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The ecological risk dynamics of pharmaceuticals in micro-estuary environments

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1 **The ecological risk dynamics of pharmaceuticals in micro-estuary**
2 **environments**

3

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5

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15 ABSTRACT

16 Micro-estuarine ecosystems have a surface area < 1 km² and are abundant in Mediterranean
17 regions. As a result of their small size, these systems are particularly vulnerable to effects of
18 chemical pollution. Due to fluctuating flow conditions of base-flow dominated by treated
19 wastewater effluents and flood events transporting rural and urban non-point-source pollution,
20 micro-estuaries are under a dynamic risk regime, consequently, struggling to provide
21 ecological services. This two-year study explored the occurrence and risks of pharmaceutical
22 contamination in the Alexander micro-estuary in Israel. Pharmaceuticals were detected in all
23 samples (n=280) at as high as 18 µg L⁻¹ in flood events and 14 µg L⁻¹ in base-flow.
24 Pharmaceutical mixtures composition was affected by flow conditions with carbamazepine
25 dominating base-flow and caffeine dominating flood events. Median annual risk quotients for
26 fish, crustaceans and algae were 19.6, 5.2, and 4.5, indicating that pharmaceuticals pose high
27 risk to the ecosystem. Ibuprofen, carbamazepine and caffeine were contribute most to the risk
28 quotients. The current work highlights that micro-estuary ecosystems, like the Alexander
29 estuary, are continuously exposed to pharmaceuticals and most likely to other pollutants,
30 placing these ecologically important systems under an elevated risk, in comparison to the more
31 frequently studied large estuarine systems.

32 INTRODUCTION

33 Surface waters around the world are contaminated with and influenced by diverse mixtures
34 of organic pollutants, such as solvents, microplastics, flame retardants, pesticides and
35 pharmaceuticals^{1,2}. Chronic exposure of stream and estuarine environments to active
36 pharmaceuticals has become a global emerging ecological risk³. The main sources of
37 pharmaceuticals in surface water include raw sewage or treated wastewater effluents⁴⁻⁶, urban
38 runoff⁷, and runoff from arable land irrigated with treated wastewater or amended with
39 biosolids or animal manures and slurries⁸. Due to water shortages, irrigation of agricultural
40 systems with treated wastewater is becoming more prevalent, resulting in the ubiquitous
41 presence of pharmaceuticals in runoff water⁷ and stream base flows⁹.

42 Flow patterns of semi-arid streams are characterized by low-discharge base flows which
43 are often dominated by, or mixed with effluents and flood events during the rainy season
44 composed of both urban and agricultural runoff^{10,11}. Such flow patterns are found in streams in
45 the Mediterranean climate zone, which includes southeastern Spain¹², southern Portugal¹³,
46 northwestern China¹⁴, southern California¹⁵ and the Middle East¹⁶. The eastern zone of the
47 Mediterranean Sea uses large amounts of treated wastewater for agricultural irrigation (87% in
48 Israel¹⁷), increasing the probability of introducing chemicals, such as pharmaceuticals, into
49 streams from cultivated fields during storm events. Thus, together with urban runoff and
50 sewage overflow, stormwater flood events are a dominant contributor to pharmaceutical loads
51 in streams⁷. While stormwater flow makes up a relatively small proportion, timewise, of the
52 annual flow, it contributes a dominant fraction of the annual water volume¹¹. Moreover, flood-
53 event frequency and magnitude are expected to increase in these regions due to climate
54 change¹⁸.

55 The presence of pharmaceuticals in both base flow and flood events results in chronic
56 exposure of aquatic habitats to pharmaceutical mixtures with changing temporal dynamics.

57 These flow dynamics change further when entering transitional water bodies, such as lagoons,
58 fjords and estuaries, where geochemical conditions and water residence time changes
59 dramatically¹¹. Estuaries, which are a zone of mixed surface and sea water¹⁹, are often
60 characterized by longer water residence times and strong gradients of salinity, temperature and
61 turbidity. These gradients are especially dynamic in micro-estuaries, which are typical to semi-
62 arid zones¹¹. These relatively small water bodies of few meters in depth, a few kilometers in
63 length, and a surface area of <1 km², are mainly governed by a sandbar at the mouth section.
64 Despite their small size, micro-estuaries are an important ecological and sociological services
65 provider¹¹. These environments are generally understudied, and although they are known to
66 frequently suffer from eutrophication¹¹, little is known on the risk they are subjected to from
67 micropollutants, such as pharmaceuticals, and on their ability to attenuate such toxicants as
68 shown for other similar vegetated water bodies²⁰.

69 The occurrence, composition and concentration dynamics of mixtures of pharmaceuticals
70 pose a potential unacceptable risk to aquatic habitats. Although the potential risk of single
71 pharmaceuticals to different aquatic compartments has been previously shown, evaluating risk
72 for mixtures is more challenging²¹. Many tools and models exist for the determination of the
73 risks of pollutant mixtures²², with the most effective for risk estimation being the concentration
74 addition model²². Although this model neglects the possible antagonistic and synergistic
75 interactions of chemicals, it can serve as an important tool for assessing mixture risk and
76 identifying dominant pollutants and threatened taxonomic groups²².

77 For a comprehensive evaluation of potential risks of pharmaceuticals in micro-estuarine
78 environments, flux dynamics must be quantified. This calls for high-temporal-resolution
79 sampling combined with comprehensive analytical analysis and the application of appropriate
80 risk models. The current study explored the biannual dynamics of pharmaceutical mixture

81 fluxes in and out of the micro-estuary and evaluates the risk posed by the pharmaceuticals to
82 aquatic organisms.

83

84 **MATERIALS AND METHODS**

85 **Site and sampling**

86 The Alexander stream main channel flows a distance of 32 km and drains an area of 550
87 km². It starts in the Samaria mountains (Palestinian Territory), crosses the Hefer valley (Israel),
88 and ends at the Mediterranean Sea (Figure S1). The Alexander stream is ephemeral throughout
89 most of its length, receiving some fountain water and mainly treated wastewater from a
90 treatment facility located ~13 km upstream from the estuary head. The Alexander estuary
91 (defined as a micro-estuary¹¹) is ~6.5 km long with a maximal depth of ~3 m and average cross-
92 sectional width of 20 m. Detailed characteristics of the Alexander micro-estuary are described
93 elsewhere^{11,23,24}. Water upstream from the estuary head (N32.375 E34.912) and adjacent to the
94 estuary mouth (N32.394 E34.869) was sampled at the depth of ~20 cm (Figure S1). Each of
95 the sampling stations was equipped with an automated water sampler (Sigma 900©, Hach
96 Company, Loveland CO; and ISCO 3700 Full-Size Portable Sampler, Teledyne, Lincoln NE)
97 with a carousel containing 24 350mL glass bottles. Samples were taken every 0.25–4 h, with
98 higher sampling frequency during the rising limb of the hydrograph and peak discharge, and
99 lower sampling frequency on the falling limb of the hydrograph. Monthly base-flow water grab
100 samples were collected with a horizontal water sampler (5 L, Model 110B, OceanTest
101 Equipment, Fort Lauderdale FL). All samples were filtered using 90 mm GF/F filters (nominal
102 pore size of 0.7 µm, MGF, Sartorius, Göttingen, Germany) and immediately frozen (-20 °C).
103 A total of 237 flood samples and 44 base-flow samples were analyzed over 2 hydrological
104 years (2016–2018).

105

106 **Analysis of pharmaceuticals**

107 Water samples were defrosted overnight and 200-mL aliquots were spiked with 10 μ L of a
108 mixture of isotopically labeled internal standards (see detailed information in SI) and
109 concentrated using SPE cartridges (Strata-X, 200 mg, Phenomenex, Torrance, CA).
110 Pharmaceuticals were quantified by LC–HRMS analysis using a Q Exactive Plus hybrid FT
111 mass spectrometer coupled with a Dionex Ultimate 3000 RS UPLC (Thermo Fisher Scientific,
112 Waltham, MA). Instrumental parameters, limits of quantification are recoveries are shown in
113 the SI and in Table S1.

114

115 **Ecotoxicological risks: Single-compounds**

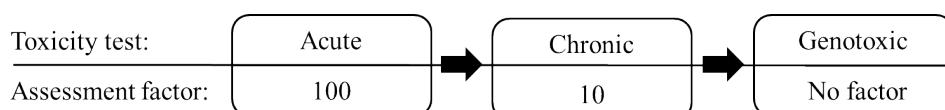
116 Experimental data on the apical effects (72 h algal growth; 21 d daphnid reproduction, 28
117 d fish growth and reproduction etc.) of the studied compounds were obtained from the
118 published literature. The values were then used alongside assessment factors proposed by the
119 European Chemicals Agency²⁵ (Table S2) to derive predicted no-effect concentrations for the
120 aquatic habitat ($PNEC_{\text{aquatic}}$) (Table 1 and Table S3)²⁵. In the case where only genotoxicity tests
121 results were available, no assessment factors were applied due to the sensitivity of such
122 experiments. Risk characterization ratios for each study compound for each sample monitored
123 were then calculated using Equation 1. A risk quotient (RQ) ≥ 1 was considered an unacceptable
124 risk for the aquatic habitat. RQs were calculated based on a single-compound approach:

125
$$RQ_p = \frac{MC}{PNEC_{\text{aquatic}}} \quad (1)$$

126 where RQ_p is the pharmaceutical RQ, and MC is the measured concentration of the
127 pharmaceutical.

128 **Ecotoxicological risks: Pharmaceutical mixtures**

129 RQs were also calculated for the mixture of the studied compounds in each sample. It would
130 be inappropriate to use a mixture of endpoints for different taxonomic groups to assess the risk
131 of the mixtures. Therefore, we initially estimated PNECs for each pharmaceutical for each
132 taxonomic group (PNEC_{fish}, PNEC_{crustaceans} and PNEC_{algae}) using the following prioritization
133 and assessment factors diagram.



135 The assessment factor for chronic tests represents the transition from laboratory conditions
136 to the field, with an additional factor applied for the conversion of the acute test to chronic
137 levels. When no acute or chronic tests were available, the more sensitive test of genotoxicity
138 was used with no assessment factor implementation. In the rare case where no experimental
139 value was available, the toxicity calculation used by the Ecological Structure–Activity
140 Relationship model (ECOSAR) was used and regarded as an acute toxicity value. PNEC values
141 determined for 16 out the 19 analyzed pharmaceuticals, with indications of data origin and the
142 applied factors, are presented in Table 1.

143 The PNECs were then used along with the measured concentration data to estimate RQs
144 for the mixture for the separate taxonomic groups using Equation 2:

145

$$RQ_{tg} = \left[\sum_{i=1}^{16} \left(\frac{MC_i}{PNEC_{i,tg}} \right) \right] \quad (2)$$

146 where RQ_{tg} is the pharmaceutical risk quotient for a specific taxonomic group tg , MC_i is the
147 measured concentration of pharmaceutical i in the sample, and $PNEC_{i,tg}$ is the PNEC of
148 pharmaceutical i for taxonomic group tg .

149

150

151 **Table 1.** Predicted no effect concentrations (PNEC; $\mu\text{g L}^{-1}$) of the studied pharmaceuticals for
 152 the aquatic environment and for fish, crustaceans and algae taxonomic groups. Assessment
 153 factors are presented in Table S3.

Name	Therapeutic activity	Aquatic	Fish	Crustacean	Algae
Carbamazepine		0.05 ²⁶	2500 ²⁷	0.05 ²⁶	0.2 ²⁸
Lamotrigine	Anticonvulsants	150 ²⁹	600 ³⁰	1000 ²⁹	750 ²⁹
Lorazepam		0.005 ³¹	0.05 ³¹	37 ³¹	20 ³¹
Acetaminophen		0.5 ³²	0.5 ³²	95 ³³	0.5 ³⁴
Diclofenac		0.1 ³⁵	0.1 ³⁵	46 ³⁶	100 ²⁷
Ibuprofen	Anti-inflammatories	0.01 ³⁷	0.01 ³⁷	3.2 ³⁸	1 ³⁴
Ketoprofen		0.03 ³⁹	0.3 ³⁹	436.5 ⁴⁰	162.1 ⁴⁰
Naproxen		15 ⁴¹	10 ⁴²	15 ⁴¹	620 ⁴¹
Metoprolol	β -Blocker	7.3 ⁴³	1000 ⁴⁴	88 ⁴⁴	73 ⁴³
Sildenafil	Erectile dysfunction	0.026 ⁴⁵	0.026 ⁴⁵	N/A	N/A
Clofibrate acid*		0.49 ⁴⁶	0.49 ⁴⁶	1 ⁴⁷	10 ⁴⁸
Bezafibrate	Lipid regulators	0.034 ⁴⁵	0.034 ⁴⁵	2.3 ⁴⁹	6000 ⁴⁹
Gemfibrozil		0.38 ⁴⁵	0.38 ⁴⁵	7.8 ⁴⁹	312 ⁴⁹
Caffeine	Stimulant	1 ³⁴	30 ⁵⁰	12 ⁵¹	1 ³⁴
Sulfamethoxazole		0.6 ²⁷	800 ²⁷	1 ²⁹	0.6 ²⁷
Sulfapyridine	Sulfonamides	0.012 ³¹	0.35 ³¹	0.012 ³¹	19.3 ⁵²

154

155 *Metabolite of Clofibrate.

156

157 RESULTS AND DISCUSSION

158 Our study focused on a typical micro-estuary in the Alexander Stream, which is in a semi-
 159 arid Mediterranean climate zone and which drains a watershed of mixed land use (agricultural
 160 and municipal) that is highly influenced by treated wastewater^{11,23}. Here we report data for
 161 base-flow and flood-event durations over 2 hydrological years (2016–17 and 2017–18) (Table

162 S4) which were within the normal annual flow-regime cycle based on measurements from 50
163 years²³. In addition to the temporal and spatial dynamics data for pharmaceuticals, our analysis
164 evaluated ecosystem health by determining risk and its drivers.

165 **Pharmaceutical occurrence and concentration: Estuary head and mouth**

166 All water samples collected at the head or mouth of the Alexander estuary during flood
167 events or under base-flow conditions were contaminated with pharmaceuticals or their
168 metabolites (Table 2). The five most frequently detected pharmaceuticals in the flood-event
169 samples were carbamazepine, diclofenac, bezafibrate, caffeine, and sulfamethoxazole. Under
170 base-flow conditions, the ubiquitous pharmaceuticals were carbamazepine, lamotrigine,
171 bezafibrate, gemfibrozil, caffeine, and sulfamethoxazole. These occurrence patterns are similar
172 to those in European rivers, where carbamazepine, diclofenac, caffeine, and sulfamethoxazole
173 are highly ubiquitous⁵³.

174 Noticeable differences in substances in the base-flow and flood conditions were found. For
175 example, ibuprofen was detected in ~90% of the flood-event samples but in only 27% of the
176 base-flow sample. In contrast, lorazepam was detected in only ~20% of flood-event samples
177 compared to ~80% in the base-flow samples. Changes between flow conditions were also
178 pronounced in terms of concentrations of pharmaceutical groups (Table 2). Anticonvulsants,
179 sulfonamides, and the erectile dysfunction drug sildenafil exhibited higher average
180 concentrations in base-flow samples than in the flood-event samples. Anti-inflammatories,
181 lipid regulators, the stimulant caffeine, and the β -blocker metoprolol showed the opposite trend
182 with higher concentrations in the flood-event vs. base-flow samples. These clear differences
183 suggest a dominant influence of pharmaceuticals' environmental stability on their transport
184 dynamics in the stream. The less environmentally persistent anti-inflammatories and caffeine
185 (as calculated by Aminot et al. based on DT₅₀ values⁵⁴) are rapidly degraded in wastewater-
186 treatment plants and are therefore present at low concentrations under base-flow conditions.

187 The more environmentally persistence pharmaceuticals, such as anticonvulsants⁵⁴, are found

188 in relatively high concentrations under both base-flow and flood conditions.

189 **Table 2.** Occurrence (%) and concentration (ng L⁻¹) of pharmaceuticals and their metabolites in water samples collected from the head and mouth
 190 of the Alexander micro-estuary during flood Events (2016-17 and 2017-18; n = 236) and base flow (2017 and 2018; n = 44).

	<u>Floods events</u>					<u>Base-flow</u>				
	Occurrence	Average	Median	Max	Min	Occurrence	Average	Median	Max	Min
Carbamazepine	100	414	180	4,953	25	100	643	644	1,947	52
Epoxy carbamazepine	100	28	15	294	1	98	57	60	179	0
trans-Dihydroxy carbamazepine	100	425	162	3,857	19	100	1,233	1,125	3,458	68
Lamotrigine	97	30	14	235	0	100	111	113	235	9
Lamotrigine-N-oxide	17	0.3	0	7	0	18	1	0	9	0
Acetaminophen	39	152	0	2,127	0	18	12	0	184	0
Diclofenac	100	126	77	730	0	80	99	46	692	0
Ibuprofen	89	297	172	3,291	0	27	71	0	592	0
Ketoprofen	60	20	17	158	0	41	9	0	34	0
Naproxen	17	40	0	1,146	0	34	25	0	174	0
Metoprolol	82	7	2	1,038	0	66	5	2	77	0
Sildenafil	51	3	1	179	0	82	10	6	139	0
Clofibrate acid	60	148	17	11,765	0	41	5	0	36	0
Bezafibrate	100	94	66	1,041	5	91	39	18	248	0
Gemfibrozil	83	263	22	4,428	0	91	140	27	2,704	0
Lorazepam	19	0.4	0	4	0	77	2	2	4	0
Caffeine	100	2,859	2,746	12,969	19	100	828	60	11,559	11
Sulfamethoxazole	100	51	14	656	0	100	115	82	656	1
Sulfapyridine	98	10	4	133	0	89	11	7	87	0

191

192 Overall, pharmaceutical concentrations found in the Alexander estuary were comparable to
193 those found in other surface-water bodies, with a slight tendency toward the upper end of the
194 concentration ranges^{2,53}. Further comparison to other estuarine environments was performed
195 using the German Environmental Agency pharmaceuticals in the environment database⁵⁵. A
196 total of 336 entries of mean and maximum values for 12 pharmaceuticals derived from 16
197 countries were considered. Average concentration of pharmaceuticals in the Alexander micro-
198 estuary was on average ~7 folds higher than compiled entries from the database with
199 metoprolol being the only compound showing a lower average concentration in the Alexander
200 estuary compared to other estuarine systems (Table S5).

201 The average pharmaceuticals cumulative concentration at the estuary head was slightly
202 higher during flood events, with 5 $\mu\text{g L}^{-1}$ compared to 4 $\mu\text{g L}^{-1}$ under base-flow conditions
203 (Figure 1). These findings contradict the assumption that dilution of the base-flow water,
204 dominated by treated wastewater, by stormwater runoff significantly reduces the overall
205 pharmaceutical concentration in flood events. Our findings suggest that pharmaceuticals in
206 runoff events originate from another source, perhaps the urbanized part of the watershed. The
207 increased concentrations of rapidly degradable compounds such as ibuprofen and caffeine
208 during flood events support the assumption of an urban influence, suggesting sewage overflow
209 mixed with urban runoff as a source for pharmaceuticals in the stream and estuary.

210 Within-season dynamics were also observed during the rainy season, where the highest
211 concentration peaks (18.6–17 $\mu\text{g L}^{-1}$) were recorded at the estuary head during the first flood
212 event for each year (F2016-17 #1, F2017-18 #1; Figure 1). The peak concentration for F2016-
213 17 #1 was dominated by caffeine (41%) and anticonvulsants (29%), whereas the two
214 consecutive peaks of F2017-18 #1 were composed mainly of caffeine (76% and 67%). We
215 assume that cumulative concentration peaks at the beginning of a rainy season are attributed to
216 the flushing of pharmaceuticals accumulated in bed sediments and pore water in the upper

217 tributaries during the wastewater base flow of the dry season. Although dilution had little
218 influence on overall dynamics, high-discharge flood events (e.g., F2018 #4) exhibited a
219 significant dilution effect resulting in low accumulation of pharmaceutical concentrations.

220 Under base-flow conditions, pharmaceutical concentrations were dominated by the
221 anticonvulsant carbamazepine and its metabolites. In general, the cumulative base-flow
222 concentrations were lower than those of the flood events with one exception, the sample
223 collected on 7 March 2018, which contained elevated concentrations of caffeine and anti-
224 inflammatories (Figure 1). This rise in concentration was composed mainly of caffeine (~12
225 $\mu\text{g L}^{-1}$) and ibuprofen (~1 $\mu\text{g L}^{-1}$), probably from a recorded malfunction in the sewage-
226 treatment facility upstream. The malfunction resulted in approximately 2 weeks of raw sewage
227 being spilled into an upper tributary, carrying the more degradable compounds into the estuary
228 and elevating the cumulative pharmaceuticals concentration. On the other hand, a sharp drop
229 in cumulative concentrations in the estuary head was observed during the dry season of 2018.
230 At that time, the base flow was redirected from the stream for irrigation, lowering the annual
231 median to 2.3 $\mu\text{g L}^{-1}$, in comparison to 3.7 $\mu\text{g L}^{-1}$ in 2017. The vast changes in pharmaceutical
232 concentrations and mixture composition from sewage spills during base flow and sewage
233 overflow during the rainy season, and on the other hand, from redirection of reclaimed
234 wastewater for irrigation, emphasize the lack of a proper watershed-management effect on
235 estuarine and coastal environmental pollution.

236 While flow types dictate the composition and concentration of pharmaceutical mixtures
237 entering the estuary, the conditions in the estuary may influence the overall pharmaceutical
238 concentration, and more importantly, cause shifts in pharmaceutical mixture composition.
239 During flood events, cumulative pharmaceuticals concentration was generally lower in the
240 estuary mouth vs. head. As an example, the peak concentration of the F2016-17 #1 event
241 decreased by >20% along its flow in the estuary to 14.3 $\mu\text{g L}^{-1}$ at the mouth (Figures 1 and 2).

242 Although the total contribution of anticonvulsants and caffeine to the cumulative concentration
243 was maintained, the proportion of caffeine decreased from 41% to 28%, while that of the
244 anticonvulsants increased from 29% to 44%. In the case of the F2017-18 #1 event, mouth
245 samples were not collected since discharge was very low (Table S4), and the flood water was
246 contained in the estuary and strongly diluted by the base flow and seawater intrusion.

247 The estuary water body holds ~300,000 m³ over a length of 6.5 km, resulting in long-
248 duration replacement of base-flow water with storm water. This enables the estuary to hold on
249 to low water volumes, associated with small flood events, prolonging their residence time. The
250 effect is on the scale of complete retention in the estuary, such as for F2017-18 #1, to hours,
251 e.g., F2016-17 #1 where the first flood wave entering the estuary arrived at its mouth after 10
252 h. Therefore, the presence of a base-flow signature of pharmaceutical mixture composition is
253 highly pronounced in the first flood-event outflow samples. In general, an increased
254 contribution of anticonvulsants and lipid regulators to the cumulative pharmaceuticals
255 concentration was recorded in outflux samples of all flood events (Figures 1 and 2).

256 A reduction in pharmaceutical concentration was observed under base-flow conditions
257 along the flow in the estuary, where anticonvulsant concentrations dropped by ~25% from head
258 to mouth during the 2017 base flow. Reduction was also pronounced in a spring 2018 water
259 sample where the elevated caffeine concentrations dropped by almost 50%. Pharmaceutical
260 addition and removal along the estuary under the different flow conditions are complex and
261 derive from the dynamics between the multiple sources, the compound characteristics and
262 persistence, and sorption–desorption processes. These changes may influence the risk potential
263 and differentiate the waters entering the estuary from those exiting to the near-shore
264 environment.

265

266

267 **Ecotoxicological risks: Single compound approach**

268 Due to dilution cycles, continuous changes in geochemical conditions, and the diverse
269 exposed taxonomic groups, there is a need for a dynamic ecological risk evaluation. We
270 focused our efforts on the changes in risk between the head and mouth of the estuary under
271 different flow conditions (Figure 3). Under base flow conditions, at the head of the estuary,
272 carbamazepine and diclofenac were found to pose an unacceptable risk, with median RQ of
273 ~15 and ~ 1 respectively. Sildenafil and lorazepam had median RQ values of 0.3 and 0.5,
274 respectively, posing minor risk. All other compounds exhibited median RQ values < 0.1
275 indicating an acceptable risk. Carbamazepine risk was also found to pose an unacceptable risk
276 at the head of the estuary during flood-events, with a median RQ of 2.5, under these conditions
277 ibuprofen and caffeine were also found to pose an unacceptable risk with median RQs of ~18
278 and ~3, respectively. Diclofenac and acetaminophen also showed RQ values higher than 1 on
279 several occasions. Our data show that flood risk is driven by more readily degradable, but still
280 toxic, compounds into the estuary that are otherwise absent from the system. Moreover, flood
281 water eventually replaces the estuarine volume to become background water, dominating the
282 habitat for weeks, and imposing elevated risk during the rainy season.

283 Under the base-flow regime, risk quotients for the estuary outflow were lower than for the
284 head of the estuary. Median RQ values for carbamazepine and diclofenac were reduced from
285 15 to 10 and from 1 to 0.1, respectively (Figure 3). These drops in RQ may be the consequence
286 of dissipation processes in the estuary due to prolonged water residence time under base-flow
287 conditions. For some pharmaceuticals, RQ values in the outflow during flood events were also
288 reduced compared to the head with the estuary with drops of 10% and 30% was observed for
289 ibuprofen and caffeine in outflow samples. On the other hand, an increase in median RQs was
290 observed for carbamazepine and gemfibrozil, from 2.5 and 0.01 to 7 and 0.6, respectively.
291 These increases in relatively environmentally stable pharmaceuticals suggest additional

292 sources of treated wastewater along the flow in the estuary, and perhaps, resuspension and
293 desorption processes in the estuary. These changes show that the risk regime expands beyond
294 temporal variations to spatial location along the estuarine flow, and reflects the complex
295 dynamics of pharmaceutical mixtures transported through the estuary.

296

297 **Ecotoxicological risks: Analysis per taxonomic groups**

298 While single-compound risk is crucial to identifying key stressors, the estuarine
299 environment is exposed to multiple pharmaceuticals which may affect each taxonomic group
300 differently. Furthermore, use of the concentration addition model to estimate cumulative risk
301 enables targeting risk assessments to specific taxonomic groups, such as fish, crustaceans and
302 algae (Figure 4). The overall annual RQ medians at the head of the estuary for fish, crustaceans
303 and algae were 20, 3.5 and 4.5, respectively. For fish, median RQ values for flood events was
304 ~22 vs. only 4.5 under base-flow conditions (Figure 4). Risk for crustaceans was elevated in
305 the base flow with median RQ values of ~11 compared to 3.5 in the flood events. For algae,
306 the RQ values for the flood and base flow conditions were relatively similar (4.5 and 3.5,
307 respectively). Only 3 inflow water samples (out of 151), collected from the base flow during
308 the peak dry season of 2018, exhibited RQ values lower than 1 (i.e., no risk) for fish (Figure
309 S2). For crustaceans, 11 samples (collected at the end of F2017 #3 and beginning of F2017 #4
310 events) showed RQ values lower than 1; and 10 samples exhibited no risk for algae, mainly
311 samples from F2017 #4 at peak discharge and the base flow in the 2018 dry season.

312 The highest RQ values of 338 and 227 were calculated for fish during the second and third
313 flood events of 2016 (F2016-17 #2 and #3), in agreement with its elevated median RQ in flood
314 events (Figure 4 and S2). Although the median RQ for crustaceans was higher during base
315 flow, peak RQ values of 32 and 27 were measured at the beginning of the first flood event of
316 each of the rainy seasons, suggesting first season wash of the upper watershed. Similar to

317 crustaceans, the RQ for algae peaked at 16 and 19 at the beginning of the first two flood events
318 of each of the rainy seasons.

319 The anti-inflammatory ibuprofen, which was less dominant in terms of concentration
320 (Figures 1 and 2), was the compound found to contribute most to the RQ for fish, (Figure S2).
321 Yet even in the absence of ibuprofen, 95% of the water samples showed unacceptable risk to
322 fish with RQ values higher than 1. In these samples, risk was mainly derived from the lipid
323 regulator bezafibrate and the anti-inflammatory diclofenac. Risk for crustaceans was derived
324 almost entirely from the anticonvulsant drug carbamazepine, in both flood events and base
325 flow, with a small contribution from the stimulant caffeine. An additional contribution to risk
326 for crustaceans was the lipid regulator metabolite clofibrate acid. Unlike risk to fish, removal of
327 the dominant contributor (i.e., carbamazepine) from the cumulative risk calculation for
328 crustaceans resulted in only ~20% of the samples exceeding an RQ value of 1. Risk to algae
329 was mostly driven by the stimulant caffeine, contributing 56% of the risk on average, followed
330 by carbamazepine, responsible for 30% of the RQ, mainly during base flow (Figure S2). Minor
331 contributions to risk for algae were from ibuprofen and sulfamethoxazole. Omitting both
332 caffeine and carbamazepine from the cumulative risk calculations for algae still resulted in
333 ~25% of the samples exceeding an RQ of 1, all from flood events.

334 The overall median RQ values for estuary mouth samples were relatively similar to those
335 for the head samples (Figure 4). The most pronounced difference in risk to crustaceans was
336 found during flood events, being over twofold higher in mouth vs. head samples, with a median
337 RQ of ~8. Median RQ for algae during flood events was slightly higher in the outflow (5.5),
338 even though the dominant toxicant, caffeine, had decreased in concentration (Figures 1 and 2).
339 This additive effect was due to the increase in carbamazepine concentration, which was also
340 found to be a main contributor of risk to algae (Figures S2 and S3). The same trend was found
341 for cumulative risk to fish during flood events, being slightly elevated in outflow samples with

342 a median RQ of 23 (Figure 4). The reduction in ibuprofen concentration was substituted by the
343 elevation in gemfibrozil concentration and risk to fish. Base-flow conditions at the mouth of
344 the estuary were similar to those at the estuary head, where the biggest recorded difference was
345 a decrease of 25% median RQ for fish. It is important to note that the elevated risk to algae
346 from caffeine, due to the sewage spill in March 2018, dropped by almost 50% from head to
347 mouth (Figures S2 and S3). The observed removal emphasizes the strong dissipation processes
348 that might occur along the estuary under long water residence times.

349

350 **Ecotoxicological risks: Effects of geochemical conditions**

351 Although geochemical factors were not included in our risk calculation, they can affect
352 directly or indirectly the ecological risk in micro-estuaries. Temperature in the micro-estuary
353 ranges between 15-30 °C, peaks in the dry season when treated wastewater flows dominates
354 the system (Figure S4). Temperature rise in dry season (i.e., base flow condition) is expected
355 to pose abiotic stress via reducing the level of dissolved oxygen. In practice, the highly nutrient
356 loaded wastewater induces eutrophication processes resulting in sever oxygen deficiency
357 throughout the micro-estuary water column (Figure S4). The continuous anoxic-hypoxic
358 conditions, which may be harmful for aquatic organisms on its own, along with low rates of
359 water replacement¹¹, and possibly reduced microbial degradation of pharmaceuticals under
360 these conditions⁵⁶, may result with elevated stress to the aquatic habitat during the dry season.

361 The reactivity of pharmaceuticals might be also affected by geochemical conditions, and in
362 turn, influence the ecological risk. In micro-estuaries, pharmaceuticals are exposed to rising
363 levels of salinity along the flow and with depth of the water column (Figure S4). This may
364 affect the potential calculated toxicity of compounds to some extent^{52,57,58}. Yet salinity may
365 more profoundly affect pharmaceuticals risk indirectly, due to alteration of sediment-water
366 partitioning. Elevated salinity is expected to increase adsorption of organic pollutants, reducing

367 the concentration in water column (and lowering the risk to organisms within that media), but
368 increasing the concentration in bed sediments and the resulted risk to benthic dwellers.
369 However, these effects are more pronounced for hydrophobic compounds rather than to the
370 studied pharmaceuticals exhibiting low $\log D$ (Table S1).

371

372 **Environmental implications**

373 Estuarine environments, such as the Alexander stream, are continuously exposed to organic
374 pollutants⁵⁹, among them biologically active pharmaceuticals^{3,23,53}. Pharmaceuticals are
375 introduced to estuarine habitats via runoff from rural and municipal zones during the rainy
376 season. During the long and dry summer periods, pharmaceuticals are introduced via
377 wastewater which made up the sole water source for these ecosystems. The composition of the
378 pharmaceutical mixtures is highly coupled to the flow patterns, thus exposing the estuary to a
379 changing risk regime.

380 The use of general PNEC_{aquatic} and RQ as risk parameters is of great value in locating
381 dominant drivers, but the use of taxonomic group PNECs allowed a more practical risk
382 assessment, especially in systems with fluctuating flow conditions. While general aquatic risk
383 showed elevated risk from caffeine during flood events, in practice, caffeine risk peaked at the
384 beginning of the flood events and mainly affected algae, which are most likely washed from
385 the system during this flood stage. On the other hand, ibuprofen is mostly toxic to fish, which
386 stays within the estuary. Nevertheless, risk to algae extends beyond the flood's first wave and
387 remains a concern as these primary producers are extremely important to micro-estuaries,
388 dictating the oxygen balance in these highly eutrophicated ecosystems.

389 Ibuprofen, carbamazepine and caffeine were the main compounds contributing to risk for
390 different taxonomic groups. However, their removal from the system is not expected to fully
391 mitigate the ecosystem, as the level of the mixture's risk remains high. Caffeine and ibuprofen

392 originate mainly from raw or semi-treated wastewater and can be controlled by preventing
393 sewage spills and overflows. Carbamazepine, on the other hand, is diffusive and more
394 persistent, and its removal from the system is more challenging. Along with lipid regulators
395 and sulfonamides, removing carbamazepine's adverse effect requires the complete removal of
396 treated wastewater from the stream flow, and therefore must include control of the arable land
397 runoff from fields irrigated with treated wastewater. The latter is of high importance since flood
398 tails water, posing potential risk to algae and elevated risk to fish, enters the estuary in flow
399 with decreasing velocities, eventually acquiring base-flow residence times of ~20 days.

400 The Alexander micro-estuary is exposed annually to chronic risk throughout its entire
401 length and under the different flow conditions. This reflects the high potential risk in
402 hypereutrophic micro-estuaries to several taxonomic groups from a mixture of pharmaceuticals
403 originating from both point and diffuse pollution sources. The current work findings highlight
404 the need to update water regulations, which do not currently take pharmaceutical mixtures into
405 account. Furthermore, watershed-management interfaces are required in semi-arid region
406 streams and estuaries to reduce pharmaceutical influx and prolong water residence time,
407 thereby enhancing the natural removal processes.

408

409 **Supporting Information**

410 Information is provided about internal standards used for LC-HRMS; selected
411 pharmaceuticals properties and analytical method parameters (Table S1). Assessment factors
412 determination for PNEC_{aquatic} are provided in Table S2 and pharmaceuticals assessment factors
413 and PNECs are presented in Table S3. Sampled flow events characteristics are listed in Table
414 S4. A map of the study site is shown in Figure S1. Risk quotient distribution between
415 pharmaceuticals during 2 hydrological years is presented in Figures S2 and S3. Geochemical

416 conditions in the Alexander micro-estuary in the two-years study duration are shown in Figure
417 S4. These materials are available free of charge via the Internet at <http://pubs.acs.org/>.

418

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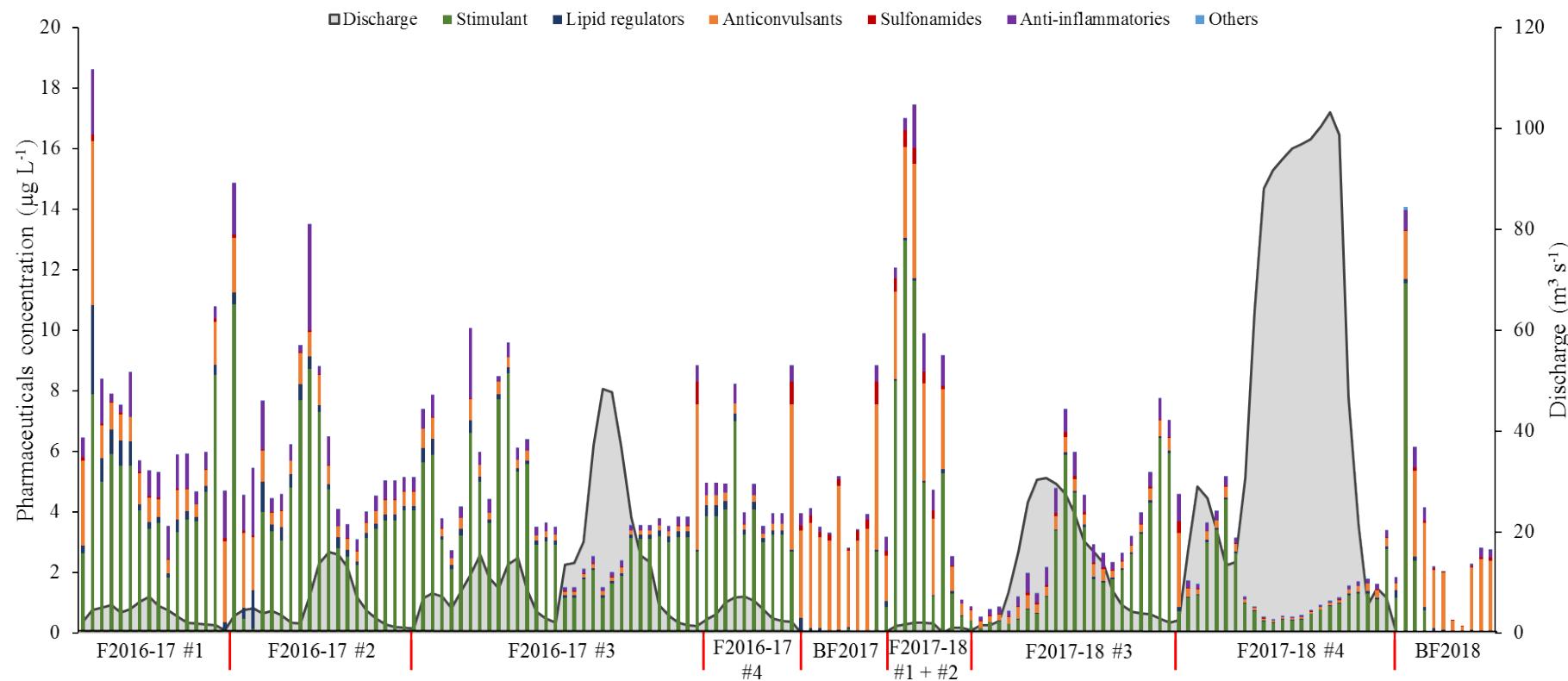
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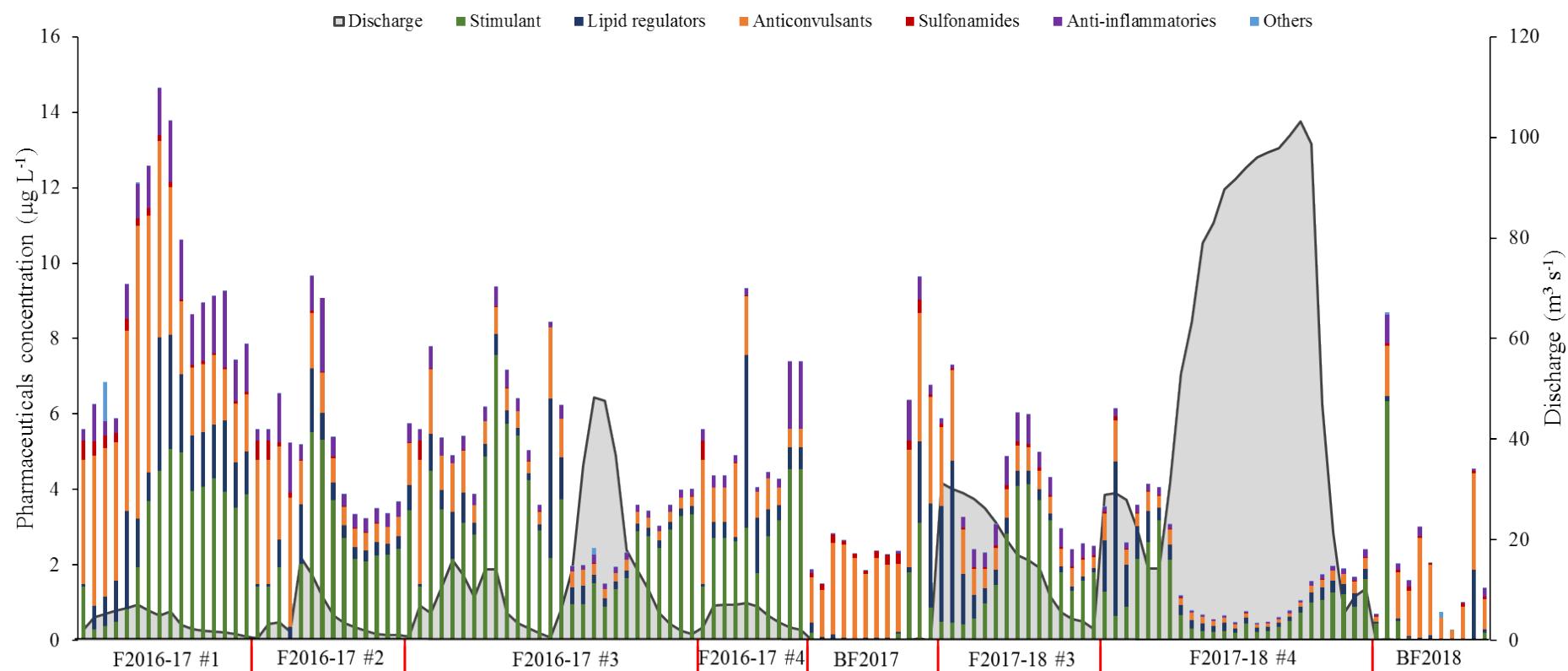
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627 **Figure 1.** Concentrations of groups of pharmaceuticals (left axis) and flow discharges (right axis) during 2 hydrological years (Table 2) measured
628 at the head of the Alexander micro-estuary. Horizontal axis presents water samples in chronological order, where F represents flood events and
629 BF represents base flow.

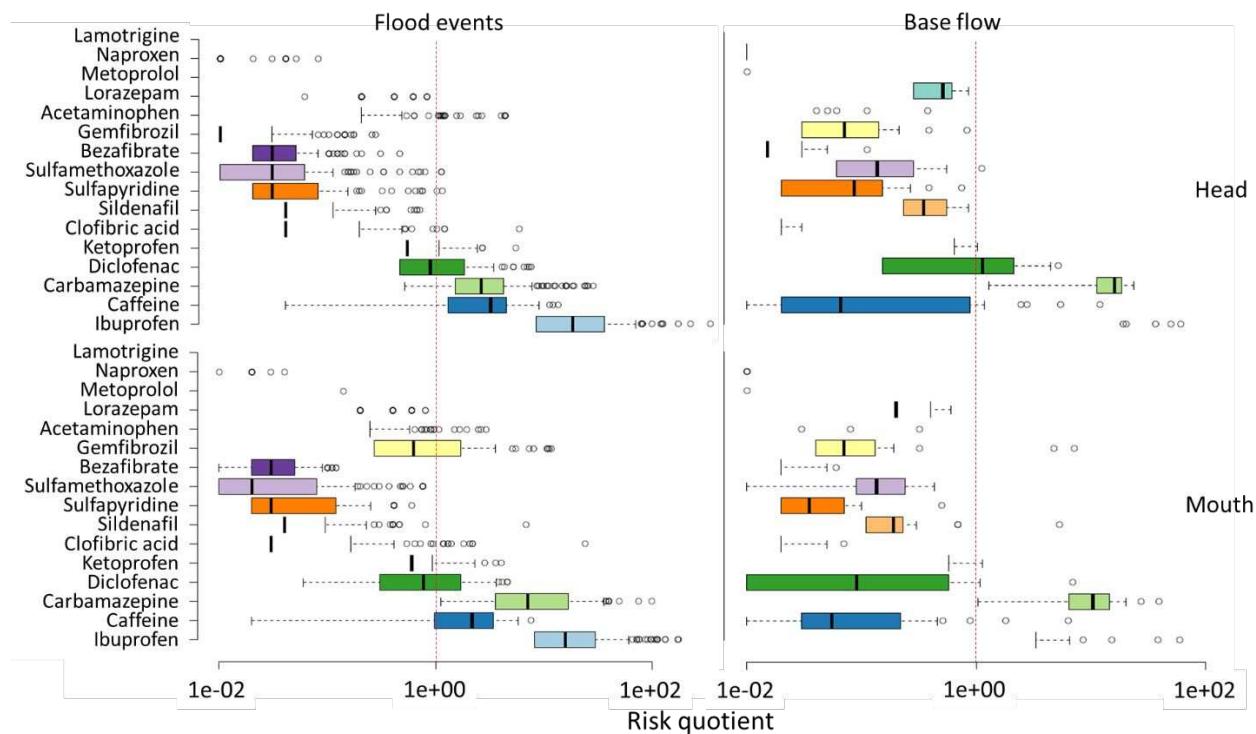
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632 **Figure 2.** Concentrations of groups of pharmaceuticals (left axis) and flow discharges (right axis) during 2 hydrological years (Table 2) measured
 633 at the mouth of the Alexander micro-estuary. Horizontal axis presents water samples in chronological order, where F represents flood events and
 634 BF represents base flow.

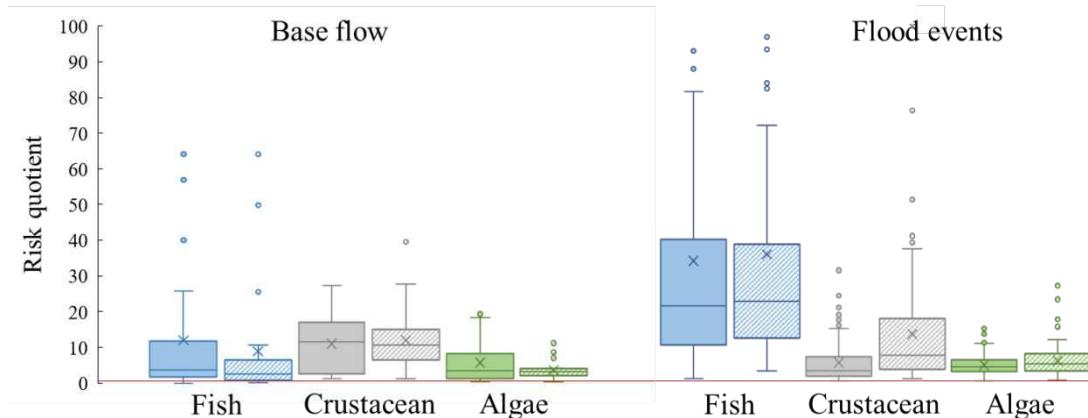
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637 **Figure 3.** Pharmaceutical risks in flood and base-flow water samples collected at the head and
 638 mouth of the Alexander micro-estuary. Risk quotients were calculated using point of no effect
 639 concentrations for the aquatic habitat ($\text{PNEC}_{\text{aquatic}}$).

640



641

642 **Figure 4.** Cumulative pharmaceutical risk in the Alexander micro-estuary. A risk quotient of
643 1 (red line) marks toxicity benchmarks (Table 1). Cumulative pharmaceutical risk to fish,
644 crustaceans and algae at the head of the estuary (solid, n = 151) and mouth of the estuary
645 (diagonal stripes, n = 130). Exceptional samples from head (7) and mouth (9) samples in flood
646 events, with risk quotients ranging from 100 to 338 for fish, were omitted.