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Exposure duration and composition are important variables to predict short-term toxicity of effluents to a tropical copepod, *Acartia sinjiensis*

Sarah Stone*[1,2], Kitty S. McKnight[1,3], Christoph Naab[1,4], Darren J. Koppel[1,5], Monique T. Binet[1], Dianne F. Jolley[1], Stuart L. Simpson[1,6]

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[1] Centre for Environmental Contaminants Research, CSIRO Land and Water, Tharawal Country, Lucas Heights, NSW, Australia

[2] School of Life Sciences, University of Technology Sydney, Gadigal Country, NSW, Australia

[3] Department of Biological Sciences, Macquarie University, Wallamattagal Country, NSW, Australia

[4] University of Augsburg, Universitätsstraße 2, D-86159 Augsburg, Germany

[5] Faculty of Science and Engineering, Curtin University, Noongar Country, Bentley, WA, Australia

[6] Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, China

***Corresponding Authors:** sarah.stone@csiro.au; stuart.simpson@csiro.au

Highlights

- Toxicity of complex effluents increased slightly with increasing 6-78 h duration
- Nauplii were more sensitive to an 18-h and 78-h exposures compared to 6-h exposure
- Time-weighted average concentrations were not suitable for predicting mixture toxicity
- Continuous exposures provided conservative toxicity estimates for pulse exposures
- Concentration-addition models provided useful prediction of mixture pulse toxicity

Abstract

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Predicting the toxicity of effluent exposures, which vary in duration, composition, and concentration, poses a challenge for ecological risk assessments. Effluent discharges may frequently result in the exposure of aquatic organisms to high concentrations of mixed contaminants for short durations. In the receiving environment effluents will undergo dilution and physical or chemical processes that further reduce contaminant concentrations at varying rates. To date, most studies comparing toxicity risks of continuous and pulsed contaminant exposures have focused on individual contaminants. In this study, the toxicity to the tropical euryhaline copepod *Acartia sinjiensis* of two complex effluents was assessed, comparing 6- and 18-h pulses and 78-h continuous exposures. Observations of larval development success and population size were completed after a 78-h incubation period, to observe for latent effects after pulse exposures. The chemical compositions of the effluents were assessed over time and different contaminants (i.e., metals, ammonia or organics) declined at differing rates. These were characterized as either a minimal, steady, or rapid decline. Nauplii development and population after 78 h were more impacted by effluent exposures following an 18-h pulse, compared to a 6-h pulse. Based on pulse-exposure concentrations, the 50% effect concentrations (EC50) were similar for continuous and 18-h exposures but up to 3-fold greater (lower toxicity) for the shorter 6-h exposures. Time-weighted average concentrations did not accurately predict toxicity from pulse exposures of the effluents. Concentration-addition toxicity modelling using toxicity data from pulse exposures of single contaminants was useful for predicting the toxicity of chemical mixtures exposed for varying durations. Recommendations for modified approaches to assessing risks of short-term effluent discharges are discussed.

Introduction

The ecological risk assessment of short-term discharges such as stormwater runoff or intermittent industrial effluent discharges to the aquatic environment can be challenging because they can contain temporally and spatially variable mixtures of contaminants. The discharge events may result in highly varied contaminant exposures to aquatic organisms that become difficult to fully characterize once effluents or runoff enters the aquatic environment.

Common approaches for assessing toxicity risks involve comparisons of chemical concentrations with water quality guideline values (WQGVs) or undertaking whole effluent toxicity (WET) tests (USEPA, 2002; Warne et al., 2018). However, these risk assessment tools were designed primarily for assessing risk of continuous, and usually chronic, exposure conditions rather than pulse exposures. Provided WQGVs exist for the chemicals present, and WET methods are suitably sensitive, both approaches can provide a desired conservative assessment of risks posed by pulse exposures (Colvin et al., 2021; Stone et al., 2021). However, overly conservative assessments may occur when assessing risk from short-term pulses. An example of this may be the risk assessment of produced waters released from oil and gas operations via permitted discharges. The long exposure times of continuous exposure toxicity tests may not accurately reflect the toxicity of discharges which are modelled to rapidly dilute upon mixing with receiving waters (Gissi et al., 2021). In such scenarios, toxicity risk assessments may result in undesirable outcomes for those responsible for the discharges and those managing the environment through higher and potentially unnecessary costs associated with management or remediation (Angel et al., 2020).

The comparison of contaminant mixture toxicity under continuous or short-term exposure conditions can improve environmental relevance of reference data and understanding of tools for assessing risk, thereby improving environmental management practices. However, a number of important knowledge gaps exist that need to be addressed to substantially improve

risk-assessment approaches for pulse exposures. Most studies investigating toxicity modifying factors relevant to short-term exposures are explored using single contaminant exposures, including factors such as exposure duration (Gama-flores et al., 2006; Hogan et al., 2013), exposure frequency (Angel et al., 2010; Diamond et al., 2006), organism life stages (Hoang and Klaine, 2007; Stone et al., 2021), recovery time and latent effects (Naddy et al., 2000, Mebane et al., 2021). Mixtures of contaminants are common exposure scenarios for effluents. Mixture toxicity is typically studied under continuous exposure conditions, and few published studies have described the toxicity caused by pulse exposures to characterized contaminant mixtures (followed by a recovery period for the remainder of test duration to observe latent endpoints) (Gissi et al., 2021; Angel et al., 2020; Mehler et al., 2020).

For some organisms and contaminants, the toxicity of continuous and short-term contaminant exposures have been observed to be similar when compared on a time-weighted average concentration (TAC) basis, where the concentration of the pulse exposure is normalized over the total test duration (Angel et al., 2017, 2015, 2010; Stone et al., 2021). For such exposures, the risk assessment of contaminant pulses may be extrapolated from comprehensive concentration-effects databases of continuous toxicity exposure. This TAC-based approach may also be effective for predicting toxicity of metal mixtures (Angel et al., 2020), but less-effective for rapidly acting chemicals such as ammonia or organic contaminants (Stone et al., 2022; Diamond et al., 2006; Burton et al., 2000). The effectiveness of the TAC-based approach for predicting the toxicity of complex mixtures containing both inorganic and organic contaminants is not well known. Understanding the chemistry (fate processes for different contaminants), toxicity (differing modes of action and toxicokinetics) and transport (environmental processes) of short-term contamination is important to enabling the development of models that can more accurately predict pulse exposure toxicity, and thus improving the risk assessment of such events.

103 In this study, it was hypothesized that pulse toxicity trends observed in individual
104 contaminant exposures may not appropriately describe how the toxicity of mixtures can
105 change under pulse conditions. The objectives of the study were to investigate: (1) if the life
106 stage of the organism affects the toxicity of a mixture, (2) if duration of exposure affects the
107 toxicity of mixtures, (3) if trends observed in single contaminant toxicity inform or predict
108 the trends observed in mixtures, and (4) if TAC-based normalisations are useful for
109 predicting the toxicity of mixtures under pulse exposure conditions. If the toxicity of mixtures
110 is affected by these factors, then toxicity-testing paradigms may be shifted to incorporate
111 these, or improved models should be developed to better assess risk in the aquatic
112 environment and improve the environmental relevance of ecotoxicity data.

113 **Methods**

114 ***General laboratory techniques and reagents***

115 All glassware and plasticware used for culturing and toxicity testing were cleaned to a
116 rigorous standard as described by Stone et al. (2021). Filtered seawater was used in culturing
117 and toxicity testing as the media base, control and diluent water. Seawater was collected from
118 Cronulla (NSW, Australia), filtered on the day of collection to 0.45 µm (Sartobran P sterile
119 midicap, Sartorius Stedium Biotech) and stored in the dark at 4 °C until use (Gissi et al.,
120 2013).

121 Physico-chemical parameters were monitored periodically, throughout testing and
122 culturing, to ensure consistent test conditions over the 78-h exposures (Table S1). The
123 monitored parameters included dissolved oxygen (% saturation), light intensity, salinity
124 (reported as practical salinity units, PSU), temperature and water pH (Table S2). Chemical
125 stock solutions used for toxicity test treatments were prepared using analytical grade reagents
126 as described in the Supplementary Information.

127 The artificial effluent mixtures (A and B) were prepared using different techniques.
128 Mixture A was prepared once by combining two wastewater samples (potential effluents)
129 from the oil and gas industry to form a complex mixture of organic and inorganic
130 contaminants typical of what might be discharged into the marine environment. Once
131 combined and mixed, Mixture A was dispensed into multiple pre-cleaned 40-mL amber glass
132 vials, capped immediately with Teflon septa-lined lids and stored in the dark at 4°C until use.
133 The dispensing of Mixture A into multiple vials and capping for later use reduced the
134 change over time of contaminant concentration (e.g., loss of volatile compounds). In-house
135 testing has shown that little, if any, change to toxicity occurs when produced waters are
136 stored in this manner (4°C, dark, without headspace) for several weeks (unpublished data).
137 The current study involved experiments conducted over 10 weeks. While effluent dilutions
138 were confirmed in each experiment by trace metals analyses, the composition of organic
139 analytes was unable to be measured in each individual exposure introducing some uncertainty
140 in the chemical composition change between exposures. The continuous toxicity exposures
141 run alongside all pulse toxicity exposures resulted in similar toxicity endpoints for Mixture A,
142 indicating that the potential change of mixture composition, and hence toxicity, was minimal
143 over the experimental period.
144 Mixture B, a synthetic mixture prepared in the laboratory, was prepared immediately
145 prior to test or pulse initiation, by spiking individual chemicals into test treatments to create a
146 nominal Mixture B composition (undiluted) of 38 µg copper/L, 6 mg propoxur/L, 28 mg
147 ammonia/L, and 28 mg SDS/L. These four contaminants were chosen to represent an effluent
148 scenario with contaminants of varying modes of action. The measured constituents of
149 Mixtures A and B are described in the Chemical characterisation section, in the Results and
150 Discussion.

151 Dissolved organic carbon (DOC) was not measured in treatments for this study. Prior
152 studies using seawater from the same source indicate a range of 1.5 ± 0.25 mg DOC/L in
153 seawater control treatments (unpublished data), and Mixture A was prepared using two
154 effluents, 1 part 280 mg DOC/L and 3 parts 12 mg DOC/L.

155 ***Chemistry-only exposure analysis***

156 An experiment was conducted with Mixture A to assess for changes in concentrations of
157 major organic contaminants over time, under the conditions used in the toxicity tests (Table
158 S1). Other contaminants of concern including metals and ammonia were analysed
159 comprehensively throughout all other toxicity tests. A dilution series of Mixture A was
160 prepared (% v/v) in seawater (0, 1, 3, 6, 22 and 36%), and a larval development toxicity test
161 was initiated following the methods described below. Due to the volume required for
162 analyses, a large portion of the volume of each replicate vial was utilized for chemistry sub-
163 sampling over time. Therefore, although organisms were added to these solutions, the effect
164 endpoints for the organisms in these tests were not assessed, as the sub-sampling and volume
165 changes were likely to impact on larval development and population, i.e., this was a
166 sacrificial experiment used for chemistry assessment only.

167 Sub-samples of Mixture A dilutions were collected for analysis of organic compounds at
168 five timepoints (0, 6, 18, 48, and 78 h) throughout test exposures. Sub-samples were from
169 individual replicates, filtered to 0.45 µm to remove test organisms (polyethersulfone,
170 Sartorius Minisart syringe filters) and stored in the dark at 4 °C in amber glass vials with
171 Teflon lids until analysis. Organic analytes were polycyclic aromatic hydrocarbons (PAHs),
172 phenols, benzene, toluene, ethylbenzene, xylene (BTEX), total recoverable hydrocarbons
173 (TRHs) and fatty acids (Table S3). Other target analytes were able to be assessed
174 comprehensively during all toxicity tests, and included a suite of metals (including copper,
175 zinc and manganese) and ammonia (See chemicals analyses section).

176 ***Larval development toxicity test***

177 The toxicity test used in this study was a chronic, static larval development toxicity test
178 using *A. sinjiensis* which is described in detail by Stone et al. (2021), modified from Binet et
179 al. (2019), and summarized here in brief. Culturing protocol is described in detail in
180 Supplementary Information for the copepods and microalgae used as feed. This toxicity test
181 assessed organism development from egg (start of test), through several nauplii stages, to the
182 test endpoint of metamorphosis into copepodite (78 h), described as the larval development
183 ratio. Quality assurance included physico-chemical parameters, measured contaminant
184 concentrations, handling controls for each pulse scenario, and reference toxicant data (Table
185 S4). Organisms were cultured under controlled conditions (Table S1), and details of the
186 methods are provided in the Supplementary Information.

187 *Continuous exposures*

188 The continuous toxicity test had a 78-h exposure period (no water renewal). All toxicity
189 tests had: (i) control treatments without contaminants added, (ii) a nickel reference toxicant to
190 assess changes in organism sensitivity over time, and (iii) a dilution series of either of the
191 artificial effluents: Mixture A or B (Table 1). Control treatments were undertaken in
192 quadruplicate, and contaminant treatments in at least triplicate for each concentration.
193 Reference toxicant treatments were run in duplicate or triplicate with four measured treatment
194 concentrations (4, 8, 12, and 16 µg Ni/L).

195 Test treatments for the nickel reference toxicant and Mixture B were prepared with
196 180 mL of warmed seawater (28–31 °C) and algal feed in 250-mL polycarbonate containers
197 (TechnoPlas). Live algae were provided as food (rinsed of nutrient rich media, and
198 concentrated) at nominal cell densities of 6.3×10^3 cells/mL *Tetraselmis chuii* and
199 5×10^4 cells/mL *Tisochrysis lutea*, with densities confirmed using flow cytometry (Binet et
200 al., 2019). The nickel (reference toxicant) and Mixture B dilutions were prepared by adding

201 aliquots of chemical stocks to the 180 mL of diluent. Test treatments for Mixture A were
202 prepared as a dilution series in seawater in bulk (% v/v) and warmed, followed by addition of
203 algae as food at the same densities as above.

204 To initiate the tests, the toxicity test treatments were randomized and inoculated by
205 pipetting from a concentrated stock of eggs collected from healthy adults isolated 24 h
206 earlier. Targeted organism density for each experiment was between 40 – 60 eggs, with initial
207 densities varying by ± 6 eggs between treatments. There were then three activities to confirm
208 the starting conditions. Firstly, test treatments were incubated at 28–31 °C, in low light
209 conditions, on an 18:6 h light:dark light cycle (Table S1). Secondly, egg numbers were
210 verified by counting eggs vials collected periodically throughout inoculation using
211 microscopy (Olympus SZx10, 6.3–100 × magnification). Finally, sub-samples were collected
212 from separate replicates in each treatment for physico-chemical analyses (pH, dissolved
213 oxygen and salinity) and for analysis of metals and ammonia (filtered to 0.45 µm upon
214 collection and stored at 4 °C until analysis). Collection of sub-samples was done using a
215 mesh (20 µm) covered pipette tip to retain all organisms in the test solutions.

216 During the test, all treatments were randomly rearranged in the temperature-control
217 cabinet twice daily and fed at 48 h to maintain feed concentrations of 3.1 – 6.3 × 10^3 cells/mL
218 *T. chuii* and 4 – 5 × 10^4 cells/mL *T. lutea*. All tests were terminated at 78 ± 2 h, when larval
219 development (nauplii to copepodite) had occurred in at least >50% of organisms in the
220 control treatments.

221 To complete the tests, sub-samples were again collected for physico-chemical and
222 dissolved metals and ammonia analyses, after which the organisms within treatments were
223 fixed and stained with formalin (0.5%) and Rose Bengal (0.02%), and then refrigerated (<4
224 °C) for a minimum of 24 h before counting. Rose Bengal stained the translucent organisms
225 pink, permitting easier microscope assessment. Organisms must be fixed with formalin

226 before counting as they move rapidly and erratically, thus cannot be counted alive at the
227 densities used in these tests. The staining/fixative solutions were sieved to collect the
228 organisms and counted microscopically (6.3 – 100× magnification) to assess the number of
229 unhatched eggs, nauplii and copepodites in each replicate.

230 To assess toxicity test endpoints, the larval development ratio (LDR) and population size
231 for each replicate were calculated as described by Binet et al. (2019) as follows:

232 $$Larval\ population, measured\ as\ total\ number\ of\ hatched\ animals = (N_t + C_t) \quad (1)$$

233 $$Larval\ development\ ratio\ (LDR_t) = C_t/(N_t + C_t) \quad (2)$$

234 where: t = time of test termination (hours), N = number of individual nauplii and C = number
235 of individual copepodites (N_t, C_t: at time t).

236 Larval development is often a more sensitive endpoint compared to population size for *A.*
237 *sinjiensis* (Stone et al., 2021 and 2022). Population is included as an endpoint, used as a
238 proxy for organism survival (Binet et al., 2019). True organism survival endpoints cannot be
239 observed following this method as organisms are fixed with formalin before counting and it is
240 not possible to tell whether organisms were alive or dead at the test termination.

241 Experimental data were only included in dose-response analyses when ≥50% LDR was
242 observed in continuous control treatments for that experiment (Table S1) and the EC50 for
243 dissolved nickel reference toxicant was within Cumulative Sum chart limits (Table S4). The
244 acceptability criterion of ≥50% LDR is commonly used for *A. sinjiensis*, and other copepod
245 species, as it allows for slower or faster development to be observed in treatments compared
246 to controls (Binet et al., 2019; Gissi et al., 2018; Kusk and Wollenberger, 2005; Stone et al.,
247 2021; Wollenberger et al., 2003).

248 *Pulse exposures*

249 Pulse exposure toxicity was assessed for the two artificial effluents: Mixture A and B
250 (Table 1). Single pulse exposures were considered in this study as *A. sinjiensis* has a rapid life

251 cycle, and it is likely that such short-lived organisms may only experience a single
252 contamination event. Each pulse exposure scenario had corresponding handling controls that
253 underwent the same treatment (i.e., initial test volumes, timing of water changes, water
254 quality measurements, and sampling over the 78-h test). Pulse durations of 6- and 18-h
255 duration are representative of typical rainfall periods as determined by Colvin et al. (2021).

256 This study employed a square profile exposure design, as is most prevalent in current
257 pulse-exposure methodology (Stone et al., 2022). This was chosen over a tapered exposure
258 design which, although possibly more environmentally relevant (Gordon et al., 2012), is less
259 practical, less reproducible, and requires significantly more chemical analyses to adequately
260 characterize.

261 For Mixture B, test treatments destined for pulse exposures were prepared and incubated
262 exactly as continuous control treatments described above with 180 mL of diluent. Pulses were
263 initiated after 24 h by adding aliquots of stocks of the four chemicals present in the mixture at
264 the correct ratio. This method was not possible for Mixture A, since the complex mixture was
265 a whole effluent, so concentrated stock contaminants could not be added in small aliquots.

266 For Mixture A, test treatments destined for pulse exposures were prepared and incubated
267 as continuous control treatments described above, except with an initial test volume of 60 mL
268 as is used in the original method by Binet et al. (2019). At the pulse initiation time (either 24
269 or 48 h after test initiation), 120 mL of a dilution series of Mixture A, which had been
270 prepared in seawater and warmed (28–31 °C), was added to the appropriate replicate. The
271 dilution series accounted for the 60 mL of seawater already present in each treatment, so that
272 the final concentration and volume (180 mL) of Mixture A in the replicate was comparable to
273 that in the continuous exposure.

274 Termination of the effluent pulse-exposures (Mixture A and B, handling controls) were
275 completed by replacing at least 99% of contaminated water with clean diluent (seawater and

276 algal feed). Contaminated water was removed by reducing the overlying water to 20 mL,
277 using a 54-µm polyester mesh sieve, and replacing with the diluent to 180 mL, completed
278 twice. For all pulse treatments, filtered sub-samples for chemical analysis and measurement
279 of physico-chemical parameters were collected immediately after pulse initiation (24 or
280 48 h), and before and after pulse termination (30, 42, 54 or 66 h). Following this process,
281 treatments were incubated for the remainder of the 78-h exposure period. To complete the
282 tests and assess the endpoints the method was the same as continuous treatments, described
283 above.

284 ***Chemical analyses***

285 Throughout toxicity testing, sub-samples were collected for analyses to chemically
286 characterize exposure scenarios. At a minimum, samples were collected at the beginning and
287 end of exposures (pulse and continuous) for analyses of metals and ammonia. While as much
288 chemical characterisation was undertaken as possible, there were unknown constituents in
289 each mixture, similarly to many effluent samples that require assessing for toxicity in a risk
290 assessment or regulatory scenario.

291 All sub-samples were taken under test conditions, and immediately stored for
292 analyses. Sub-samples for metals analysis were collected in acid-washed 5-mL polypropylene
293 vials (Technoplas, Australia), filtered, acidified to 0.2% with nitric acid (Tracepur, Merck)
294 and stored at <4 °C until time of analyses. Sub-samples for ammonia analysis were filtered
295 and frozen (-20 °C). Sub-samples for organics analysis were collected as described in the
296 chemistry-only exposure section.

297 Organic analytes were measured using gas chromatography-mass spectrometry
298 (National Measurement Institute [NMI], NSW, Australia). In-house methods varied for each
299 group of analytes; polycyclic aromatic hydrocarbons (NGCMS_1111), phenols

300 (NGCMS_1111), BTEX (NGCMS_1121), total recoverable hydrocarbons (NGCMS_1112 or
301 1121) and fatty acids (NGCMS_1131).

302 Thawed seawater samples were analysed for total ammonia (mg NH_3-N/L) using a
303 discrete analyser (AQ400 Colorimetric nutrient analyser, SEALS Analytical, USA) or
304 segmented flow analyser (NMI, in-house method NWD17). Seawater samples destined for
305 ammonia analysis were diluted 10-fold with ultrapure water. The quality assurance for this
306 analysis included blanks, duplicate samples, spiked duplicate samples and an independent
307 standard after every 10th analysis.

308 A suite of metals was analysed, including copper, nickel, manganese, and zinc.
309 Measured metal concentrations were consistent in the dilution series, confirming the nominal
310 %-dilution of each mixture. Metals samples were analysed using a combination of
311 inductively coupled plasma-atomic emission spectrometry (ICP-AES, Agilent 720ES) and
312 ICP-mass spectrometry (ICP-MS, Agilent 7900). For each metal, nominal concentrations >4
313 µg/L were analysed using ICP-AES. All other samples were analysed using ICP-MS. Quality
314 assurance involved seawater calibration standards and blanks (acidified to 0.2% HNO_3).
315 Signal concentration drift was observed and corrected for using a multi-element standard
316 (QCS27, Analytical West Inc.). Duplicate and spiked duplicate samples were run after every
317 10 samples, only during ICP-MS analyses. Both the CSIRO and NMI laboratories were
318 accredited (National Association of Testing Authorities (NATA)).

319 ***Statistical analyses***

320 Data analyses and graphics generation were completed using the statistical environment R
321 studio (version 1.1.423, RStudio Team, 2020) with extension packages *drc* for toxicity
322 modelling (Ritz et al., 2015), and *ggplot2* (Wickham, 2016) or *ggpubr* (Kassambara, 2020) to
323 generate all figures.

324 *Exposure concentrations*

325 Exposure concentrations in this study were calculated as the pulse-exposure concentration
326 (PeC) and time-weighted average concentration (TAC). The PeC was equivalent to the
327 concentration of contaminant present during the pulse-exposure period only, while the TAC
328 was calculated as the exposure concentration normalised over the entire test duration (78-h).

329 TACs were calculated as per Angel et al. (2010) where the product of pulse concentration
330 (P_C) and pulse duration (P_D) were added to the product of the residual concentration (R_C) and
331 the duration the residual concentration was present (i.e., the test duration (T_D) minus duration
332 before pulse initiation (I) and pulse duration). This exposure concentration is then divided by
333 the test duration to normalise the exposure, as shown in Equation 3:

334 $$TAC = \frac{((P_C \times P_D) + R_C \times (T_D - I - P_D)}{T_D} \quad (3)$$

335 *Concentration-addition modelling*

336 Concentration-addition modelling was applied as a conservative approach in assessing
337 mixture toxicity (Cedergreen et al., 2008). This approach utilized toxic units, calculated for
338 ammonia, copper and propoxur by dividing the measured (copper and ammonia) or nominal
339 (propoxur) concentration of the contaminant in the mixture by the modelled individual EC50
340 for that contaminant derived from 6-, 18- and 78-h exposures, as per Equation (4) (Figure S1)
341 (Koppel et al., 2018).

342 $$\sum TU_i = \sum_{i=1}^{n} \frac{C_i}{EC50_i} \quad (4)$$

343 Here: n = the number of measured mixture components, C_i = the concentration of the
344 individual mixture component, and $EC50_i$ = the concentration of the component i that causes
345 a 50% effect at the equivalent duration of exposure.

346 For each mixture and exposure duration, the sum of toxic units was calculated using
347 contaminants where individual toxicity data was available at the relevant exposure duration to
348 determine whether concentration-addition was a suitable predictor of toxicity under

349 continuous and pulse conditions. This approach utilized individual contaminant toxicity data
350 collected for copper, ammonia and propoxur in previous studies (Stone et al., 2021 and
351 2022). The response data were modelled with concentration expressed as $\sum TU_i$ (Koppel et
352 al., 2018).

353 *Toxicity estimates*

354 Reported toxicity estimates were based on nominal dilution series that were verified by
355 measured concentrations. Larval development rates or population size as a percent of
356 handling controls were used to derive all toxicity values. The endpoints were normalized to a
357 percent of control by dividing the treatment endpoint by the average endpoint observed in
358 control treatments. The toxicity estimates for each exposure scenario were calculated as the
359 effect concentration (EC) values and their 95% confidence limits. Data were pooled from
360 repeat experiments. These toxicity estimates were produced using models fitted to each
361 dataset (Table S5). Models of best fit were determined using the *mselect* function in the *drc*
362 package, comparing the Akaike Information Criterion values. The models used were either
363 log-logistic two-parameter, or Weibull (type 1, three-parameter or type 2, two-parameter).

364 The continuous and pulse toxicity of the individual contaminants copper, ammonia and
365 propoxur were described by Stone et al. (2021 and 2022) and were collated in the current
366 study for comparison purposes. Toxicity test conditions were identical to those described in
367 the current study.

368 The EC values were compared between different exposure scenarios to determine
369 significant differences. This was done using the *EDcomp* function in *drc* (Ritz et al., 2015,
370 Table S6). The LDR endpoint was compared between exposure conditions using a two-way
371 ANOVA test, followed by a Tukey's Test to make pair-wise comparisons (base R,
372 unbalanced designs, $p<0.05$).

373 **Results and Discussion**

374 ***Chemical characterisation***

375 The artificial effluents Mixture A and Mixture B were chemically characterized
376 throughout the exposure periods. Analytes detected in Mixture A (21%-effluent at 0 h) were:
377 13 µg Cu/L, 280 µg Mn/L, 2.9 mg NH_3-N/L, 67 µg naphthalene/L, 3.4 µg fluorene/L, 4 µg
378 phenanthrene/L, 400 µg phenol/L, 250 µg 2-methylphenol/L, 260 µg 3-& 4-methylphenols/L
379 and 290 µg 2,4-dimethylphenol/L, 47 µg benzene/L, 18 µg toluene/L, 71 µg *m,p*-xylene/L, 34
380 µg *o*-xylene/L, 170 µg TRH C6-C9/L and 580 µg TRH C10-C14/L (Table S3, Table S7).
381 Mixture B (7.6% at 0 h) contained 1.8 µg Cu/L, 1.9 mg NH_3-N/L, 460 µg propoxur/L
382 (measured) and 2.1 mg sodium dodecyl sulfate/L (nominal) (Table S7).

383 The test organism *A. sinjiensis* is sensitive to copper, ammonia and propoxur with 78-h
384 LDR EC50s of 1.7 µg Cu/L (Stone et al. 2021), 2.7 mg NH_3-N/L, and 260 µg propoxur/L
385 (Stone et al., 2022). Thus, significant toxicity was expected to be observed after continuous
386 exposure of *A. sinjiensis* to the two mixtures.

387 Test exposure concentrations are most commonly reported at either one time point
388 (usually the start of tests) or averaged between test initiation and termination (USEPA, 2002;
389 Warne et al., 2018). The different chemicals analysed (including metals, ammonia and
390 organics in both Mixture A and B) showed differing profiles of declining concentration over
391 the exposure duration (example shown in Figure 1; Actual data are provided in Figure S2,
392 Table S3 and S7). Concentrations of different contaminants declined by 0-82% over a 6-h
393 pulse exposure, by 1-100% over an 18-h pulse exposure duration, and by 35-100% over the
394 78-h test duration. This resulted in a discrepancy between the arithmetic mean (average of 0-
395 and 78-h measurements) and 78-h TAC, with the arithmetic mean up to 370% greater for
396 organic compounds (Table S3).

397 These results reiterate the findings of Simpson et al. (2003), where concentrations
398 declines were attributed to adsorption to test vessels, uptake by test organisms and food,

399 volatilisation, or degradation, and the choice of exposure concentration metric used to
400 calculate a test endpoint was shown to significantly change the reported EC values. Ideally,
401 where contaminant loss over time is significant, the TAC should be calculated using multiple
402 measurements over the exposure duration to ascertain the exposure concentration with
403 sufficient accuracy (e.g., ±20%). A single measurement at the start of tests, or end of tests, or
404 even start and finish for rapidly declining compounds may result in inaccuracy of the TAC of
405 >50%.

406 The measurements enabled three classes of chemical loss to be identified, broadly
407 classified as those that (i) are relatively stable over the test duration (e.g., manganese,
408 phenols, ammonia), (ii) display a relatively steady decline over the entire 78-h test duration
409 (e.g., copper, propoxur), and (iii) steep decline within the first 18 h of exposure (e.g.,
410 benzene, naphthalene, toluene) (Figure S2, Table S3 and S7). This means that the
411 composition of the mixture that larvae may be exposed to over 78 h may be significantly
412 different to what the larvae are exposed to in short 6- or 18-h pulses (example in Figure 1),
413 which is an important consideration for ecotoxicological testing of pulse scenarios. The
414 mixture composition changed over the exposure durations, which is likely to occur in the
415 environment as organisms will be exposed while effluent or run-off dilutes with receiving
416 waters.

417 The toxicity of contaminant mixtures was therefore assessed using concentration-
418 response curves based on the nominal PeC expressed as %-effluent dilution of the mixtures
419 (Table 2). Chemistry data for individual contaminants were generally not used to model
420 concentration-response curves due to the varying concentration-decline profiles. The
421 chemistry data were utilized in calculating toxic units for concentration-addition modelling.

422 ***Continuous exposure toxicity assessment***

423 Dissolved nickel was used as a reference toxicant to observe potential changes in
424 organism sensitivity over time. The 78-h nickel EC50 for LDR in each experiment fell within
425 two standard deviations of the prior 20 exposures, with a mean EC50 for LDR of 9.1 ± 0.7 µg
426 Ni/L (Table S4). During the 78-h test durations, physico-chemical parameters were all within
427 acceptable limits, with the mean pH at each time point ranging from 8.1–8.2, salinity from
428 34–35 PSU and DO from 94–100% (Table S8).

429 Trends in toxicity data were evaluated and discussed primarily by comparing the nominal
430 EC50 toxicity estimates (% effluent) due to greater reliability of EC50 values than lower
431 effects levels (e.g., EC10 values) when working with variable data. Comparisons of data are
432 made using both EC10 and EC50 toxicity values, as EC10 values are the preferred toxicity
433 estimate for risk assessment processes, including species sensitivity distributions used to
434 derive water quality guidelines.

435 Mixture A and B had LDR EC50 values of 3.3 (2.9–3.8)% and 2.8 (2.6–2.9)% of each
436 mixture, respectively. Slopes of the concentration-response curves were very steep, with LDR
437 EC10 values for both Mixture A and B at 1.6 (1.1–2.1)% and 1.8 (1.6–2.1)% of each mixture,
438 respectively (Figure 2, Table 2), being only slightly less than the respective LDR EC50s.

439 Considering EC50 values, LDR was a more sensitive endpoint than population size for
440 Mixture A, with an EC50 of 3.3 (2.9–3.8)% for each mixture, compared to >6.4% based on
441 population size. LDR and population were equivalently sensitive endpoints for Mixture B,
442 with an EC50 of 3.2 (2.8 – 3.6)% using population size and 2.8 (2.6-2.9)% using LDR (Table
443 2).

444 ***Pulse exposures***

445 There was a slight but significant effect of the pulse exposure handling process, as
446 indicated by the differences in the LDR and population size of the controls that were handled
447 to simulate the pulse without added toxicant compared to non-handled (static) controls. The

448 exception was the 18-h pulse LDRs, which were not significantly different to non-handled
449 controls (Figure S3). All pulse toxicity values were normalized to a percent of the respective
450 handling control. Pulse exposure trends are discussed on a PeC basis, unless otherwise stated.

451 *Influence of organism age on toxicity*

452 To assess for sensitivity differences between 24-h or 48-h old nauplii (where the nauplii
453 are developing through several nauplii stages and have a range of body sizes), different pulse-
454 initiation times were assessed with Mixture A (Table 1). Considering larval development
455 EC50 values, the toxicity of 6-h and 18-h pulse exposures were within the range of 9.1–9.7%
456 and 2.9–6.3%, respectively for Mixture A. For population size, the less sensitive endpoint,
457 EC50s ranged from 16–20% after 6-h pulse exposures and 7.3–8.7% after 18-h pulse
458 exposures. The 24-h old nauplii were slightly, but not significantly, more sensitive than 48-h
459 old nauplii based on the calculated EC10s and EC50s for LDR and population size,
460 respectively (Figure 2, Table 2). This is consistent with prior research with this species that
461 indicated that 24-h and 48-h old nauplii had similar sensitivities to an 18-h pulse exposure of
462 copper (Stone et al., 2021). The 24-h old nauplii were equivalently sensitive, or slightly more
463 sensitive than 48-h old nauplii for Mixture A as well as nickel and copper as individual
464 contaminants. Therefore, pulses for Mixture B were initiated to coincide with only the 24-h
465 old nauplii, as the more conservative life-stage (Table 1).

466 *Influence of pulse duration on toxicity*

467 Aquatic organisms are generally more sensitive to individual contaminants as exposure
468 duration increases (Colvin et al., 2021; Diamond et al., 2006; Gordon et al., 2012). The
469 current study supports such trends with continuous exposures observed to provide
470 conservative or equivalent estimates of toxicity risks of 6-h or 18-h pulse exposures (Figure
471 2).

472 When comparing LDR EC10 and EC50 values, the toxicity to larval development of an
473 18-h exposure was not significantly different to toxicity observed in a continuous 78-h
474 exposure for either Mixture A or Mixture B (Figure 2). The larvae were more sensitive to an
475 18-h pulse exposure or 78-h continuous exposure of each mixture compared to a 6-h pulse
476 (Figure 2). For Mixture A, the 6-h pulse EC50 values for LDR were 3-fold greater than
477 EC50s of continuous exposures. Similarly, based on population size, the larval population
478 was more sensitive to an 18-h pulse exposure of Mixture A than a 6-h pulse (Figure 2, Table
479 2).

480 For Mixture B, no significant differences were detected between LDR EC50 values for 6-
481 h and 18-h pulse exposures or 78-h continuous exposures. This was likely due in part to the
482 variability of the data, as there was nearly a 2-fold difference in EC50 value between 6-h
483 pulse and 78-h continuous exposure. The stochastic variability of working with sensitive
484 organisms (and reproductive endpoints) can often mask significant differences, thus trends in
485 such data are still important to investigate. No significant effect on EC50 was detected using
486 the population size endpoint, i.e., 6-h and 18-h pulses and continuous exposures displayed
487 similar toxicity (Figure 2D). Notably, at similar concentrations of Mixture B, some larvae
488 developed normally following a 6-h pulse, whereas an 18-h pulse and continuous exposures
489 caused complete inhibition of larval development (Figure 2B).

490 *Toxicity on a time-weighted average concentration (TAC) basis*

491 One approach proposed for assessing the risk of toxicity from pulse exposures, and to
492 compare magnitudes of toxicity across varying exposure durations, has been to use the TAC
493 to account for different exposure-concentration profiles of contaminants over time. If toxicity
494 is proportional to contaminant exposure 'concentration × duration', this would allow the risk
495 of short-term discharges to be assessed against existing WQGVs (developed from continuous
496 exposures) when compared on a TAC-basis.

497 Applying the TAC approach, the EC50s of Mixture A under a 6-h and 18-h pulse
498 exposure were up to 5-fold lower than the EC50 calculated from the continuous exposure.
499 Similarly, for Mixture B under a 6- and 18-h pulse condition, the EC10s were 6 and 3-fold
500 lower, respectively, than a continuous exposure EC10 (Table S6, Figure S4). Hence,
501 predictions of short-term pulse toxicity based on TAC calculations from continuous toxicity
502 data would be underestimated for these mixtures (i.e., based on a Mixture B continuous
503 exposure 78-h LDR EC50 of 2.8%, a predicted 18-h pulse exposure would give an EC50 of
504 12%, however experimentally it was 2.3%). Therefore, TAC is not a suitable predictor of
505 short-term exposure risk to the environment for complex mixtures such as those in the current
506 study.

507 These findings contrast with those of past studies for individual metals and metal-
508 mixtures where the TAC approach has been found to be useful (Angel et al. 2015, 2020;
509 Stone et al., 2021). It has recently been found that the TAC approach was not suitable for
510 predicting risk of ammonia and propoxur to *A. sinjiensis*, where PeC was shown to be a better
511 concentration metric for assessing the risk and magnitude of toxicity of these contaminants
512 (Stone et al., 2022). Equivalent TACs causing different toxicities in organisms may be
513 influenced by changes in organism metabolism, rates of uptake and depuration of
514 contaminants, or incomplete recovery of organisms (Angel et al., 2018). A TAC approach
515 (derived from continuous exposure data) may not be effective for predicting toxicity of pulses
516 of mixtures where contaminant concentrations and hence, mixture compositions change
517 rapidly over time (Figure 1) or have a very rapid mode of toxicity (e.g., ammonia) (Batley
518 and Simpson, 2009; Diamond et al., 2006; Milne et al., 2000).

519 ***Comparing trends of individual contaminants and mixtures***

520 The results of the current study concur with past studies, observing that aquatic organisms
521 may tolerate or recover from short (e.g., 1 – 12 h) exposures to contaminants. As exposure

522 duration increases (e.g., 18 – 26 h), toxicity after the contaminant pulse exposure can
523 converge with toxicity observed during conventional continuous exposures (e.g., 72 – 96 h).
524 Such toxicity-exposure duration relationships, primarily based on studies of individual
525 contaminants, may indicate that the uptake of the contaminant is more rapid than the
526 depuration, or that the mode of toxicity is immediate and irreversible (Colvin et al., 2021;
527 Stone et al., 2022; Gordon et al., 2012).

528 Colvin et al. (2021) exposed mysid shrimp (5-day old juveniles) and purple sea urchins
529 (embryos) to short 6-, 12- and 26-h pulses of copper and zinc and compared toxicity to a
530 continuous 96-h exposure of these metals. They found for both organisms and both metals
531 that toxicity increased with increasing duration of exposure, and that toxicity (EC50 values)
532 observed after a 26-h pulse exposure was not significantly different to a continuous 96-h
533 exposure (on a pulse-exposure basis).

534 Toxicity thresholds were also observed for *A. sinjiensis* after a pulse exposure of 18-h to
535 ammonia and propoxur as individual contaminants and as effluent mixtures (A and B)
536 (Figure 3). The toxicity results of continuous 78-h exposures provided only slightly
537 conservative or equivalent estimates of toxicity observed from 18-h pulse exposures (Figure
538 3B and C), suggesting a fast-acting or irreversible mode of toxicity within 18-h for these
539 contaminants. This is contrary to trends observed with copper as a single metal exposure
540 (Figure 3A), where organisms may have the capacity to better regulate the uptake and
541 depuration of metals as they are naturally occurring at trace concentrations in the aquatic
542 environment.

543 Recent studies indicate that *A. sinjiensis* may be able to tolerate shorter 6-h contaminant
544 pulses or recover rapidly after exposure, compared to longer 18-h pulses or 78-h continuous
545 exposures. The LDR EC50 values of copper, ammonia and propoxur (based on PeC, not
546 TAC) were assessed after a 6-h pulse (Stone et al., 2021 and 2022). For ammonia and copper,

547 the EC50s were 7- and 6-fold greater (less toxic) after a 6-h pulse compared to a continuous
548 78-h exposures. Exposure to propoxur in a 6-h pulse resulted in an EC50 > 460 µg/L
549 compared to a continuous EC50 of 170 µg/L.

550 In the current study, such differences were less for contaminant mixtures after a 6-h pulse,
551 being 3-fold and 1-fold (on a %-effluent basis) for Mixture A and B, respectively (Figure 3),
552 indicating that *A. sinjiensis* could not tolerate a 6-h pulse exposure to contaminant mixtures to
553 the same extent as individual contaminant exposures. This is possibly due to the simultaneous
554 exposure to contaminants with varying modes of toxicity.

555 While many previous studies have generally shown that aquatic organisms can
556 tolerate significantly higher concentrations of contaminants for shorter durations, the current
557 study showed that for *A. sinjiensis*, these trends are less pronounced and generally not
558 proportional to concentration-time for exposure to effluents containing contaminant mixtures.
559 Such trends are likely determined by the specific contaminant mode of toxicity, and rates of
560 uptake and depuration.

561 ***Concentration-addition toxicity predictions***

562 The use of toxic units (TU) to predict the toxicity of the two effluents (Mixtures A and B)
563 was assessed, utilising toxicity-threshold data determined previously for ammonia, copper
564 and propoxur (Figure S1), while noting there were contaminants present for which the
565 effects-threshold data was not available. For Mixture A, this approach was expected to fail,
566 but was completed to consider how copper and ammonia toxicity may be affected by the
567 other mixture constituents in Mixture A. The duration-specific sum of TUs calculated using
568 PeCs of ammonia and copper for Mixture A; and ammonia, copper and propoxur for Mixture
569 B; explains toxicity well for all exposure durations (6-, 18- and 78-h exposures) (Figure 4).

570 For Mixture A under 6-h pulse exposure conditions (Figure 4A), as expected the observed
571 toxicity was greater than predicted based on copper and ammonia concentrations alone. This

572 was attributed to the presence of a range of other organic compounds that were present, but
573 not included in the cumulative TUs calculation (since the toxicity of those to this species are
574 not known). However, in longer 18-h pulse exposures, many of these compounds had
575 volatilized, so that toxicity predictions based on copper and ammonia matched the observed
576 responses (Figure 4C). For *A. sinjiensis,* 24-48-h old nauplii have been found to be more
577 sensitive to contaminant pulses than earlier life stages (Stone et al. 2021). In this study, these
578 life stage would be exposed to significantly reduced concentrations of volatile organics,
579 during a continuous exposure, due to contaminant decline over time. It is likely that these
580 volatile organics did not contribute significantly to toxicity in continuous exposures.

581 For Mixture B, under continuous 78-h exposure conditions, the observed toxicity between
582 control response and the EC50 was less than that predicted by the model that considered just
583 copper, ammonia and propoxur (Figure 4F). Around the EC50 range, the predicted and
584 observed toxicity converges. Propoxur concentrations can decrease by up to 50% over a 78-h
585 exposure period (Stone et al., 2022), and this is not accounted for here, with only initial
586 propoxur concentrations being considered. This likely explains the deviation between
587 predicted and observed toxicity, particularly at lower mixture concentrations.

588 This is an example of concentration-addition modelling only being suitable for predicting
589 the toxicity exposures of varying durations when the toxic units can be calculated from the
590 corresponding exposure duration for the individual contaminant, i.e., the duration specific
591 toxicity of the individual contaminants within the mixture must be known to enable accurate
592 predictions. The trends observed here, despite data gaps, indicate that mixture modelling is
593 worth investigating comprehensively in future pulse toxicity studies.

594 ***Risk-assessment and regulatory considerations***

595 For ammonia and propoxur as individual contaminants and for the two effluents
596 (Mixtures A and B), despite observing differences of up to 3-fold between EC10 values for 6-

597 h pulses and continuous exposures (PeC), there were no significant differences in EC10
598 statistical estimates across all exposure durations tested due to the large uncertainty in the
599 EC10 values (i.e., large 95% CLs) (Figure S5). From a regulatory perspective, this is an
600 important observation, as EC10s are most often applied in risk-assessment and regulation (no
601 observed effect concentration (NOECs) used when suitable dose-responses are not available).
602 For example, EC10s or NOECs are the preferred statistical estimates for the derivation of
603 guideline values (Warne et al., 2018), deriving safe dilution factors (Adams et al., 2008), and
604 for calculating hazard quotients (Kortenkamp et al., 2009).

605 Assessing the risk of short-term discharges requires improved understanding of how
606 the chemical composition of effluent and the duration of exposure modifies the toxicity of
607 contaminants to aquatic organisms. There are several ways that the current understanding of
608 pulse toxicity has been suggested to be applied to a regulatory setting including TACs or
609 modification of direct toxicity assessments (Diamond et al., 2006; Colvin et al., 2021; Angel
610 et al., 2020). Angel et al. (2020) discusses the use of TAC to relax safe-dilution limits by a
611 factor less than or equivalent to the TAC to prevent highly conservative outcomes and
612 acknowledges that this may not be a suitable approach where chemicals act rapidly. This
613 study has shown that TACs have limited applicability to mixtures containing fast acting
614 toxicants or in mixtures where effluent composition may change rapidly over time, and the
615 calculation of TAC can become complex if not only considering the nominal %-effluent
616 dilution. The modification of direct toxicity assessments to reduce exposure duration may
617 provide a more environmentally relevant estimate of toxicity, as recommended by Colvin et
618 al. (2021) and Gissi et al. (2021). Generally, conventional continuous exposures provide a
619 conservative or equivalent estimate of toxicity, as confirmed in the current study.

620 Further short-term toxicity testing with varied species and contaminants (individual
621 and mixtures) is required to investigate the consistency and applicability of pulse toxicity

622 trends in risk assessment of short-term discharges of contaminants to the aquatic
623 environment. This study provides useful reference data in scenarios where conventional
624 ecotoxicological testing may need to be modified, or continuous ecotoxicity data extrapolated
625 to short-term contamination scenarios.

626 **Conclusion**

627 In the current study, a number of toxicity-modifying factors were investigated, using two
628 artificial effluents containing complex contaminant mixtures, including organism life-stage
629 and exposure duration. To assess application in a regulatory space, this data was compared to
630 pulse toxicity trends for individual contaminants and compared on a PeC (pulse-exposure)
631 and TAC (time-averaged) basis. The difference in toxicity between continuous and short-term
632 exposures was greater for individual contaminant toxicity compared to mixture toxicity on a
633 PeC basis. The study showed that risk of toxicity from short-term exposures is highly
634 dependent on the chemical composition of the effluent and is affected by the duration of
635 exposure. The toxicity test outcomes from conventional continuous exposures provide the
636 ability to derive guidelines that conservatively protect against the risk of short-pulse
637 exposures, and comparisons of toxicity on a TAC basis were not adequately conservative for
638 guideline application. Concentration-addition toxicity modelling was suitable for predicting
639 the toxicity of short-term exposures of mixtures when toxic units were calculated from
640 individual toxicity data (PeC) at the relevant exposure duration, but this requires a level of
641 chemical measurement that would not normally exist for a monitored effluent discharge. The
642 factors of exposure duration and chemical composition of effluent can greatly affect observed
643 toxicity and thus risk-assessment and toxicity modelling. These data provide insights to
644 improve approaches to the regulation of short-term exposures in aquatic environments.

645

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Table 1. Continuous and pulse exposure scenarios assessing the toxicity of Mixture A and B to *A. sinjiensis* (pulses within 78-h total test period).

Contaminant	Exposure type	N	Pulse (h)		Nominal dilution range (%-effluent)
			Initiation	Duration	
Mixture A	Continuous	3	0	78	0.3 – 6.4
	Pulse	1	24	6	2.4 – 47
	Pulse	2	24	18	0.7 – 21
	Pulse	1	48	6	2.5 – 50
	Pulse	2	48	18	0.7 – 22
Mixture B	Continuous	2	0	78	0.5 – 7.6
	Pulse	1	24	6	0.5 – 8
	Pulse	2	24	18	0.5 – 7.6

N = number of toxicity tests.

Table 2. Toxicity of Mixture A and B to *Acartia sinjiensis* under varying exposure conditions, based on nominal %-effluent PeCs (endpoints of either 78-h larval development ratio or population size). 95% confidence limits are presented in brackets.

Effluent	Endpoint	Exposure scenario	Init.[a] (h)	EC10 (%-effluent)	EC50 (%-effluent)
Mixture A	LDR	6-h pulse	24	3.0 (0.0–6.6)	9.7 (5.4–14)
		6-h pulse	48	2.0 (0.0–4.7)	9.1 (5.0–13)
		18-h pulse	24	0.5 (0.0–1.1)	2.9 (1.6–4.2)
		18-h pulse	48	2.1 (0.7–3.5)	6.3 (5.0–7.5)
		78-h continuous	0	1.6 (1.1–2.1)	3.3 (2.9–3.8)
Mixture B		6-h pulse	24	0.7 (0.0–1.5)	3.6 (2.3–4.8)
		18-h pulse	24	1.4 (1.0–1.8)	2.3 (2.1–2.5)
		78-h continuous	0	1.8 (1.6–2.1)	2.8 (2.6–2.9)
Mixture A	Population	6-h pulse	24	2.4 (1.1–3.6)	16 (11–21)
		6-h pulse	48	7.6 (4.9-10)	20 (15–25)
		18-h pulse	24	1.5 (0.3–2.6)	7.3 (5.6–9.1)
		18-h pulse	48	1.1 (0.1–2.0)	8.7 (6.5–11)
		78-h continuous	0	1.4 (-0.9–3.8)	> 6.4
Mixture B		6-h pulse	24	> 8	> 8
		18-h pulse	24	1.5 (0.6–2.5)	5.1 (3.9–6.4)
		78-h continuous	0	3.0 (2.8–3.2)	3.2 (2.8–3.6)

[a]Init. = initiation time, PeC = Pulse-exposure concentration.

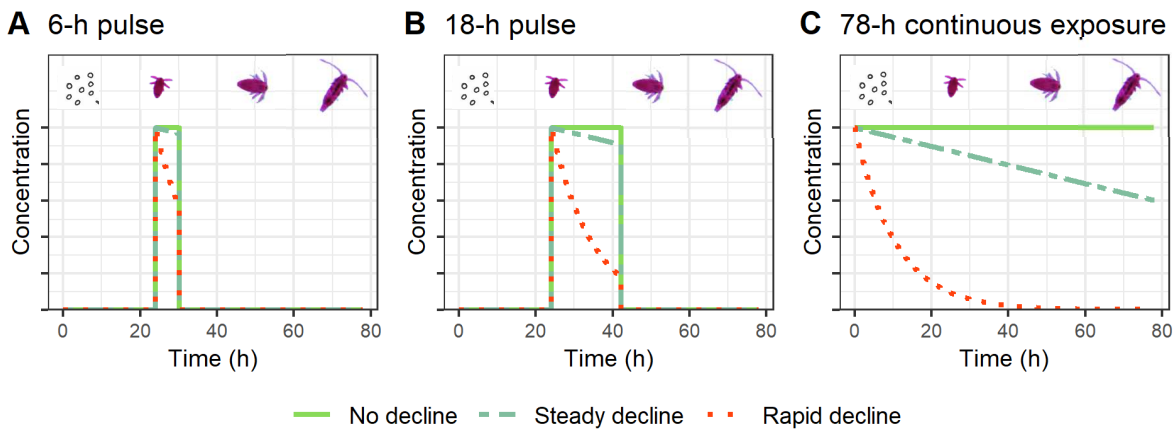


Figure 1. Example of mixture composition change over A) a 6-h pulse exposure, B) an 18-h pulse exposure or C) a 78-h continuous exposure, where the mixture has three components, one chemical that experiences no decline (full line), one chemical that exhibits steady decline over exposure duration (dashed line), and another chemical that rapidly declines within the first 18-h of exposure (dotted line).

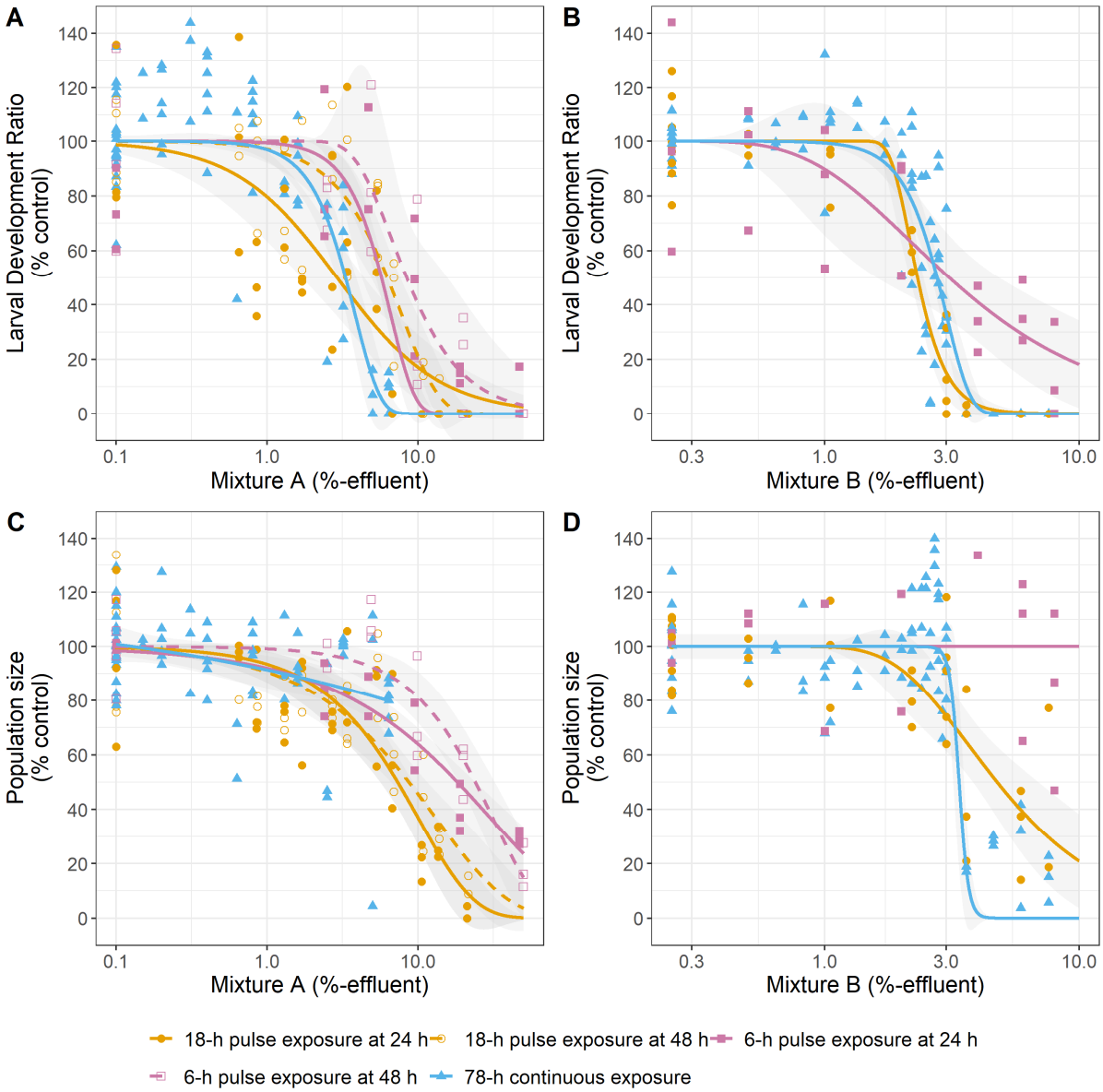


Figure 2. Concentration-response curves indicating the effect of three effluent exposure durations of 78-h continuous (blue triangle), 18-h pulse (orange circle) and 6-h pulse (purple square), with two pulse-initiation times of 24 h (closed shape, solid line) and 48 h (open shape, dashed line) on the two test endpoints of larval development ratio (LDR) (A and B) and population size (C and D) of *A. sinjiensis* following exposure to two effluents Mixture A (A and C), and Mixture B (B and D). The data and calculated models used nominal pulse-concentration % effluent. Grey shaded area represents the 95% confidence intervals. Each data point represents one replicate, data were pooled from separate experiments.

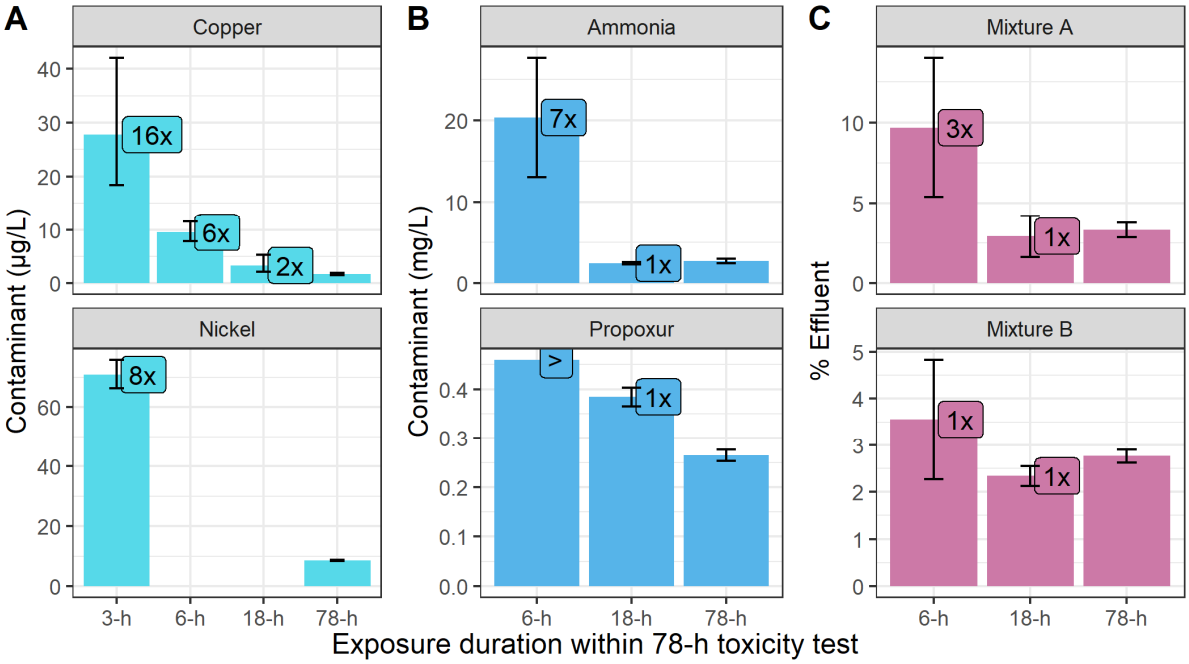
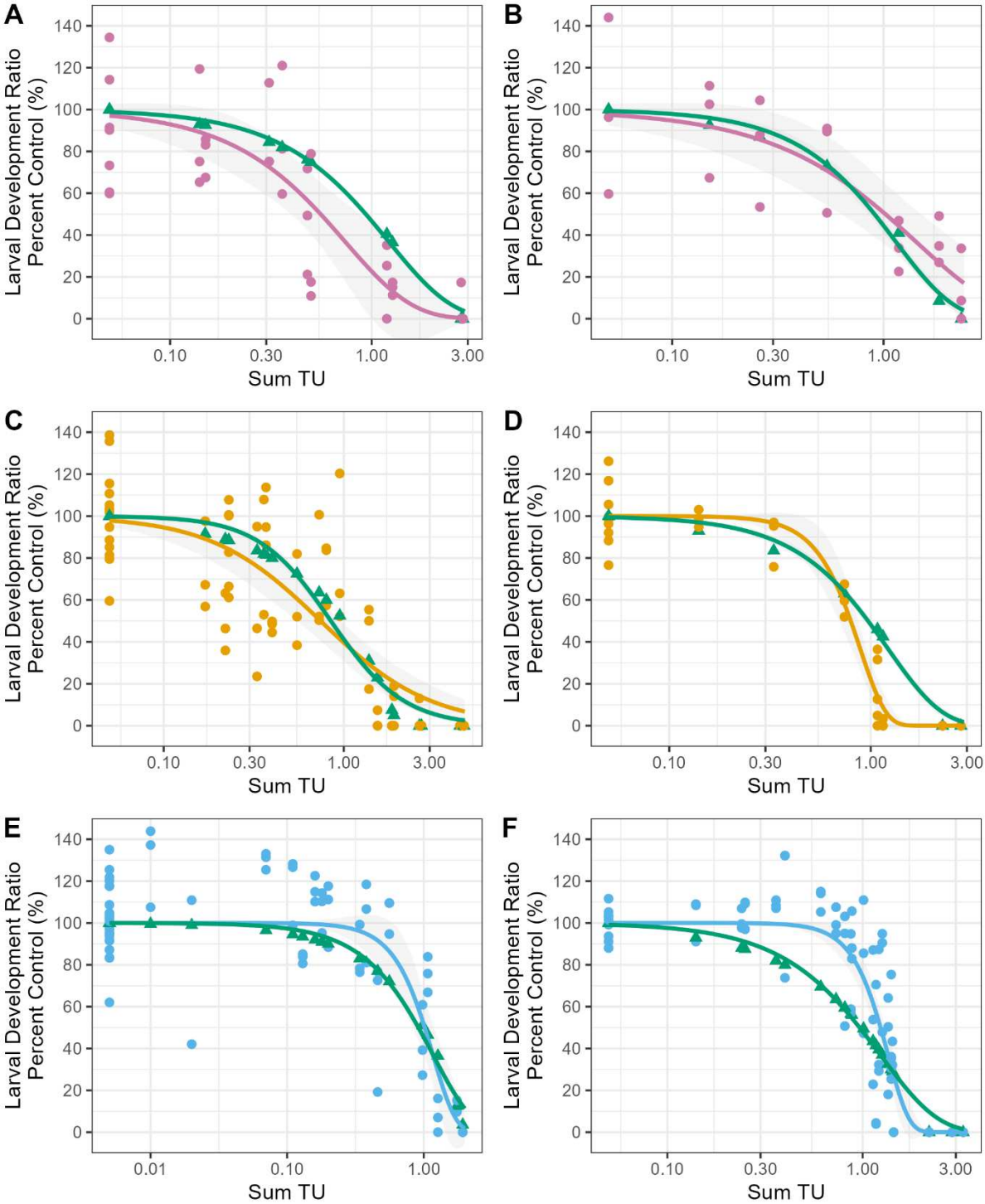


Figure 3. Comparison of EC50 values for larval development ratio (LDR) (based on PeCs) for short-term 3-h, 6-h or 18-h pulses to the most sensitive 24-h old nauplii compared to continuous 78-h exposures to: A) individual metals, copper and nickel; B) individual contaminants; ammonia and propoxur; and C) contaminant mixtures Mixture A and B. The factor difference between short-term and continuous EC50 values is labelled on the bar-plot, or '>' is labelled where the EC50 value exceeded the highest concentration tested. Data for A and B are from Stone et al. (2021 and 2022). Error bars represent 95% confidence limits of modelled toxicity value.



812

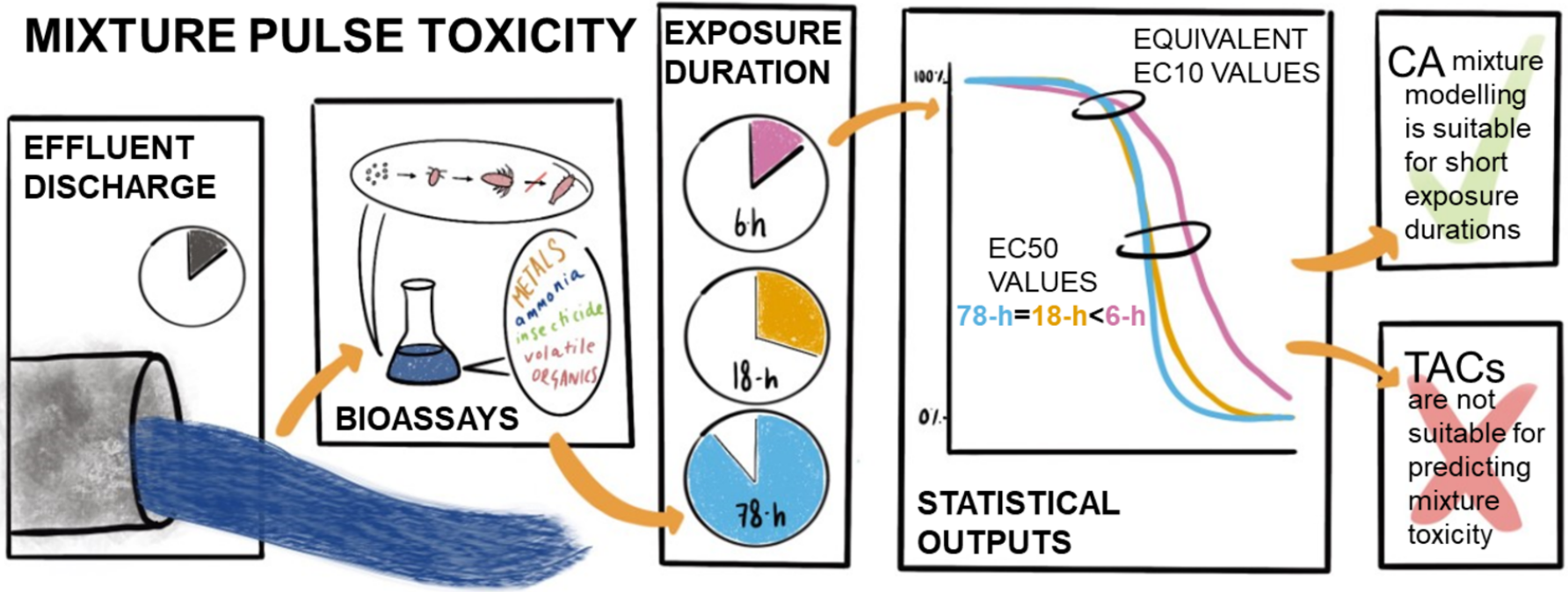
813 **Figure 4.** A comparison of toxicity (larval development ratio) based on toxic-unit (TU)

814 concentration-addition modelling (green triangles) and observed toxicity for: A and B) 6-

815 h pulse exposures (purple circles), C and D) 18-h pulse exposures (orange circles), and E

816 and F) 78-h continuous exposures (blue circles) of Mixture A (A, C and E) and Mixture B

(B, D, and F). The sum of TU was composed of copper and ammonia for Mixture A and copper, ammonia and propoxur for Mixture B (Figure S1).


MIXTURE PULSE TOXICITY
EFFLUENT DISCHARGE
BIOASSAYS
METALS
ammonia
insecticide
volatile
ORGANICS
EXPOSURE DURATION
6-h
18-h
78-h
EQUIVALENT EC10 VALUES
100%
0%
EC50 VALUES
78-h=18-h<6-h
STATISTICAL OUTPUTS
CA mixture modelling is suitable for short exposure durations
TACs are not suitable for predicting mixture toxicity