 1980; Cerrato et al., 1991), we used shell length as a sorting category as other studies with this species have done (Frouin et al., 2007; Pariseau et al., 2009; Greco et al., 2011).

Next, the cross contamination of hexazinone detected in the atrazine and At/Ind treatments makes analysis of the effects of atrazine on clams difficult to interpret. However, in other studies examining environmentally relevant concentrations of atrazine on other bivalves (i.e., mussels, oysters), a variety of sub-lethal effects were observed (Nogarol et al., 2012; Juhel et al., 2017; Britt et al., 2020). In an acute study with the Brazilian mussel *Diplodon expansus*, mucus secretion, a protective mechanism, and cilia loss were observed on gill structures indicating negative effects from potential loss of food uptake and respiration (Nogarol et al., 2012). A study by Juhel et al. (2017) observed genotoxicity, impacts on the immune system, and inhibitions on endocrine systems during an acute one-week study in the green mussel (*Perna viridis*) at concentrations at and *below* environmentally detected limits. At concentrations as low as 3 μg/L, significant effects were observed on the microbiome and growth of the eastern oyster (*Crassostrea virginica*) in a two-month study (Britt et al., 2020). These studies provide a diverse range of observations on the negative effects of atrazine on bivalves at environmentally relevant concentrations.
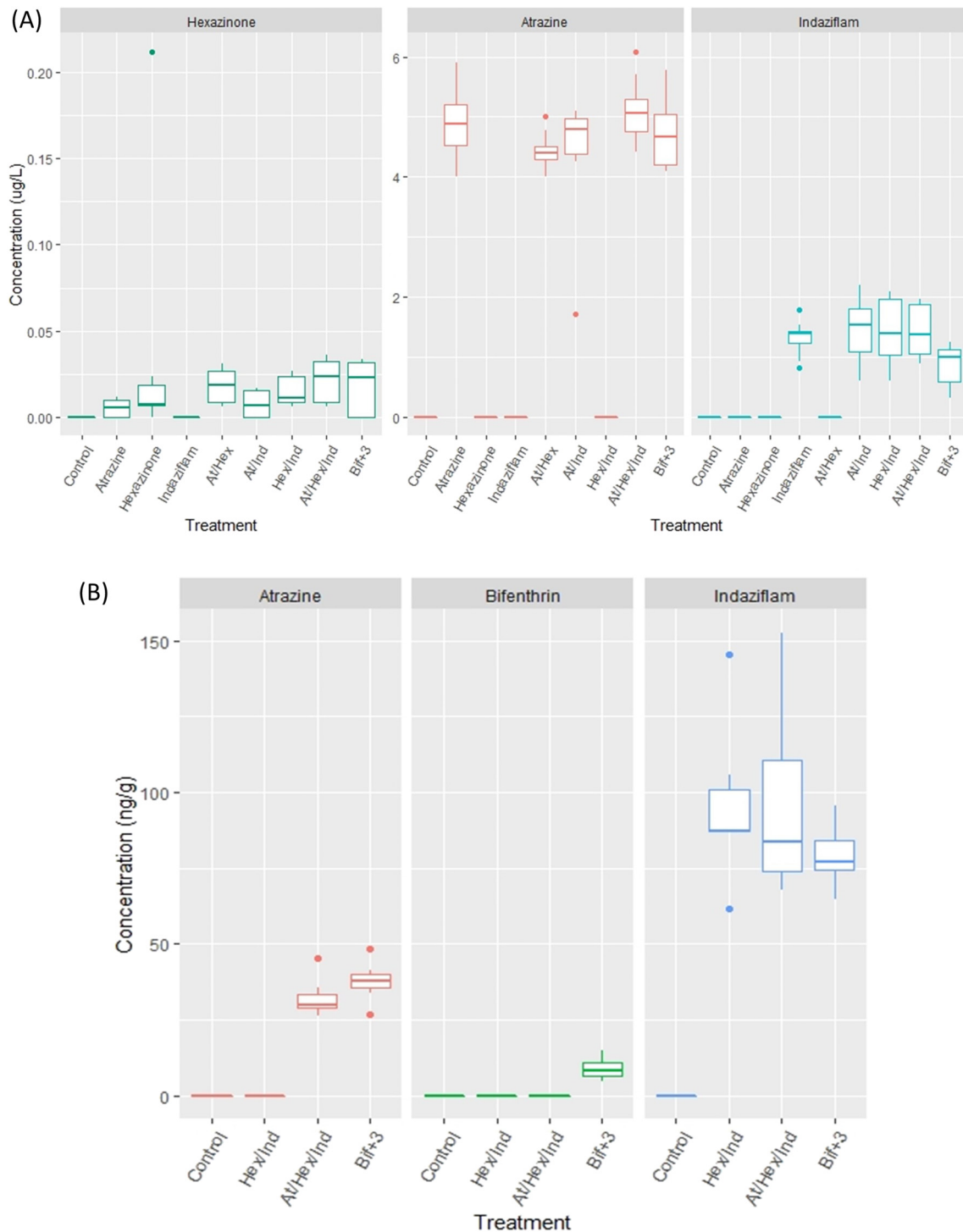
Fig. 6. Concentrations of A) hydrophilic and moderately hydrophobic compounds detected in water from each treatment, separated by compound tested (hexazinone plot separated due to magnitude difference in detection - note y-axis label) and B) moderately hydrophobic and hydrophobic compounds detected in clam tissue from four selected treatments, separated by compound tested. Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values with abnormal outliers represented by black dots.

Finally, in addition to the cross-contamination, limited information on forestry herbicide applications from industry partners made the dosing regime less precise than in exposure studies wherein data were available to mimic runoff events from multiple users of a watershed (Hasenbein et al., 2016; Britt et al., 2020). Environmentally relevant concentrations applied in this study reflect the combined exposure from potentially multiple land use types. Additionally, while information on exact compound concentrations was not available for determining experimental dosing concentrations, compounds detected in tissue samples at the end of the experiment did not exceed those detected in recent field sampling by Scully-Engelmeyer et al. (2021). Future studies would benefit from stronger data collaborations with industry to include more precise concentrations in

Table 2

Summary table of significant effects by treatment as compared to control; increase in parameter indicated by + and decrease indicated by −.

Treatments	Growth	Condition Index	Clearance Rates	Mortality
Atrazine				+
Hexazinone		−		+
Indaziflam			−	+
At/Hex	+		−	+
At/Ind				+
Hex/Ind		−		
3-Way (At/Hex/Ind)			−	+
Bif + 3				+

experimentation. While size variation affected statistical significance and a hexazinone contamination was detected, the patterns we observed across treatments at low concentrations highlight the need to address contaminant exposures with potential synergistic effects in aquatic systems. Despite experimental limitations, this study provides valuable information for management decision-making and highlights priority research needs to address environmentally relevant concentrations of aquatic contaminants.

5. Summary/conclusion

Herbicide combinations used in forestry pose a previously unquantified environmental risk to downstream shellfish populations. The herbicide indaziflam was particularly notable in this study as it negatively affected clams, when applied individually and in combination with other herbicides, and was detected in tissue samples at concentrations higher than those previously predicted from other studies (Tompkins, 2010; Brabham et al., 2014). These detected concentrations were much higher than those of atrazine, despite atrazine's known bioaccumulation properties (Jacomini et al., 2006; Flynn and Spellman, 2009; Scully-Engelmeyer et al., 2021; Fig. 6b). The dosing concentrations of both compounds were equal, pointing to higher bioavailability and/or lack of metabolism of indaziflam. This study is unique in reporting accumulated tissue concentrations of lipophilic compounds following known and environmentally relevant dosing concentrations during chronic (3-month) exposure.

Studies focusing on effects of one or two compounds over short periods of time can miss important details about environmental exposure including sub-lethal effects of chronic exposure or combined effects of multiple compounds. As pesticide fate varies based on physical and land use factors as well as the physio-chemical properties of the pesticides themselves (Arias-Estevez et al., 2008), organisms may be exposed to many combinations of these compounds from numerous sources. Therefore, studies on multiple chemical stressors, such as this, benefit from collaborations across multiple sectors. Future research should focus on combined effects of these and other compounds on diverse non-target organisms incorporating uses across a spectrum of industries to better understand community-level effects.

CRediT authorship contribution statement

Alexandra G. Tissot: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Elise F. Granek:** Conceptualization, Methodology, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition. **Anne W. Thompson:** Validation, Resources, Writing – review & editing. **Michelle L. Hladik:** Validation, Resources, Writing – review & editing. **Patrick W. Moran:** Conceptualization, Methodology. **Kaegan Scully-Engelmeyer:** Conceptualization, Software, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

During measurements, clams were kept on towels that had been soaked in cold seawater and were not exposed for more than ten minutes to avoid further stress.

The flow cytometer produced a graph for each sample comparing chlorophyll and forward scatter light particles to calculate a cell count; these graphs were analyzed using FloJo software (Version 10) whereby a window was created around particles to be selected for counting using the aforementioned particle definition (Fig. A2).

Table A1

Average and maximum concentrations of compounds detected in Oregon (U.S. Geological Survey, 2020).

Compound	Concentration	Average	Max
Atrazine	ng/L	80.00	6110
Bifenthrin	µg/kg	24.26	436
Hexazinone	ng/L	9.42	339

Table A2

Baseline algal density post-mixing, quantities in cells per mL.

Treatment	Timepoint										
	0	1	2	3	4	5	6	7	8	9	10
At/Hex	28337	25558	16172	17289	13171	12484	15900	10623	13952	8057	9365
At/Hex/Ind	23499	28061	15167	19376	10555	10644	15113	14282	10527	12812	7015
At/Ind	23559	16713	12097	18411	13299	11071	16638	9823	11057	8574	6740
Atrazine	18494	20583	12030	14919	16192	16401	18546	8625	12772	10452	6143
Bif + 3	20813	22905	13063	18092	14905	8991	10550	8744	9715	9294	5998
Control	20948	15656	10098	12509	16255	11218	15934	9774	7732	9135	5875
Hex/Ind	12657	14974	8992	9778	11763	9873	12963	9643	9832	7213	6479
Hexazinone	14161	17052	12330	14607	16357	13749	18296	9656	10553	13272	6683
Indaziflam	24684	24140	15097	20264	14856	9282	15270	10907	12261	9793	8647

Table A3

Mean detected concentrations of dosed compounds $\pm$ SE by treatment. Detections in water (w) reported in μg/L, detections in tissue (t) reported in ng/g; non-detected compounds noted ND. Cross contamination points highlighted, and samples that were not tested are marked by $\neq$.

Treatments	Atrazine (w)	Hexazinone (w)	Indaziflam (w)	Atrazine (t)	Indaziflam (t)	Bifenthrin (t)
Control	ND	ND	ND	ND	ND	ND
Atrazine	4.92 ± 0.189	0.005 ± 0.002	ND	$\neq$	$\neq$	$\neq$
Hexazinone	ND	0.032 ± 0.023	ND	$\neq$	$\neq$	$\neq$
Indaziflam	ND	ND	1.31 ± 0.099	$\neq$	$\neq$	$\neq$
At/Hex	4.43 ± 0.107	0.018 ± 0.003	ND	$\neq$	$\neq$	$\neq$
At/Ind	4.41 ± 0.352	0.008 ± 0.002	1.50 ± 0.178	$\neq$	$\neq$	$\neq$
Hex/Ind	ND	0.015 ± 0.003	1.45 ± 0.188	ND	95.7 ± 9.68	ND
3-Way (At/Hex/Ind)	5.09 ± 0.179	0.022 ± 0.004	1.44 ± 0.135	32.4 ± 2.42	96.3 ± 11.6	ND
Bif + 3	4.74 ± 0.187	0.019 ± 0.005	0.894 ± 0.113	37.8 ± 2.50	79.2 ± 3.73	9.05 ± 1.35

Fig. A1. Experimental tank set-up in laboratory at PSU, clams were maintained in sand in 12 L tanks within larger 64 L closed-system tanks to reduce further stress and mimic natural conditions.
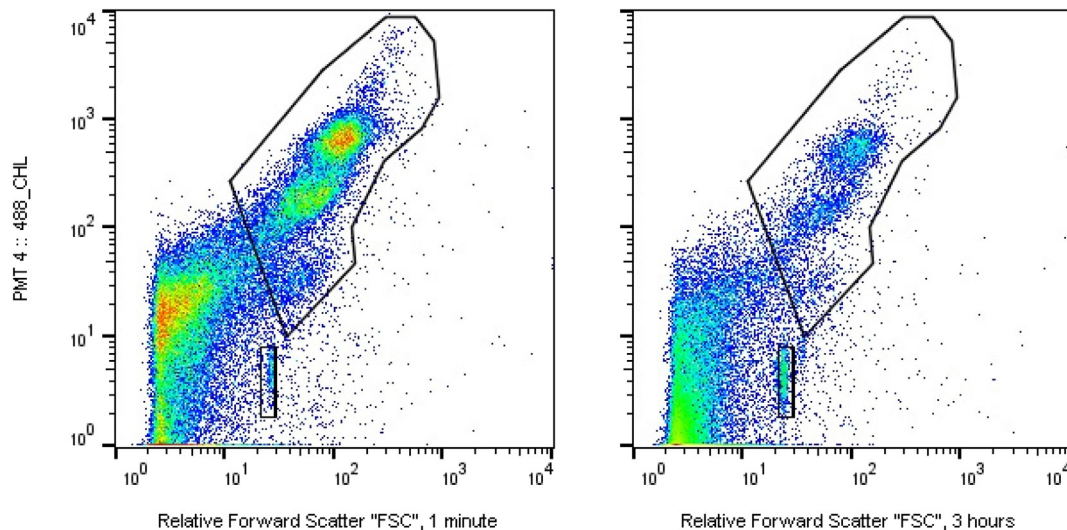
Fig. A2. Flow cytometer cell count selection window; initial concentration vs final. Color indicates density of cells, lower selection window chosen for size calibration using micron beads with upper window chosen for cell counts.
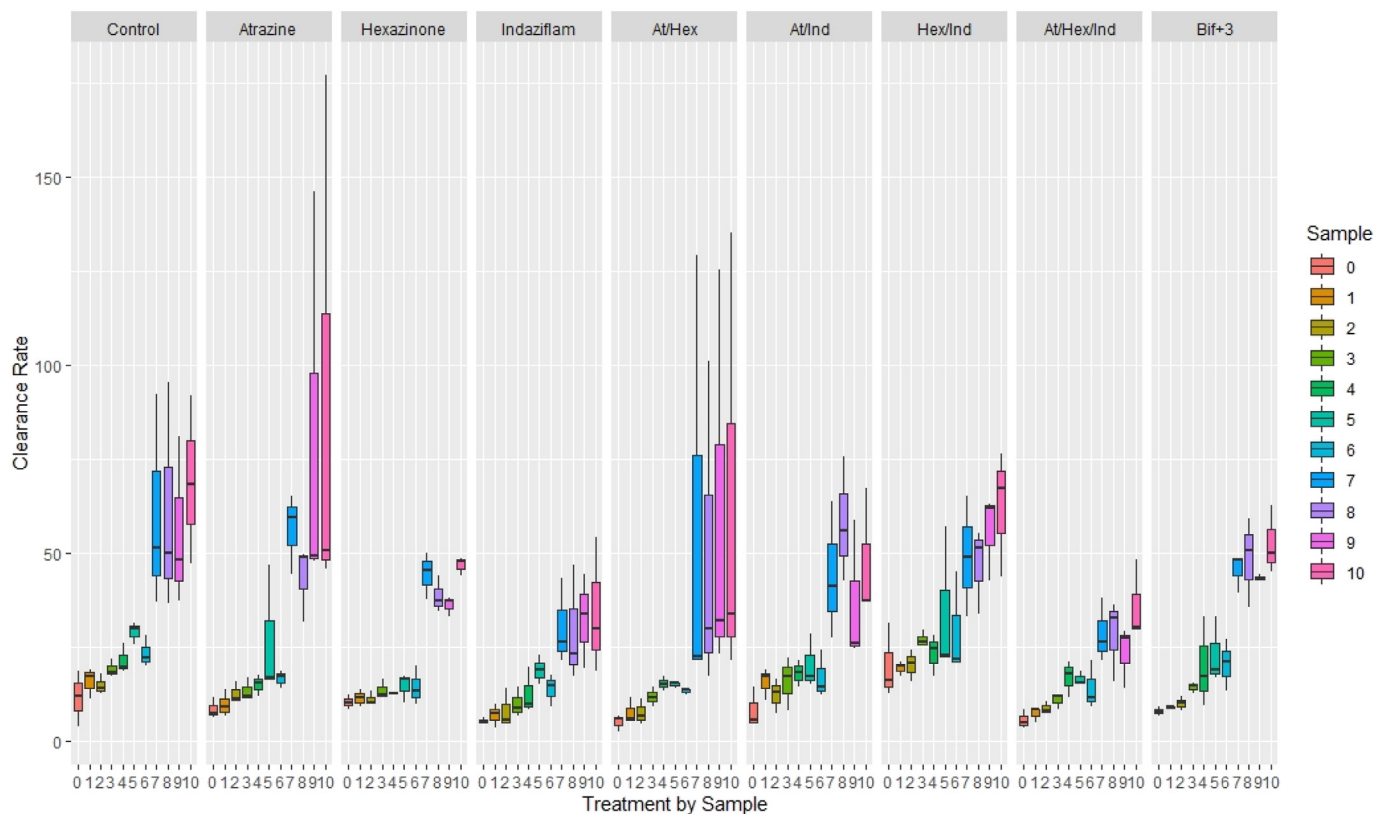

Fig. A3. Algal clearance rates throughout experiment, separated by treatment and showing sample point. Box plots represent distribution of data with thick black lines representing median values and upper and lower quartiles forming the box. Boxes missing upper or lower quartile are indicative of median being identical to upper or lower quartile, respectively. Lines outside of the box represent the range of "normal" lowest and highest values.

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