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Complex chemical cocktail, containing insecticides diazinon and permethrin, drives acute toxicity to crustaceans in mountain lakes

Authors: Oliver Machate^{1,2}, Dirk S. Schmeller³, Adeline Loyau^{3,4}, Albrecht Paschke⁵, Martin Krauss¹, Eric Carmona¹, Tobias Schulze¹, Alessandra Moyer⁶, Kurt Lutz⁶, Werner Brack^{1,7}

Institutions:

1. Helmholtz Centre for Environmental Research – UFZ, Department of Effect Directed Analysis, Permoserstrasse 15, 04318 Leipzig, Germany
2. University of Leipzig, Department of Biology, Johannisallee 21-23, 04103 Leipzig, Germany
3. Laboratoire écologie fonctionnelle et environnement, Université de Toulouse, Toulouse INP, Université Toulouse 3 - Paul Sabatier (UPS), Toulouse, France
4. Department of Experimental Limnology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Alte Fischerhütte 2, Stechlin, D-16775, Germany
5. Helmholtz Centre for Environmental Research – UFZ, Department of Ecological Chemistry, Permoserstrasse 15, 04318 Leipzig, Germany
6. Department of Biology, San Francisco State University, San Francisco, CA 94132-1722
7. Goethe University Frankfurt, Department of Evolutionary Ecology and Environmental Toxicology, Max-von-Laue-Strasse. 13, 60438 Frankfurt am Main, Germany

Corresponding author:

Abbreviations:

POPs: Persistent organic pollutants; PAHs: Polycyclic aromatic hydrocarbons; HCHs: Hexachlorocyclohexanes; HCB: Hexachlorobenzene; DDT: Dichlorodiphenyltrichloroethane; PCBs: Polychlorinated biphenyls; LC-HRMS: Liquid chromatograph-high resolution mass spectrometry; GC-HRMS: Gas chromatograph-high resolution mass spectrometry; TU: Toxic unit; SI: Supporting information; PSD: Passive sampling device; PRC: Performance reference compound; SI: supplementary item; MDLs: Method detection limits; AF: Additional File; EC: Effect concentration.

*Dirk S. Schmeller: ds@die-schmellers.de

Email addresses:

*Oliver Machate: oliver.machate@gmail.com

Dirk S. Schmeller: ds@die-schmellers.de

Adeline Loyau: al@adeline-loyau.net

Albrecht Paschke: albrecht.paschke@ufz.de

Martin Krauss: martin.krauss@ufz.de

Eric Carmona: eric-carmona.martinez@ufz.de

Tobias Schulze: tobias.schulze@ufz.de

Alessandra Moyer: alessandramoyer@gmail.com

Kurt Lutz: kurtrobertlutz@gmail.com

Werner Brack: werner.brack@ufz.de

ORCID:

Oliver Machate: 0000-0002-4247-8358

Dirk S. Schmeller: 0000-0002-3860-9933

Adeline Loyau: 0000-0003-4230-5892

Albrecht Paschke: 0000-0003-2824-2194

Martin Krauss: 0000-0002-0362-4244

Eric Carmona: 0000-0002-1834-8342

Tobias Schulze: 0000-0002-9744-8914

Werner Brack: 0000-0001-9269-6524

1 **Complex chemical cocktail, containing insecticides diazinon and**
2 **permethrin, drives acute toxicity to crustaceans in mountain lakes**

3 **Abstract:**

4 Mountain lakes have long been perceived as pristine environments. However, atmospheric
5 deposition of persistent organic pollutants (POPs) have been shown to expose these
6 sensitive ecosystems to chemical pollution. Little is known on how this pollution impacts
7 aquatic ecosystems at high altitudes. We combined passive sampling with liquid and gas
8 chromatography high resolution mass spectrometry (LC- and GC-HRMS) to screen the water
9 of eight lakes in three different regions of the French Pyrenees. In total, we screened for 479
10 organic chemicals including POPs, polycyclic aromatic hydrocarbons (PAHs), legacy and
11 current-use pesticides, biocides, and musk fragrances. We detected a complex cocktail of
12 151 individual chemicals and used their toxic unit summation (ΣTU) to assess toxicity for
13 crustaceans and algae. While risks for algae never reached chronic risks, this was always
14 the case for crustaceans. Acute toxic risk thresholds for crustaceans were even exceeded in
15 several of our sites. At sites with acute toxic risk levels ($> 0.1 \Sigma TU$) crustaceans were
16 completely absent or showed a low abundance. We conclude that crustaceans were at least
17 partly impacted by the high toxic risks driven by the insecticides diazinon and permethrin.
18 These drugs are widely used to protect livestock from blue tongue disease transmitted by
19 sucking insects, suggesting free roaming livestock as local source. Our results provide
20 important evidence on toxic chemical pollution in relatively remote mountain areas, with
21 important consequences for aquatic mountain ecosystems.

22

23

24 **Keywords:**

25 Organic Micropollutants; Passive Sampling; Risk Assessment; Planktonic Toxicity; Pyrenees

1. Introduction

Due to their remoteness, mountains and their lakes are perceived as pristine, healthy environments. This perception has been proven wrong by a number of studies on chemical pollution in remote mountain environments. Persistent organic pollutants (POPs) have been found to undergo atmospheric long-range transport and accumulate in remote polar regions (Wania and Mackay, 1996), but may also impact remote montane lakes (Daly and Wania, 2005; Fernandez and Grimalt, 2003). Polycyclic aromatic hydrocarbons (PAHs) were among the first pollutants to be found in European mountain lakes. However, other substances, such as hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), have been frequently detected in mountain lakes of the Alps, Pyrenees and Caledonia (Vilanova et al., 2001). The number of substances detected in mountain lakes has continued to grow and now also includes brominated flame retardants, synthetic musks (Schmid et al., 2007), and some current-use agricultural pesticides such as terbuthylazine, chlorpyrifos and pendimethalin (Ferrario et al., 2017; Santolaria et al., 2015). Long- and medium-range atmospheric transport have been intensively investigated to understand pollution pathways in remote areas (Wania and Westgate, 2008). In contrast, knowledge on the role of local activities, such as traffic (Blumer et al., 1977), tourism, and pastoralism (Gassner et al., 1997; Weissinger et al., 2018), have been little studied as pollution sources in mountains. Although impacts of pollution on mountain organisms have been investigated (Garcia-Reyero et al., 2005; Quirós et al., 2007; Sparling and Fellers, 2009), the contamination of mountain lakes with organic micropollutants, pollution sources, and resulting toxic risks remain little understood.

Mountain lake ecosystems may be particularly sensitive to adverse effects of chemical pollution, as living conditions at high altitudes are extreme (Catalan et al., 2006), causing environmental stress to aquatic organisms. Due to this strong selective pressure, aquatic communities in mountain lakes, are often simple and consist primarily of microorganisms,

53 phyto- and zooplankton, macroinvertebrates, and a few amphibian species (Sánchez-
54 Hernández et al., 2015). These species show life-history traits particular to mountain
55 organisms, i.e. increased fatty tissue and lifespan, which make them prone to accumulate
56 organic pollutants. In combination with the increased sensitivity due to the high level of
57 prevailing environmental stress and the potential funnelling of organic pollutants in mountain
58 lakes (Daly and Wania, 2005; Kallenborn, 2006; Liess et al., 2016), mountain lake
59 ecosystems are particularly vulnerable to adverse effects of chemical pollution. While toxicity
60 at lower altitudes is known to be an important limiting factor of aquatic ecosystem quality
61 (Machate et al., 2021; Posthuma et al., 2020), little is known about the extent and impact of
62 pollution induced toxicity in mountain lakes (Schmeller et al., 2018).

63 In this study, we provide a first overview on the exposure of mountain lakes of the French
64 Pyrenees to a broad range of chemicals and related toxic risks. We combined passive
65 sampling with liquid and gas chromatography high resolution mass spectrometry (LC- and
66 GC-HRMS) to screen for 479 different compounds. These compounds include those known
67 to undergo atmospheric transport and substances that might be introduced via local sources.
68 We report potential impacts on freshwater plankton communities using a toxic unit (TU)
69 approach.

70

71

72 **2. Material and Methods**

73 **2.1 Study area**

74 Eight permanent mountain lakes of the French Pyrenees in three regions, (1) Lescun, (2)
75 Neouvielle, and (3) Bethmale, were investigated (figure 1, table 1). Target lakes were
76 selected to cover a wide geographic range to include different altitudes and contamination
77 sources (tourism, pastoralism, and atmospheric deposition (table 1)). All lakes are positioned
78 at altitudes ranging from 1714 to 2400 m and are not directly adjacent to urban areas or field

79 crops. We therefore assume that the direct input of organic micropollutants from domestic
80 wastewater, industry, and field crops was negligible. In general, however, all lakes are freely
81 accessible to livestock and tourists, but to retrieve exact data on tourist numbers and
82 livestock is difficult, as tourists do not pay an entrance fee and most livestock flocks roam
83 freely, even across the Franco-Spanish border. The lakes are often the only source of water
84 for livestock, which may stay close to the lakes. Livestock can move from one lake to another
85 and their activity radius is difficult to assess or map. We developed indices for tourism and
86 pastoralism as proxies for exposure pressure, as exact numbers of livestock or tourists are
87 not available for our sites (table 1). The tourism index was calculated considering the
88 following factors: (a) proximity of the GR10, the most popular hiking trail in the Pyrenees,
89 does not lead to the sample site = 0 points, GR 10 leads to the sample site = 1 point, (b)
90 proximity of secondary hiking trails to the lakes (0 = absence, 1 = presence), (c) the distance
91 to the closest parking lot (distance >7.1km = 1 point, 5.1 to 7km = 2 points, 3.1 to 5km = 3
92 points, 1.1 to 3 km = 4 points, between 0 and 1km = 5 points), (e) the presence or signs of
93 camping material and/or tents close to the lakes (absence = 0, presence = 1 point), observed
94 during the sampling.

95 The pastoralism index simply considers the complete absence of either horses, sheep or
96 cattle (0 points), presence of one of the species (1 point), two of the species (2 points) or all
97 three species (3 points).

98

99 **2.2 Passive sampling**

100 Silicone rubber passive sampling devices (PSDs) were used to investigate the pollution
101 status of the lakes to avoid shortcomings of traditional whole water grab sampling. These
102 include the lack of representativeness in time, logistic challenges and limitations in pre-
103 concentration of hydrophobic chemicals such as pyrethroids (Brack et al., 2017). PSDs are
104 light in weight, accumulate pollutants from the water phase and allow for a time-integrated

105 sampling (Booij et al., 2016; Schulze et al., 2011). Therefore, PSDs are the ideal sampling
106 technique when investigating the pollution of mountain lakes.

107 The PSDs were derived from translucent AlteSil™ silicone sheets, with a size of 30 x 30 cm
108 and a thickness of 5 mm, obtained from Altec Products Ltd., Victoria/UK. They were
109 prepared for sampling as described by Smedes and Booij (2012). Briefly, using a template,
110 the silicon sheets were cut into smaller silicon stripes of the size of 5.5 x 9 cm² and pre-
111 cleaned via Soxhlet extraction with ethyl acetate. To ensure a sufficient removal of oligomers
112 and other impurities, each sheet was extracted for at least 40 h in total. Consecutively, the
113 sheets were stored in methanol until spiking with performance reference compounds (PRCs)
114 (supplementary item (SI) 1). For spiking we equilibrated the silicon rubber in a methanol-
115 water mixture for one week (Chepchirchir et al., 2017). The spiked membranes were dried
116 with lint-free tissue. They were subsequently placed on a polypropylene rope used for the
117 deployment in the field. For transport, they were wrapped in aluminium foil, vacuum packed
118 in a plastic bag, stored in a freezer at -20°C until deployment and transported on dry ice to
119 the respective mountain lake.

120 To cover potential temporal differences in pollutant exposure the PSDs were deployed in the
121 lakes at a depth of 0.3 to 1 m from late June to August (period 1) and August to September
122 (period 2) of 2018 (SI 2). The two sampling periods represent mountain summer and autumn,
123 respectively. They were chosen to cover most of the extent of the ice-free season, during
124 which pollutant input from the surrounding environment is possible. Exposure times varied
125 between 38 and 49 days. Given the difficult logistics, no standard cage was used to deploy
126 the PSDs. The PSDs were attached in equidistant spacing on a polypropylene rope
127 (Chepchirchir et al., 2017). The rope was stretched between two stainless steel rods that
128 could be anchored into the embankment of the lake to keep the PSDs immersed in water
129 during the sampling period. Before placing the PSDs back into their respective transportation
130 box and on dry ice, they were carefully rinsed with lake water to remove excess fouling and
131 debris. In the lab, the samplers were stored at -20°C until extraction.

132

133 **2.3 Extraction of passive samplers**

134 Two silicon sheets per site were extracted twice at room temperature with 100 mL of
135 methanol for 24 h. This mild extraction procedure was chosen to avoid the loss of
136 compounds due to volatilisation and extraction of oligomers (Brockmeyer et al., 2015). The
137 extracts were concentrated to approximately 2 mL using a rotary evaporator after adding
138 nonane as a keeper. Care was taken that the samples did not fall dry. The concentrate was
139 split into two aliquots, one for analysis by LC-HRMS and one for GC-HRMS. A volume of 20
140 mL ethyl acetate was added to the GC fraction before reducing the volume to approximately
141 2 mL. Finally, both extracts were transferred into evaporation fingers and evaporated under
142 the gentle stream of nitrogen to a volume of 0.5 mL.

143

144 **2.4 Chemical analysis and data evaluation**

145 The extracts were screened for 479 target compounds using LC- and GC-HRMS. Targets
146 included chemicals known to undergo long-range atmospheric transport (e.g. PCBs, PAHs
147 and legacy pesticides like DDT and HCB). As novelty, we also included compounds
148 assumed to not undergo atmospheric transport . Such compounds have not been
149 investigated in the context of mountain lakes. The latter include current-use agricultural
150 pesticides and biocides, personal care products, and industrial chemicals.

151 The semi-volatile hydrophobic analytes were comprised mainly of PAHs, PCBs and
152 pyrethroid insecticides (N=142). They were measured using a QExactive GC system
153 (Thermo) equipped with Thermal Desorption Unit (TDU-2; Gerstel, Mulheim/Ruhr, Germany)
154 and a cooled injection system (CIS; Gerstel). The remaining analytes (N=337), primarily
155 including herbicides, fungicides, insecticides and personal care products, were analysed
156 using an UltiMate 3000 LC system (Thermo) coupled to a QExactive Plus MS (Thermo) with
157 a heated electrospray ionization source (SI 3).

158 A set of method-matched calibration and internal standards, consisting of 9 (GC) and 40 (LC)
159 isotope-labelled compounds (SI 4), were used for quantification. Six levels of calibration
160 standards of concentrations of 2.5, 10, 50, 100, 250, and 1000 ng/mL were prepared. These
161 standards in ethyl-acetate or methanol (GC or LC compound mix, respectively) were spiked
162 on top of two silicon sheets. After letting the compound mixes and sheets equilibrate
163 overnight, the sheets were subjected to the same extraction procedure as the PSD field
164 samples. Target substances were allocated to their respective internal standards based on
165 similarity in retention time. Method detection limits (MDLs) were determined based on the
166 lowest measurable concentration of the respective standard compound (Additional file (AF),
167 sheet 1). To check for potential contaminations from storage, transport and handling of the
168 sheets, laboratory and field blanks were prepared and analysed simultaneously.

169 LC-HRMS raw data files were converted to mzML data using the ProteoWizard MSConvert
170 tool (v3.0.18265; Chambers et al., 2012). MZmine (v2.40.1) was used for peak detection,
171 sample alignment and target compound annotation (Pluskal et al., 2010) according to
172 Beckers et al. (2020). Finally, a blank correction, calibration and quantification of the
173 annotated target compounds were performed using the in-house R package MZquant
174 (v0.6.1) (SI 5). GC-HRMS-detected compounds were quantified with the software
175 TraceFinder 4.1 (Thermo Scientific). To visualize the data, the ggplot R package v3.2.1 was
176 used (Wickham, 2016).

177

178 **2.5 Calculation of water concentrations and toxicological risk assessment**

179 The toxic risk to algae and crustaceans was assessed by means of TU summation
180 (Ginebreda et al., 2014; Sprague and Ramsay, 1965). To this end, the measured PSD
181 concentrations were transformed into time-integrated freely dissolved water concentrations
182 based on the uptake rates of the analytes (Smedes and Booij, 2012) (SI 6). Preferably
183 experimentally determined partitioning coefficients between PSD and water (K_{PW}) were used
184 (Smedes, 2018; 2019). If these data were not available, the UFZ-LSER database (Ulrich et

185 al., 2017) was used to model missing partitioning coefficients via poly-parameter linear free
186 energy relationships (pp-LFERs) (AF, sheet 1).

187 Toxic units were summed up according to the mixture toxicity model of concentration addition
188 (Backhaus and Faust, 2012) for algae (ΣTU_{Alg}) and crustaceans (ΣTU_{Crust}) (equation 1):

189
$$\Sigma TU = \sum_{i=1}^n \frac{c_{i,water}}{EC_{x_i}} \text{ (equation 1)}$$

190 where $c_{i,water}$ is the concentration of the analyte in water and EC_{x_i} is the effect concentration
191 (EC) at level x . Lethal and sublethal EC-values for both organism groups were retrieved from
192 the US EPA ECOTOX Knowledgebase (09/12/2020) and used to calculate the 0.05 quantiles
193 ($Q_{0.05}$) for each compound (Busch et al., 2016; Schulze et al., 2021). If no data were
194 available, EC_{50} -values for algae and crustaceans were estimated by using baseline ECOSAR
195 1.0 (Mayo-Beana et al., 2009; Nabholz et al., 1998) type model in ChemProp 6.3 (UFZ
196 Department of Ecological Chemistry, 2016) (SI 7; AF, sheet 3 & 4). For algae, 33% and for
197 crustaceans 37% of the EC-values were extracted from the ECOTOX Knowledgebase and
198 67%, respectively 63%, were modeled via ECOSAR. The risk thresholds were set to 0.1 TUs
199 for acute toxic risk to algae and crustaceans, and 0.02 and 0.001 TUs for a chronic toxic risk
200 to algae and crustaceans, respectively (Malaj et al., 2014).

201

202 **2.6 Zooplankton abundances**

203 Due to their central role in aquatic food webs and their high sensitivity to toxic pressures
204 (Beketov et al., 2013; Wolfram et al., 2021), crustaceans are frequently used as indicators of
205 pollution (Hanazato, 2001). We sampled zooplankton while the passive samplers were
206 retrieved. Two hundred litres of lake water were filtered through a plankton net with a mesh
207 size of 25 μm . The obtained filtrate was transferred into a 50 mL vial and preserved by
208 adding 3 mL of 30% formalin. In the laboratory, the zooplankton was stained with
209 erythrosine, identified and counted under a microscope in a counting wheel. We used counts
210 of crustaceans, counts of rotifers and total counts of both species' groups, number of species

211 (= richness) combined and separate for crustaceans and rotifers to test pollutant impact on
212 plankton communities.

213

214 **2.7 Statistical analysis**

215 All statistical analyses and data computation were performed in R v3.5.1 (2018-07-02; R
216 Core Team, 2018). As the dataset was relatively small and data were not normally
217 distributed, non-parametric tests were used. We used a paired Wilcoxon signed rank test to
218 compare pollutant concentrations between the two sampling periods, and a Kruskal-Wallis
219 ANOVA to compare concentrations between regions. $\sum TUS_{Crust}$ was compared between
220 sites with and without cattle using a paired Wilcoxon signed rank test. We used a Spearman
221 rank correlation between $\sum TUS$ (or pollutant concentrations) and the number of crustaceans,
222 rotifers observed, and species richness.

223

224 **2.8 Source attribution**

225 To evaluate the contribution of the atmosphere and local activities as the source of pollution,
226 especially for the detected insecticides, we grouped the contaminants according to their use
227 classes (AF; sheet 1). POPs (legacy pesticides, PCBs), PAHs, and agricultural plant
228 protection products (current-use herbicides and fungicides) were assumed to be deposited
229 from atmospheric transport. Insecticides used in non-agricultural applications (to treat
230 animals and as insect repellents for human use) were assumed to be introduced via local
231 sources. As huge differences in concentrations between the different pollutant groups
232 occurred, the additive concentrations were normalized to the maximum concentration
233 observed within the group, resulting in values between 0 (no detection) to 1 (maximum
234 concentration). Results are displayed in form of a heatmap (R package “ComplexHeatMap”;
235 function “heatmap”; Gu, 2016).

236

237 **3. Results**

238 **3.1 Water contamination**

239 In total, 151 compounds were detected in the PSDs (AF; sheet 1). For 141 compounds,
240 freely dissolved water concentrations were quantified (AF; sheet 2) (details on excluded
241 substances in SI 8). These compounds include: 37 POPs (eleven PCBs, ten legacy
242 pesticides), 16 PAHs, 44 current-use pesticides and biocides (nine insecticides and
243 repellents for human and veterinary use, 24 fungicides and eleven herbicides), and 60
244 diverse compounds (e.g. those used in fragrances, cleaning products and personal care
245 products (SI 9). Four compounds were detected in all samples at concentrations higher than
246 10 ng/L. They include the fungicide azoxystrobin with concentrations of up to 41 ng/L (factor
247 of 15 higher than lowest concentration), the herbicide terbuthylazine with a maximum
248 concentration of 12 ng/L (40 times higher than lowest concentration), benzothiazole with its
249 highest concentration of 90 ng/L (70 times higher than lowest concentration) and the insect
250 repellent DEET (chemical name: N,N-diethyl-meta-toluamide) with a maximum concentration
251 of 627 ng/L (900 times higher than lowest concentration). In addition to these ubiquitous
252 compounds, 24 compounds exhibited a detection frequency of more than 70%, including
253 eight fungicides, three herbicides, the insect repellent icaridin, the legacy pesticide HCB,
254 PCB 18, and 10 compounds of diverse use. The highest maximum concentration was found
255 for diphenyl phosphate (19 µg/L), a compound used as an additive in paints and coatings. It
256 was detected in a total of three samples. Higher concentrations were also detected for
257 benzenesulfonic acid (6.6 – 10 µg/L), used in household products and as a surfactant, and
258 the transformation product terbuthylazine-desethyl-2-hydroxy of the herbicide terbuthylazine
259 (2.1. 3.9 µg/L). Concentrations of the same magnitude were detected in six of the 16
260 samples. Higher concentrations were also found for the phytochemical genistein with 1.7
261 µg/L, which is probably of natural origin, and DEET with 627 ng/L.

262 Lakes in the Neouvielle region showed the lowest number of detected pollutants (average
263 number of pollutants detected across lakes: summer = 40; autumn = 47; AF sheet 1). In the
264 regions Lescun (summer = 67; autumn = 64) and Bethmale (summer = 68; autumn = 58) the

265 number of detected pollutants was consistently higher, corresponding to the higher
266 precipitation in those regions as compared to Neouvielle (table 1). In our summer samples,
267 there was a significant difference of pollutant concentrations between regions for PAHs ($K_{2,7}$
268 = 5.396, $p = 0.025$), UV-filters ($K_{2,7} = 5.000$, $p = 0.037$), and compounds classified as other
269 ($K_{2,7} = 6.250$, $p = 0.005$), with the Neouvielle sites being the least polluted, except for
270 compounds classified as other. Such differences were not significant for the autumn samples
271 alone. When pooling summer and autumn samples, we observed significant regional
272 differences in the concentrations of PAHs ($K_{2,15} = 5.867$, $p = 0.023$), fungicides used in
273 biocidal applications ($K_{2,15} = 6.870$, $p = 0.011$), veterinary insect repellents ($K_{2,15} = 4.776$, $p =$
274 0.045), and UV-filters ($K_{2,15} = 6.195$, $p = 0.019$). Sites in the Neouvielle region were the least
275 polluted across all our samples from 2018 (AF sheet 1).

276 Concentrations between sampling periods ($n = 8$) were only significantly different for insect
277 repellents applied on humans (summer: 185 +/- 80 ng/L; autumn: 108 +/- 49 ng/L; $S_{15} =$
278 3416, $p = 0.023$) and legacy pesticides (summer: 0.72 +/- 0.26 ng/L; autumn: 1.4 +/- 0.3
279 ng/L; $S_{15} = -18$, $p = 0.008$) with higher concentrations in summer and autumn, respectively.

280

281 **3.2 Risks to plankton communities**

282 The toxic risk for algae ($\sum TUS_{Alg}$) (AF, sheet 3) ranged from 1×10^{-3} to $0.01 \sum TUs$. The
283 highest $\sum TUS_{Alg}$ were observed in Puits ($0.01 \sum TUS_{Alg}$) in both periods, followed by Gourg
284 de Rabas ($6 \times 10^{-3} \sum TUS_{Alg}$), and Madamete Bas (summer, $6 \times 10^{-3} \sum TUS_{Alg}$; autumn, 4×10^{-3}
285 $\sum TUS_{Alg}$) of the Neouvielle region, and the lakes of the Bethmale region during summer ($8 \times$
286 $10^{-3} \sum TUS_{Alg}$) (figure 2A). Neither the threshold of $0.1 \sum TUS_{Alg}$ for acute toxic effects nor the
287 threshold of $0.02 \sum TUS_{Alg}$ for chronic toxic effects was exceeded. Main drivers of toxicity
288 were the agricultural herbicides terbuthylazine and atrazine, and the fungicides spiroxamine
289 and azoxystrobin. Terbuthylazine and atrazine contributed $0.02 \sum TUS_{Alg}$ and $0.003 \sum TUS_{Alg}$,
290 respectively, while spiroxamine and azoxystrobin contributed $0.02 \sum TUS_{Alg}$ and $0.01 \sum TUS_{Alg}$

291 to the overall toxicity for algae. These four compounds accounted for 66.6 +/- 4.4% of TUS_{Alg}
292 and were present in our lakes at all times.

293 The toxic risk for crustaceans ($\sum TUS_{Crust}$) (AF, sheet 4) ranged from 1×10^{-3} to $0.25 \sum TUS$
294 and exceeded the threshold for chronic toxic effects of 1×10^{-3} TUs at all sampling sites in
295 both sampling periods (figure 2A). The threshold of 0.1 TUs for acute toxic risk was
296 exceeded four times, twice in Ayes, once in Acherito (summer) and Ansabere (autumn). The
297 two insecticides diazinon and permethrin, which are used to protect livestock from sucking
298 insects, predominated $\sum TUS_{Crust}$ ten of our 16 samples. In total, diazinon and permethrin
299 cause 0.37 and 0.44 $\sum TUS_{Crust}$, respectively. When present, permethrin contributes 66.5 +/-
300 7.2%, and diazinon 37.1 +/- 9.9% to TUS_{Crust} . Generally, $\sum TUS_{Crust}$ was about four times
301 higher in sites with cattle present (0.088 +/- 0.096) as compared to sites without (0.017 +/-
302 0.036; $U_7 = 50$, $p = 0.065$).

303 With increasing $\sum TUS_{Crust}$, we observed a decreasing count of crustaceans ($R_{14} = -0.629$, $p =$
304 0.011). The correlation was driven by the concentrations of diazinon ($R_{14} = -0.745$, $p =$
305 0.001) and permethrin ($R_{14} = -0.681$, $p = 0.005$), but not that of $\sum TUS_{Crust}$ of the other
306 compounds ($R_{14} = -0.028$, $p > 0.05$). The richness of rotifers declined with increasing
307 $\sum TUS_{Alg}$ ($R_{14} = -0.554$, $p = 0.028$) (table 2).

308

309 **3.3 Indication for atmospheric transport**

310 Compounds expected to be introduced to the mountain lakes via atmospheric deposition
311 (PCBs, PAHs and field crop pesticides) were omnipresent in the mountain lakes under
312 investigation. In contrast, veterinary insecticides, for which an atmospheric input is much less
313 plausible and local sources are available, were only detected in certain lakes (figure 3).
314 Similar to atmospheric pollutants, insect repellents used on humans could also be detected
315 in all samples, but higher concentrations were only detected in the lakes Ayes, Acherito,
316 Ansabere and Coueyla Gran. In the same lakes, trace amounts of diazinon and permethrin
317 were detected. Compounds expected to be introduced via atmospheric deposition exhibited

318 a different distribution, with their greatest concentrations in Puits, Ansabere and Gourg de
319 Rabas.

320 **4. Discussion**

321 We performed an extensive chemical screening, including more than 400 compounds, in
322 mountain lakes of the French Pyrenees. We found a complex cocktail of 141 chemicals
323 including POPs, pesticides, biocides, and synthetic musk compounds at mostly background
324 levels. Except for POPs and some of the musk compounds, most of the compounds reported
325 here were detected for the first time in mountain aquatic ecosystems. In particular, the two
326 veterinary biocides diazinon and permethrin have been found at concentrations expected to
327 cause toxicity to crustaceans. The toxic concentrations of these compounds may have
328 caused substantial changes in zooplankton communities with likely repercussions for the
329 overall functioning of aquatic mountain ecosystems. We also detected a range of biocides
330 toxic to algae, but did not observe chronic toxic risks, suggesting that the risk for primary
331 producers might be low. However, rotifer species richness was negatively correlated to
332 biocides toxic to algae, suggesting an indirect effect through selective algal survival.

333 Our results agree with previous observations of the impact of insecticides on the composition
334 of zooplankton communities and food webs (Giddings et al., 2001; Groner and Relyea, 2011;
335 Hanazato, 2001). Larger, probably more sensitive taxa, such as crustaceans, occurred in
336 lower abundances or were absent in lakes with acute toxic risks. In contrast smaller, less
337 sensitive plankton taxa, such as algae, rotifers and ciliates, did not show a clear association
338 with $\sum TU_{Scrust}$. Particularly, diazinon and permethrin seem to play an important role in the
339 toxicity in our study sites. Due to its very high toxicity to crustaceans, diazinon is frequently
340 identified as a driver of invertebrate toxicity in waste and surface waters as soon as
341 concentrations exceed the detection level (Beckers et al., 2018; Kandie et al., 2020; Moschet
342 et al., 2014). Since 2007, the EU has banned diazinon, an acetylcholinesterase inhibitor,
343 from plant agricultural use. However, diazinon is still allowed in veterinary applications to
344 treat pets and livestock against a large range of ectoparasites. Similarly, permethrin is not

345 registered for applications in agriculture and forestry in the European Union since 2000, but
346 is still used in veterinary and human medicine against acarian, lice, fleas and mosquitoes
347 (Lewis et al., 2016) and can also be found in insect repellents for human use. We suspect an
348 entry to our study lakes by livestock and/or tourists. Atmospheric transport appears unlikely,
349 due to the lack of use for field crop and the deviating spatial distribution of both compounds
350 as compared to compounds introduced via the atmosphere (e.g. current-use plant protection
351 products; Ferrario et al., 2017; Santolaria et al., 2015; Silva et al., 2017). Both insecticides
352 are applied directly to livestock, including sheep, cattle, and horses via ear tags or sprays
353 (Bhushan et al., 2017; Reeves et al., 2010), from where they emit into the environment and
354 cause long-lasting contaminations (Gassner et al., 1997).

355 An increased use of insect repellents containing diazinon and permethrin for the protection of
356 cattle from sucking insects appears to be likely, linked to outbreaks of the blue tongue
357 disease. Blue tongue disease is a vector-borne viral disease of livestock transmitted via
358 sucking insects, and has occurred in Europe since the early 2000s. Its presence is linked
359 back to climate change, and has caused the death of millions of animals at huge financial
360 costs (Jones et al., 2019). Particularly, at the beginning of the blue tongue panzootic in
361 Europe in 2006, there were no vaccines available against bluetongue virus serotype 8. The
362 strategy followed by France and across the EU was then based on animal movement
363 restrictions and epidemiological surveillance of infected areas, combined with disinsectization
364 operations of cattle and stables. In 2021, the blue tongue disease was still a notable disease
365 in the EU (European Parliament, 2016) and cattle must be protected against insect vectors.
366 Free roaming livestock therefore are suspected to act as a major source of permethrin and
367 diazinon input into the studied mountain lakes.

368 Our study is the first that detected a wide range of current-use pesticides in mountain lakes.
369 Previous studies reported the presence of the low volatility herbicide terbutylazine in
370 mountain lakes of the Pyrenees and glaciers in the Italian Alps (Ferrario et al., 2017;
371 Santolaria et al., 2015). The reported concentration levels ranged from 0.32 ng/L (August

372 2014) to 2.27 ng/L (July 2011) in the water of the Pyrenean lake Sabocos and reached a
373 maximum level of 1.98 ng/L in the melting waters of the Lys Glacier (Italy). These levels are
374 similar to those observed in our Pyrenean sites. Here we contribute to increasing evidence
375 that current-use, low volatility pesticides, traditionally deemed unavailable for atmospheric
376 transport (SI 10), may undergo aerial transport from field crops into remote regions (Silva et
377 al., 2017). Atmospheric transport from lower altitudes to our high altitude lakes in the
378 Pyrenees seems likely, as the detected pesticide inventory is in agreement with observations
379 in the Ebro River basin (located at the foothills of the Spanish Pyrenees; Ccancapa et al.
380 2016), and other waterbodies of Europe (Fang et al., 2019; table 3). . However, peak
381 concentrations in agricultural areas are several orders of magnitude higher than in mountain
382 lakes (Fang et al., 2019). Such peaks in pesticide concentrations occur at times of pesticide
383 application when surface runoff from areas of plant production or peak discharges of
384 wastewater occur. These peaks often dominate risks in agricultural streams at low altitudes
385 (Liess et al., 2021). One may therefore assume that the risk for mountain lakes by field crop
386 pesticides is lower than for water bodies at lower altitudes. However, since mountain
387 organisms are likely more sensitive to chemical pollutants than organisms at lower altitudes
388 (Kallenborn, 2006) a detailed impact analysis is needed to better understand changes driven
389 by chemical pollution of mountain lakes.

390 In addition to insecticides from veterinary applications, we also detected the insect repellents
391 DEET and icaridin used in human applications. They have been detected in most of our
392 samples and in highly variable concentrations ranging from 0.7 to 627 ng/L for DEET and 0.4
393 to 31 ng/L for icaridin. While icaridin is not well investigated, the measured DEET
394 concentrations extend from background contamination levels to levels similar to those
395 observed in surface waters close to urban and field crop regions (Costanzo et al., 2007). The
396 broad distribution in the Pyrenees may hint to a local impact by tourists combined with
397 atmospheric deposition. More detailed studies are needed to better discern between local
398 and atmospheric sources of pollution in a mountain context. Our study, however, suggests
399 that the lower diversity of chemical pollutants found in the Neouvielle region as compared to

400 the other two regions is caused by the different precipitation schemes in the different regions.
401 This strengthens our notion that chemical pollution in the French Pyrenees is driven by a
402 combination of local sources and atmospheric transport.

403 **5 Conclusion**

404 Our study demonstrates that remote mountain lakes are not only impacted by the
405 atmospheric transport of POPs, but suffer from a more general introduction of pollutants,
406 including local sources. While overlooked so far, free roaming livestock may introduce
407 considerable levels of contaminants such as the two highly toxic insecticides diazinon and
408 permethrin. The introduced quantities are sufficient to cause acute or chronic toxic stress to
409 and are one of the drivers of changes in planktonic communities. Such stress and changes
410 may further be enhanced by local pollution sources, such as tourists. In more general terms,
411 the pollution-induced changes in the planktonic community may impact on ecosystem
412 functioning and ecosystem health, with a high potential to also reduce e.g. water quality
413 (Schmeller et al., 2018). More specifically, the observed impact of diazinon and permethrin
414 on zooplankton may also harm a known biological barrier to the amphibian pathogenic
415 fungus *Batrachochytrium dendrobatidis* (Deknock et al., 2020; Schmeller et al., 2014). The
416 changes in the zooplankton community induced by chemical pollution may therefore also
417 allow a deadly pathogen to increase its impact on amphibians, key biological elements in
418 mountain lakes. The high diversity of chemical compounds detected in our study highlights
419 the need to consider chemical mixtures as drivers of biological change (Machate et al. 2021;
420 Posthuma et al. 2020) with potentially far reaching risks for animal and human health
421 (Bernardo-Cravo et al., 2020; Schmeller et al., 2020). An improved large-scale environmental
422 monitoring, including remote areas, must be set up to allow better prediction of
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424

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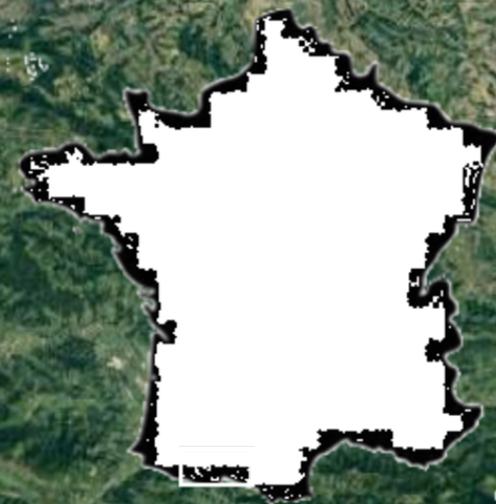
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Figure Titles and Legends

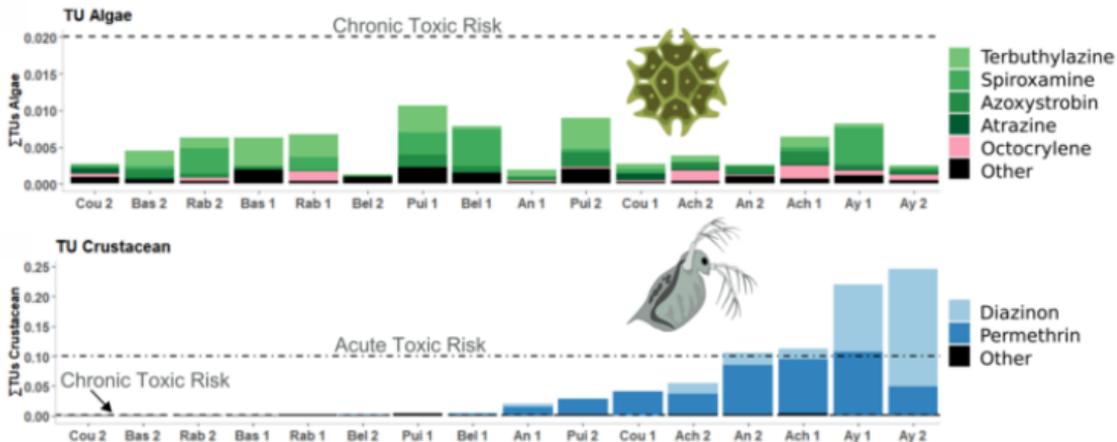
Figure 1: Location of the study regions and lakes under investigation in the French Pyrenees. Excerpts show the relief and exact position of the lakes within their respective study regions Lescun, Neouvielle and Bethmale. Abbreviations: An = Ansabere; Ach = Acherito; Pui = Puits; Rab = Gourg de Rabas; Bas = Madamete Bas; Coueyla = Coueyla Gran; Ay = Ayes; Bel = Belonguere.

Figure 2: Toxic risk and abundance of zooplanktonic organisms of the focal lakes. A) Barplot of Σ TUs for crustacean and algae, as well as of the abundance of crustacean and rotifers, at the different sampling sites. Sites are ordered according to the amount of TUs caused towards crustacean (high = right; left = low). Compounds displayed in colour contribute most of the observed TUs. Dashed lines represent thresholds for acute and chronic toxicity. B) Number of individuals in 200 L of lake water.

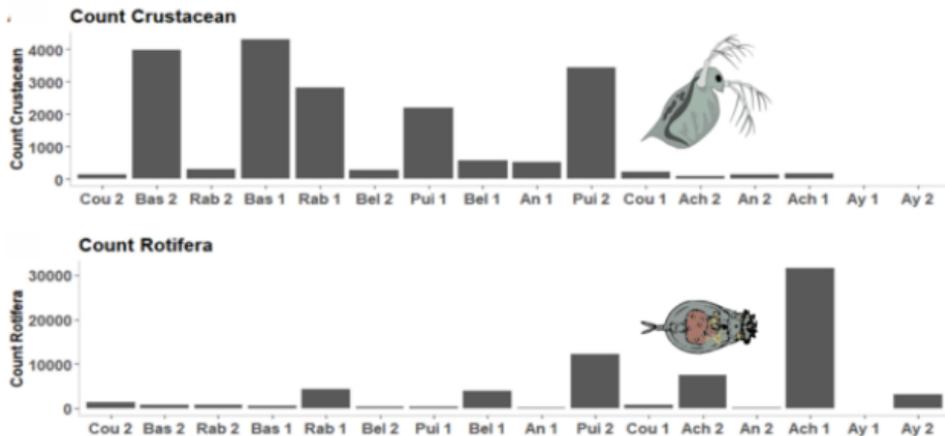
Figure 3: Heat map of sampling sites and maximum value-normalized sum concentrations of compound groups originating from the atmosphere and used in biocidal applications. Lakes ordered according to burden with veterinary insect repellents (declining from top to bottom). Abbreviations: Other.InRep.Vet= Veterinary insect repellents other than diazinon and permethrin; InRep.Hum= Insect repellents of human use. MNC = Magnitude of Normalized Concentrations.



A Predicted Toxicity

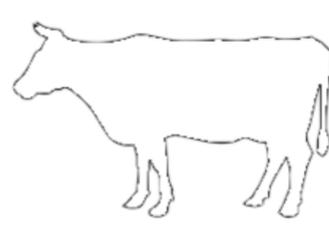


B Abundance Zooplanktonic Organisms





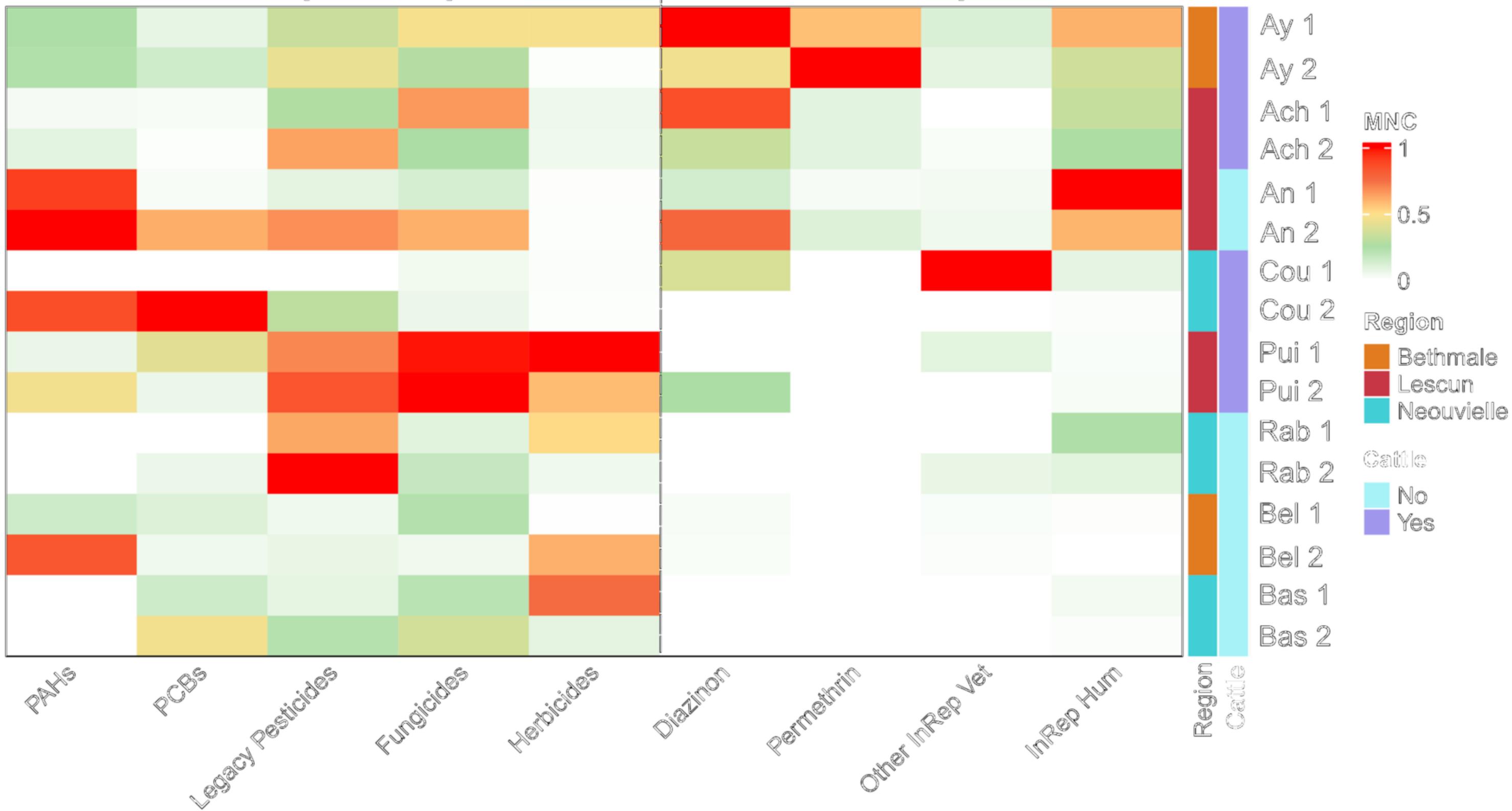
Atmospheric Input



Local Input



Lake & Sampling Period



MNC



Region



Cattle

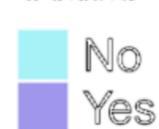


Table 1: Characteristics and environmental variables of the studied lakes in the French Pyrenees.

Lake name	Abbreviation	Region	Latitude	Longitude	Size [ha]	Catchment [ha]	Annual count of wet days [#]	Max depth [m]	Conductivity [μ S]	pH	Water temperature [§] [°C]	Pastoralism	Tourism	TC [μ g/l]	TN [μ g/l]	TP* [μ g/l]	Cattle ¹
Ansabere	An	Lescun	42.887780	-0.708850	0.20	13.7	39 days	~ 1.5	86.2	8.4	19.7	2	5	3.133	0.668	0.000	No
Acherito	Ach		42.878250	-0.709200	6.89	49.7	39 days	> 6	111.4	8.4	20.0	1	6	4.695	1.039	0.007	Yes
Puits d'Arious	Pui		42.864030	-0.633430	0.24	NA	39 days	> 3	11.8	7.8	20.1	2	2	3.960	0.865	0.022	Yes
Gourg de Rabas	Rab	Neouvielle	42.852500	0.145000	1.39	32.7	56 days	> 6	7.0	7.5	15.9	1	6	3.121	0.694	0.000	No
Madamate bas	Bas		42.868400	0.143620	0.14	94.4	56 days	~ 1.0	19.1	7.6	21.0	1	5	5.404	1.008	0.026	No
Coueyla Grand	Cou		42.875060	0.152760	1.04	49.6	56 days	> 4	36.6	8.5	15.6	1	7	2.707	0.147	0.000	Yes
Ayes	Ay	Bethmale	42.844080	1.064790	1.81	146.2	40 days	> 4	111.8	8.3	16.2	2	6	6.555	0.181	0.006	Yes
Belonguere	Bel		42.840290	1.063390	0.68	57.3	40 days	~ 0.8	181.5	8.3	16.9	1	3	3.977	1.185	0.005	No

[§]Average of surface water temperature measured in spring, summer and autumn

[#] days of precipitation of at least 10mm, based on WorldClim data

*all of our lakes are ultraoligotrophic following the classification based on total Phosphorus (TP < 4.7 μ g L⁻¹); oligotrophic (4.7 < TP < 9.3 μ g L⁻¹); mesotrophic (9.3 < TP < 31 μ g L⁻¹)

TC = Total Carbon; TN = Total Nitrogen; TP = Total Phosphorus

¹Based on field observations

Table 2: Overview of measured permethrin and diazinon concentrations above the minimum detection limit (MDL) of 2.5 ng/g silicon for diazinon and permethrin, and biological indicators used to characterize planktonic community. $N_{\text{species group}}$ = counts of individuals per species group, SR = species richness, number of species; $\text{Ratio}_{\text{counts}}$ = Ratio of counts of Rotifers/Crustaceans, Ratio_{SR} = Ratio species richness of Rotifers/Crustaceans, Shannon = Shannon diversity Index, Simpson = Simpson diversity Index,

Region	Lake	Date	Season	Permethrin (ng/L)	Diazinon (ng/L)	$N_{\text{Crustaceans}}$	N_{Rotifers}	$\text{SR}_{\text{Crustaceans}}$	$\text{SR}_{\text{Rotifers}}$	SR_{Total}	$\text{Ratio}_{\text{Counts}}$	Ratio_{SR}	Shannon	Simpson
Lescun	Acherito	15-Aug-18	Summer	0.091	0.017	180	31572	1	3	4	175.40	3.00	0.07	1.02
		27-Sep-18	Autumn	0.035	0.017	80	7416	4	7	11	92.70	1.75	0.80	2.01
	Ansabere	15-Aug-18	Summer	0.014	0.005	529	152	5	6	11	0.29	1.20	1.10	2.14
		21-Sep-18	Autumn	0.082	0.020	144	212	6	5	11	1.47	0.83	1.88	4.98
	Puits	15-Aug-18	Summer	<MDL	<MDL	2192	380	4	4	8	0.17	1.00	0.91	1.69
		27-Sep-18	Autumn	0.026	<MDL	3448	12128	4	4	8	3.52	1.00	1.25	2.58
Neouvielle	Coueyla	06-Aug-18	Summer	0.040	<MDL	228	864	2	14	16	3.79	7.00	2.42	8.95
		24-Sep-18	Autumn	<MDL	<MDL	144	1392	6	12	18	9.67	2.00	1.55	2.48
	Madamete bas	06-Aug-18	Summer	<MDL	<MDL	4324	524	5	9	14	0.12	1.80	0.97	1.88
		24-Sep-18	Autumn	<MDL	<MDL	3992	828	3	1	4	0.21	0.33	0.73	1.66
	Gourg de Rabas	07-Aug-18	Summer	<MDL	<MDL	2820	4292	2	5	7	1.52	2.50	0.79	2.01
		24-Sep-18	Autumn	<MDL	<MDL	299	777	3	5	8	2.60	1.67	0.88	1.96
Bethmale	Ayes	04-Aug-18	Summer	0.105	0.112	16	6	2	3	5	0.38	1.50	1.06	2.03
		21-Sep-18	Autumn	0.048	0.196	16	3146	4	9	13	196.63	2.25	0.18	1.07
	Belonguere	04-Aug-18	Summer	0.002	<MDL	584	3912	6	9	15	6.70	1.50	0.83	1.47
		21-Sep-18	Autumn	0.002	<MDL	287	333	4	10	14	1.16	2.50	1.98	5.44

Table 3: Comparison of pesticides detected in our study, the River Ebro at the foot of the Spanish Pyrenees, and the rest of Europe. Stated are concentration range (in brackets) and median (our study and for Europe) or mean concentration in ng/L (River Ebro).

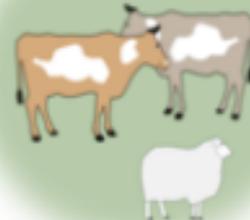
	Mountain Lakes (This study)		River Ebro (Ccanccapa et al., 2016)		Europe (Fang et al., 2019)	
Diazinon	(0.05 - 2)	0	(<MDL - 5.7)	4.00	(1.6 - 43)	10.1
Terbutylazine	(0.3 - 12)	2.4	(0.11 - 10)	2.20	(1 - 630)	13.6
Metolachlor	(0.2 - 2.1)	0.5	(1.1 - 4.9)	0.55	(0.8 - 17.6)	2.0
Tebuconazole	(0.2 - 9.3)	0.8	(1.7 - 15)	2.36	(2.6 - 86)	8.6
Azoxystrobin	(2.7 - 41)	12.0	-		(14 - 120)	45.0
Boscalid	(0.1 - 3.3)	0.4	-		(0.9 - 55)	6.5
Myclobutanil	(0.07 - 1.5)	0.1	-		(2.1 - 15)	10.5

**Unravel Chemical Mixture
&
Assess Toxic Risk**

Toxic Risk by Insecticides

 Diazinon
 Permethrin

Source



**Atmospheric
Transport**

Tourism

Pastoralism

GR10

