Global variation in freshwater physico-chemistry and its influence on chemical toxicity in aquatic wildlife

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ABSTRACT

Chemical pollution is one of the major threats to global freshwater biodiversity and will be exacerbated through changes in temperature and rainfall patterns, acid-base chemistry, and reduced freshwater availability due to climate change. In this review we

show how physico-chemical features of natural fresh waters, including pH, temperature, oxygen, carbon dioxide, divalent cations, anions, carbonate alkalinity, salinity and dissolved organic matter, can affect the environmental risk to aquatic wildlife of pollutant chemicals. We evidence how these features of freshwater physico-chemistry directly and/or indirectly affect the solubility, speciation, bioavailability and uptake of chemicals [including *via* alterations in the trans-epithelial electric potential (TEP) across the gills or skin] as well as the internal physiology/biochemistry of the organisms, and hence ultimately toxicity. We also show how toxicity can vary with species and ontogeny. We use a new database of global freshwater chemistry (GLORICH) to demonstrate the huge variability (often >1,000-fold) for these physico-chemical variables in natural fresh waters, and hence their importance to ecotoxicology. We emphasise that a better understanding of chemical toxicity and more accurate environmental risk assessment requires greater consideration of the natural water physico-chemistry in which the organisms we seek to protect live.

Key words: alkalinity, antimicrobials, dissolved organic carbon (DOC), environmental protection, hardness, herbicides, metals, pharmaceuticals, persistent chemicals, pesticides.

CONTENTS

I. INTRODUCTION

Pollution is considered one of the major threats to global fresh waters, and derives from a diversity of sources, including domestic and industrial effluents, agriculture, road run-off, mine tailings and leakage from landfill sites (Reid, MacBeath & Csatádi, 2013). Discharges contain complex mixtures of chemicals that can include persistent organic compounds, pesticides, pharmaceuticals, inorganic nitrogen compounds and metals; more than 30,000 chemicals are in widespread use (US EPA, 2019). Climate change is causing dramatic changes in thermal regimes and rainfall patterns, and this, together with an overall reduction in freshwater availability, is predicted to exacerbate the adverse effects of chemical pollutants (Balbus *et al.*, 2013; Benateau *et al.*, 2019; Bunke *et al.*, 2019). Most countries globally are challenged by the availability of fresh water (IUCN, 2020). There are, however, especially high risks due to the combined effects of pollution and freshwater flow reductions in Tunisia, Israel, Moldova, Syria, Hungary, Macedonia, Germany, Netherlands, Czech Republic, Algeria, Pakistan, South Africa, India, Spain, Nepal, Afghanistan, Korea, Bangladesh, Madagascar and Iraq (IUCN,

2009; Vörosmarty *et al.*, 2010). Furthermore, freshwater biodiversity is considered to be most threatened in the Czech Republic, Luxembourg, Kuwait, Belgium, Tunisia, Germany, Moldova, Syria, Slovak Republic and Spain (IUCN, 2009; Vörosmarty *et al.*, 2010). In the case of fishes, their biodiversity is most threatened in Indonesia, Mexico, United States, India, Australia, Tanzania, China, Malaysia, South Africa and Turkey (IUCN, 2020).

The impact of a chemical, including synthetic organic molecules, metals and other inorganic toxicants, on an organism depends on the innate toxicity of the chemical, its persistence, bioavailability, the exposure level, and the presence of other toxicants (Ibanez *et al.*, 2007). The route of exposure, life stage, health status and sex of the organism are also important (Ibanez *et al.*, 2007) as is the ability to metabolise and excrete toxicants. Other influential factors include the organisms' ecological niche (the physical environment it inhabits and its trophic position) (Windsor, Ormerod & Tyler, 2018). Importantly, the physico-chemical environment can also directly and/or indirectly affect both the toxicant bioavailability and potentially its form/innate toxicity. This is well illustrated for metals such as copper (Cu), where the presence of organic matter (De Schamphelaere *et al.*, 2004; Boeckman & Bidwell, 2006), temperature (Boeckman & Bidwell, 2006), and acid-base status (H^+, OH^-, HCO_3^- and CO_3^{2-}) (Long, Van Genderen & Klaine, 2004; Grosell, 2011) all affect metal speciation which in turn determines Cu bioavailability and toxicity. In addition, some cations compete with copper uptake processes in freshwater animals (Na^+, Ca^{2+}, and H^+ at pH<6) or reduce gill permeability (Ca^{2+}) thereby reducing the toxic impacts of copper (Grosell, 2011). For metals, the Biotic Ligand Model (BLM), a computational regulatory tool, is now widely used in environmental guideline generation that takes into account many (but not all) of the water chemistry variables we consider in this review. This enables the

generation of site-specific predictions of toxicity for many metals (Di Toro *et al.*, 2001). For chemicals more generally however, until recently the effects of water physico-chemistry on their toxicity has received relatively little study.

Chemicals are regulated in order to minimise their risks to the health of humans and wildlife populations, and avoid adverse ecological impacts (Gunnarsson *et al.*, 2019). The approach for evaluating chemical risk in the environment operates by identifying the potential hazard of chemicals, assessing exposure risk (predicted environmental concentration; PEC), characterising the ecological hazard (predicted no-effect concentration; PNEC), and then assessing the likely risk through calculation of the risk quotient (PEC/PNEC), which for wildlife species uses the endpoints of growth, development and/or reproduction (Amiard & Amiard-Triquet, 2015; Gunnarsson *et al.*, 2019). For approval of new chemicals in the European Union (EU) and the United States (US), an environmental risk assessment (ERA) is mandatory. These ERAs and ecotoxicological studies are carried out in accordance with guidelines from the Organisation for Economic Co-operation and Development (OECD) and the US Environmental Protection Agency (US EPA) (Ruden *et al.*, 2017). Even so, of the 5,000 new high-production-volume chemicals synthesised since the 1950s, less than half have undergone robust environmental safety assessments (Landrigan *et al.*, 2018). Furthermore, these ecotoxicity tests are very basic, assessing only the effects on mortality, growth and reproduction of single species; development and behaviour are not considered, yet these are fundamental life processes that can affect individual fitness with population-level consequences (Gunnarsson *et al.*, 2019). It is also the case that standardised procedures for chemical testing can vary among countries (Norberg-King *et al.*, 2018), and they use a very narrow species range. For the aquatic environment, the test species include representatives of algae, invertebrates and fish taxa. The most

commonly used species for chemical testing are the green alga *Scenedesmus obliquus*, the planktonic crustacean *Daphnia magna*, and the zebrafish, *Danio rerio*. For fish, other species, notably the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) have also strongly influenced regulatory guidelines. These species may not necessarily represent the diversity of freshwater biota within any given taxonomic group. As an example, the sensitivity of growth inhibition in response to antibiotic exposure can vary by orders of magnitude among species of cyanobacteria, influenced by the antibiotic modes of action (MoA; Le Page *et al.*, 2019). Tests also tend to focus on a single route of exposure, normally *via* the water, whilst ignoring the dietary route or the fact that most organisms are simultaneously exposed to complex chemical mixtures in nature. Further challenges in extrapolating between chemical effects from laboratory-based exposures and wildlife populations relate to possible differences across the life stages used, and the limited concentration ranges normally tested (Amiard & Amiard-Triquet, 2015). These tests also do not consider the environmental degradation of compounds or the possibility for acclimation or adaptation of organisms in polluted environments. Moreover, and importantly, these standardised tests do not account for the considerable variation that occurs in the physico-chemistry of natural fresh waters, which is increasingly recognised as being fundamentally important in understanding chemical toxicity to aquatic organisms.

This review investigates physico-chemical characteristics of natural fresh waters, including pH, temperature, oxygen, carbon dioxide, divalent cations, anions, carbonate alkalinity, salinity, and dissolved organic matter that affect the nature, form and bioavailability of chemicals and assesses how this may impact on their toxicity to aquatic organisms from algae to invertebrates and fish (Fig. 1). We consider what the

150 implications are for these environmental influences on chemical risk. In the final part of
151 the review we discuss approaches to identify global areas of concern for the interactive
152 effects of selected water physico-chemistry parameters and pollutant toxicity.
153
154 **II. WATER PHYSICO-CHEMISTRY AND THE RISK OF CHEMICALS**
155 **(1) pH**
156 The absorption and toxicokinetics of chemicals in fresh waters are directly related to the
157 acid dissociation constant (pK_a) and the ionic arrangement of the molecule (i.e. whether
158 it is ionized or not), with the non-ionized (neutral) form, which is lipophilic, passing
159 more easily through cell membranes. These factors are all affected by the pH of the
160 surrounding water.
161 In the natural environment, the pH of fresh waters varies widely (from pH 2 to
162 11) reflecting differences in regional geomorphology, hydrology, climate and/or
163 anthropogenic influences (Valenti *et al.*, 2009; Hartmann, Lauerwald & Moosdorf,
164 2014, 2019; Fig. 2A). Illustrating this, in the Rio Negro, the largest tributary of the
165 Amazon, extremely low buffering capacity and large quantities of organic acid from
166 decaying vegetation create naturally low pH values, between 4 and 5 in the main river,
167 and as low as pH 2.5 in forest streams (Walker & Henderson, 1996). Acidic pH values
168 (4–5) are also now common since the 1970s in large areas of northern Europe and
169 eastern Canada (Schindler, 1988), and more recently in China (Liu *et al.*, 2020). This
170 acidification occurs where poor buffering capacity is coupled with inputs of
171 anthropogenic ‘acid rain’, derived from fossil fuel emissions with high content of
172 sulphur and nitrogen oxides that generate strong acids (sulphuric and nitric) in rainwater
173 (Schindler, 1988). At the other end of the spectrum, there are endorheic lakes in arid
174 regions that can exceed pH 10 but these are also highly saline (‘soda lakes’ > 17 ppt

175 salinity) and so not strictly fresh water (e.g. Lake Magadi, Kenya and Lake Van,
176 Turkey; Wilkie & Wood, 1996). However, a few lakes and rivers with salinities <5 ppt
177 have pH values consistently above 9.5 (e.g. Pyramid Lake, Nevada; River Ganga in
178 Rishikesh, India) due to extremely high carbonate alkalinity (HCO_3^- and CO_3^{2-} ions)
179 released by dissolution of minerals in the unusual local geology (Wilkie & Wood, 1996;
180 Haritash, Gaur & Garg, 2016). Considerable temporal and spatial changes in pH (Blume
181 *et al.*, 2010; Rothwell *et al.*, 2010; Nienie *et al.*, 2017) can also occur in freshwater
182 systems due to both natural (e.g. catchment geology, land cover, primary production,
183 soil characteristics and water source contributions) and anthropogenic factors (e.g.
184 eutrophication *via* farmland irrigation, livestock densities, mining, domestic and
185 industrial sewage) (Rothwell *et al.*, 2010; Feng *et al.*, 2017; Varanka & Hjort, 2017).
186 However, more than 95% of surface fresh waters globally are between pH 6 and 9, and
187 99.8% are between pH 4 and 9.5 (GLORICH database; Hartmann *et al.*, 2014, 2019;
188 Fig. 2A).

189 The pK_a of each chemical identifies whether it is a weak acid or base and
190 whether it will be neutral or charged at a given water pH, which therefore affects its
191 bioavailability through chemical speciation (Stehly & Hayton, 1990) and potentially
192 predicts toxicity. When the chemical species is ionized (e.g. HA^+) it is more easily
193 dissolved in water relative to a non-ionized species (e.g. A), and the proportion present
194 as HA^+ increases as pH decreases (more acidic) below the pK_a value (Nakamura *et al.*,
195 2008). By contrast, molecules that are non-ionized are more lipid soluble and can,
196 therefore, diffuse more easily through biological membranes, increasing their uptake
197 into living cells independently of specific membrane transport systems (Erickson *et al.*,
198 2006*a*,*b*; Saparov, Antonenko & Pohl, 2006; Karlsson *et al.*, 2017). This is exemplified
199 by the example of ammonia (NH_3)/ammonium (NH_4^+), whereby higher pH increases the

200 proportion present as non-ionized ammonia (NH_3), leading to greater internal
201 accumulation and toxicity even when the external water total ammonia concentration
202 (i.e. [$NH_3 + NH_4^+$]) is constant (US EPA, 2013).

203 Speciation effects of pH have been studied extensively for metals, where it
204 influences bioavailability and/or toxicity based on the proportion of chemical species
205 generated. For example, aluminium metal species have amphoteric properties which
206 depend on pH; when in acidic water (pH <6.0) aluminium becomes bioavailable in the
207 form of Al^{3+}, in neutral environments it becomes $Al(OH)_3$ which is insoluble, while at
208 basic pH (>8.0) it presents as $Al(OH)_4^-$ (Namiesnik & Rabajczyk, 2010; Wilson, 2011).
209 More recent studies demonstrate interactions of environmental pH with the toxicity of a
210 wider range of chemicals, which we now illustrate.

211 In the algae *S. obliquus*, interactions have been observed between pH and the
212 chlorophenols (weak acids), with 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol
213 (2,4,6-TCP) and pentachlorophenol (PCP) found to be more toxic (lower EC50) at
214 lower pHs (6.5 > 7.5 > 9.0) and a similar pattern is observed in the invertebrate, *D.*
215 *magna* (Xing *et al.*, 2012). The toxicity and uptake of triclosan also changes greatly
216 with pH in *Navicula* sp. diatoms (Ding *et al.*, 2018), the green alga *Chlorella*
217 *ellipsoidea* (Khatikarn *et al.*, 2018) and freshwater shrimp, *Gammarus pulex* (Rowett,
218 Hutchinson & Comber, 2016), with the highest toxicity and bioaccumulation occurring
219 at pH ≤7.5 due to a preponderance of non-ionized species which diffuse more readily
220 into cells. The toxicity of hydroquinone to *Pseudokirchneriella subcapitata* was
221 maintained for longer at pH 7, with higher pHs leading to oxidation of hydroquinone
222 and loss of toxicity (Bahrs, Putschew & Steinberg, 2013).

223 In addition to greater bioavailability for uptake, neutral forms, under certain
224 conditions, can generate charged fractions in the cell cytoplasm (Fahl *et al.*, 1995). In

the algae *Scenedesmus vacuolatus*, which are capable of maintaining internal pH independent of the external environment, the pharmaceuticals fluoxetine, norfluoxetine, propranolol, and trimipramine (which contain a protonated basic amino group and are positively charged at internal physiological pH) showed an enhanced toxicity at higher media pH (tested at between 7.5 and 10.0) suggesting that the change in toxicity was due an effect of speciation of the basic compounds (Neuwoehner & Escher, 2011).

The toxicity of chloroquine (a weak base) has been shown to increase with increasing pH in *D. magna* due to ionization behaviour. This is true for the weak base pharmaceuticals fluoxetine, paroxetine and citalopram across a range of freshwater invertebrate species including in larval *Aedes aegypti* (Insecta: Diptera), *Cypridopsis vidua* (Crustacea: Ostracoda), and *Hydra vulgaris* (Cnidaria: Hydrozoa) (Sundaram, Smith & Clark, 2015). Here, toxicity was related to both the ionization state and membrane permeability to the uncharged form (Sundaram *et al.*, 2015). Similarly, studies on *D. magna* have shown that as pH increases toxicity changes in opposite directions depending on whether pharmaceuticals are weakly acidic (toxicity decreasing for naproxen, diclofenac, ibuprofen and ketoprofen) or weakly basic (toxicity increasing for fluoxetine and sertraline) (Bostrom & Berglund, 2015). Similarly, for other weakly acidic pharmaceuticals (acetaminophen, enrofloxacin, and sulfathiazole), toxicity decreases as the pH of the water rises and the non-ionized fraction decreases (Kim *et al.*, 2010).

Similar effects of pH on pharmaceutical toxicity are observed in vertebrates. For example, increasing water pH enhances toxicity of the weakly basic drugs fluoxetine (in larval medaka), and sertraline (in fathead minnow) (Nakamura *et al.*, 2008) including effects on feeding, growth and survival (Valenti *et al.*, 2009). These findings are also

observed for sertraline, fluoxetine, and diclofenac, in zebrafish; for sertraline the uptake was higher with increasing water pH (Alsop & Wilson, 2019).

The toxicity of the β-blockers metoprolol and propranolol (weak bases) has also been shown to increase with increasing water pH in zebrafish embryos (Bittner *et al.*, 2018). Sublethal effects (e.g. on heart rate and behaviour) of these drugs were associated with the neutral fraction (Bittner *et al.*, 2018). Interestingly, the speciation of the compound may only be relevant to its absorption (toxicokinetics) and not for its intrinsic toxicity, as freshwater pH was not found to be related to the effective internal concentration (IEC) (Bittner *et al.*, 2018).

The majority of these studies use physico-chemical parameters (including pH) set out in standardised test (e.g. OECD) guidelines. However, these test media conditions may not be applicable to the pH range observed in nature where either the study species or the chemicals being tested are relevant. Therefore, some effects of speciation and toxicity may be underestimated or overestimated – directly influencing ecological risk assessments (Valenti *et al.*, 2009; Bostrom & Berglund, 2015). We therefore emphasise the importance of understanding the link between water pH and the toxicity of the chemical in risk assessment.

Water pH also exerts a much lesser known effect directly on the trans-epithelial electrical potential (TEP) across the outer surface of aquatic animals (McWilliams & Potts, 1978; Wood *et al.*, 1998; Fig. 3A) which in turn can potentially affect their sensitivity to chemical effects. The change in TEP with water pH is of sufficient magnitude (>50 mV range) to influence the electrochemical gradient of any charged molecules (both inorganic and organic) and hence their uptake *via* the gills and skin. Ecotoxicological studies have not yet addressed this concept. However, given the large pH range in fresh water globally (Hartmann *et al.*, 2014, 2019; Fig. 2A) it likely plays

an important role in determining the uptake and hence toxicity of any charged chemicals. We recommend that future laboratory-based testing should consider the environmentally relevant water pH range when designing media for determining toxic impacts, chemical speciation and other aspects that influence uptake (such as TEP).

(2) Temperature

Temperature has a strong influence on a wide range of physiological processes in aquatic poikilotherms, whilst also affecting the bioavailability, adsorption, elimination and relative toxicity of chemicals (Kim *et al.*, 2010; Patra *et al.*, 2015; Op de Beeck *et al.*, 2017). The temperature effects on chemicals may occur directly through altering their physico-chemical behaviour (e.g. degradation and volatilisation), their transport, transfer, deposition and their fate between the water, suspended organic matter and sediments (MacDonald, Harner & Fyfe, 2005). Effects of temperature on the toxicity and bioavailability of toxicants vary with the type of chemical, and may differ among algae, invertebrate, and vertebrate species, as well as among ontogenetic stages. In many natural cases multiple effects of temperature will influence the toxicity of a chemical to aquatic organisms.

Temperature can modulate the rate of uptake of a chemical into aquatic organisms through directly affecting the chemical's mobility. At elevated temperatures chemical molecules diffuse more quickly, resulting in faster rates uptake into the organism. In turn the toxicological threshold for a chemical may be reached more rapidly. In natural systems this could determine whether a toxic effect occurs where the exposure is relatively short-lived, for example as a consequence of a chemical spill into a river. Temperature can also have a direct effect on the toxicity of a chemical through affecting the rate of its degradation. In the green algae *P. subcapitata*, for example, the

herbicide diuron has been shown to have a lower toxicity (on growth and suppression of photosystem II activity) at higher (20, 25 and 30 ºC) *versus* lower (10 and 15 ºC) temperatures due to enhanced rates of chemical degradation/volatilisation at the warmer temperatures (Tasmin *et al.*, 2014). Similarly, in the damselfly *Ischnura elegans* there was a lower toxicity for exposure to the pesticide chlorpyrifos at 24 °C *versus* 20 °C, due to a higher rate of biodegradation, which produces compounds that are less toxic (Op de Beeck *et al.*, 2017). By contrast, a faster rate of biotransformation of the organophosphate insecticide chlorpyrifos induced by higher temperatures increases its toxicity to the benthic invertebrate *C. dilutes*, in this case because the biotransformation products are more toxic to the organism (Harwood, You & Lydy, 2009).

The metabolic rate in poikilotherms is strongly influenced by temperature with an approximately twofold increase for a 10 ºC change in water temperature (Q10 effect). The metabolic rate of an organism will in turn affect the rate at which a chemical is taken up and the rate (and how) the chemical is metabolised (and excreted). Enhanced chemical uptake at elevated temperatures has been shown for a wide range of chemicals and aquatic organisms. Examples include mefluoride in the zebra mussel *Dreissena polymorpha* at 22 *versus* 17 ºC (Del Piero, Masiero & Casellato, 2012), the fungicide pyrimethanil in larval stages of *Chironomus riparius* and *D. magna* at 26 *versus* 14 °C (Seeland, Oehlmann & Müller, 2012) and the pharmaceuticals diclofenac, ibuprofen and carbamazepin in the invertebrate *Atyaephyra desmarestii* at 25 *versus* 20 ºC (Nieto *et al.*, 2016). In the cyprinid fish *Spinibarbus sinensis*, greater uptake of perfluorooctane sulfonate (PFOS; a fluorosurfactant) at 28 *versus* 18 ºC has been shown to cause a marked reduction in its ability to respond to a predatory attack (Xia *et al.*, 2015). In the above examples, higher rates of ventilation with associated higher

diffusion and/or active transport across the gills at higher temperatures are often associated with accumulation of these contaminants.

In some cases higher exposure temperatures result in effects on the organism's metabolic ability to reduce a chemical's toxicity. For example, in the snail *Physella acuta*, the fungicide pyrimethanil caused a less inhibitory effect on hatching at 25 and 20 °C *versus* 15 °C due to a more rapid chemical metabolism, as well as an enhanced capability for cell repair in embryos (Seeland *et al.*, 2013). Similarly, lower rates of mortality and higher swimming performance have been reported in the epibenthic amphipod *Hyalella azte*ca exposed to the pyrethroid insecticide bifenthrin at 20 ºC *versus* both 12 and 16 ºC (Hasenbein, Poynton & Connon, 2018) and this was related to enhanced metabolism of the insecticide (Narahashi, 2002). In the benthic invertebrate *Chironomus dilutes* toxic responses to the organochlorine dichloro-diphenyl-trichloroethane (DDT) and the pyrethroids permethrin and lambda-cyhalothrin were lower at 23 ºC *versus* 13 ºC due to reduced nerve sensitivity at the higher temperature (Harwood *et al.*, 2009).

In some cases temperature can affect a specific metabolic enzyme to enhance a chemical exposure effect. For example, in the case of the herbicide diuron (which causes thyroid disruption) exposure of tadpoles of *Lithobates catesbeianus* at 34 ºC *versus* 28 ºC accelerated tadpole metamorphosis due to a temperature-induced increase in gene expression of the key enzyme, iodothyronine deiodinase II (Freitas *et al.*, 2016). In studies on zebrafish an increase in temperature from 28 °C to 33 °C enhanced the gonadal feminising effects of the aromatase inhibitor clotrimazole (Brown *et al.*, 2015). A further illustration of how temperature can affect enzyme processes to alter a toxicological response has been shown in fathead minnow exposed to the steroidal oestrogen oestrone (E1). At a lower temperature (15 °C *versus* 18, 21 and 24 ºC) there

was a greater effect of E1 on escape performance and larval foraging due to slower degradation and elimination of the steroid (Ward, Cox & Schoenfuss, 2017). For the antibiotic, florfenicol, which inhibits protein synthesis, exposure of *D. magna* at warmer temperatures (from 20 ºC to 25 ºC) was increasingly toxic due to greater inhibition of protein biosynthesis repair mechanisms (Martins, Guimarães & Guilhermino, 2013).

The life stage of an organism can also influence how temperature affects a chemical's toxicity. Juvenile life stages of the copepod *Eucyclops serrulatus* have been shown to be more sensitive than adults to the effects of ammonia, imazamox (an herbicide), and a mixture of these pollutants, at 18 ºC *versus* 15 °C due to the greater effect of temperature on metabolic rate in juveniles, resulting in greater uptake (Di Lorenzo *et al.*, 2015). Juvenile life stages of some invertebrates also have less-efficient mechanisms for detoxification, one example being juveniles of the prawn *Macrobrachium tenellum*, which are less able to detoxify ammonia-N, and as a result are more susceptible to its toxic effects compared with adults (Figueroa-Lucero, Hernández-Rubio & Gutiérrez-Ladrón De Guevara, 2012).

In some cases the effects of temperature on chemical toxicity appear to be particularly complex. For example, in the freshwater snail *Potamopyrgus antipodarum*, where temperature is directly involved with the reproductive process, exposure to the oestrogenic endocrine disrupter Bisphenol A, has been shown to have greater reproductive effects at the lower and higher temperatures tested (7 and 25 °C) compared with at 16 °C (Sieratowicz *et al.*, 2011), but the underlying mechanism(s) have not been established.

Different populations of the same species may show differences in how temperature influences chemical toxicity. For example, populations of the damselfly *I. elegans* adapted to lower latitudes (warmer temperatures) suffer less toxicity in

exposures at high temperatures than populations adapted to higher latitudes (cooler temperatures) (Op de Beeck *et al.*, 2017). Thus, the local thermal ranges of the populations being studied should be considered, as their prior thermal adaptations may have a significant bearing on how temperature affects toxic responses to pesticides in invertebrates.

Temperature can also affect community-level responses to chemicals. As an example, exposure to the pesticide esfenvalerate had greater negative long-term effects on *Daphnia* sp. at higher temperatures as a result of altered competition across the wider zooplankton community, potentially due to increased sensitivity to competition in warmer water (Knillmann *et al.*, 2013).

An important but neglected issue is that OECD test guidelines and environmental regulations generally adopt a fixed temperature regime that does not allow for the fluctuations that individual organisms in their natural environment can experience daily, seasonal and/or annually, which, as illustrated in the examples above, may have a major impact upon the toxic effects of chemicals. This illustrates an important difference between standardised laboratory toxicity test regimes and the natural habitats of the organisms we are seeking to protect, as well as when accounting for future climate change.

(3) Oxygen

Oxygen is necessary for aerobic metabolism, including respiration in aquatic algae, plants, invertebrates and vertebrates. Fresh water holds 20–40 times less oxygen than atmospheric air when they are fully equilibrated (Cameron, 1986). Hence, water-breathing organisms are compromised relative to their terrestrial counterparts in terms of their potential capacity for aerobic metabolism. In addition, the availability of oxygen

in freshwater habitats can show considerable daily oscillations, affected by temperature and light (driving respiratory and photosynthetic rates), the presence of organic matter (a resource for microbial respiration) and various other factors (Fig. 2F). In some cases, these natural factors can lead to hypoxia. The incidence of hypoxia in freshwater environments has been increasing over the last three centuries due to anthropogenic nutrient release (eutrophication), and has been accelerated further by climate change (warming) which directly reduces dissolved oxygen levels (Diaz & Breitberg, 2009; Jenny *et al.*, 2016). These changes in oxygen availability can alter the behaviour and physiology of an organism, which in turn can have a major bearing on chemical toxicity. For example, under hypoxic conditions gill ventilation rate in fish and aquatic invertebrates increases to maintain oxygen uptake rate. However, the greater rate of water movement over the gills will also increase the uptake rate for some xenobiotics dissolved in water (McKim, Schmieder & Veith, 1985; Randall, 1990; McKim & Erickson, 1991; Yang *et al.*, 2000; Schiedek *et al.*, 2007). Hypoxia can also lead to an increase in the functional surface area of fish gills and a reduction in the mean diffusion distance between blood and water. These changes can occur quite rapidly, for example by redirection of blood flow pathways within the gills, and increases in haemoglobin concentration and its affinity for O_2, and over the longer term (days/weeks) due to reversible gill remodelling. The latter can include an interlamellar cell mass (present in some species in normoxia) which atrophies in hypoxic conditions revealing a much greater lamellar area (Nilsson, Dymowska & Stecyk, 2012; Wood & Eom, 2021). These factors that enhance functional gill area and gas exchange during hypoxia may simultaneously also enhance xenobiotic uptake rates (Sundin & Nilson, 1998; Val, 2000; Du *et al*., 2018; Gilmour & Perry, 2018; Saari *et al*., 2020).

Despite this knowledge, few studies have evaluated the combined effects of hypoxia and chemicals in the aquatic environment. In one such study on the three-spined stickleback (*Gasterosteus aculeatus*), exposure to diclofenac under hypoxic conditions (2.0 ± 0.2 mg l^{-1} dissolved oxygen) has been shown to result in the upregulation in the liver of cytochrome P4501A (CYP1A) activity, which mediates chemical biotransformation (Prokkola *et al.*, 2015). However, in contrast, downregulation of *cyp1a1* (and *hsp90*) occurs in the gills of *G. aculeatus* (Lubiana *et al.*, 2016) under the same exposure conditions. Thus, oxygen availability can potentially affect toxicity *via* both chemical uptake and responses in the enzyme detoxification system and this may vary between different tissues and result in differences in tissue sensitivities to the affects of hypoxia (Prokkola *et al.*, 2015; Lubiana *et al.*, 2016).

Chemical test guidelines for oxygen levels vary. For example, OECD test guidelines for algae do not provide recommendations for the concentration of dissolved oxygen [e.g. OECD Test No. 201 (OECD, 2011*a*)]. Yet for *D. magna* [Test Nos 202 and 211 (OECD, 2004, 2012*a*)] levels above 3 mg l^{-1} of dissolved oxygen in water are recommended, and for fish the recommendations are for more than 60% [Test Nos 203, 210, 212, 215, 229, 234 (OECD, 2019, 2013*a*, 1998, 2000, 2012*b*, 2011*b*)] or above 80% dissolved oxygen saturation [Test No. 236 (OECD, 2013*b*)]. We see a major knowledge gap in understanding and assessing toxicity of chemicals to aquatic organisms under varying environmental oxygen concentrations, as well as in understanding how these affect chemical solubility and the formation of possible toxic by-products. Research into how hypoxic conditions affect the physiology of aquatic organisms and their ability to cope with chemical toxicity is much needed given the large (and increasing) expanse of surface waters experiencing such conditions. Recent

studies on interactions between hypoxia and metal toxicity in zebrafish (*D. rerio*) and three-spined stickleback highlight the potential for major impacts of environmental oxygen (Fitzgerald *et al.*, 2016, 2019; Fitzgerald, Katsiadaki & Santos, 2017). For example, hypoxia halved the toxicity of copper during a continuous 4-day exposure in zebrafish embryos. This effect was highly dependent on developmental stage; once the larvae hatched hypoxia increased copper toxicity (Fitzgerald *et al.*, 2016).

(4) Carbon dioxide

Most laboratory ecotoxicology studies are conducted under conditions where the partial pressure of gases is close to equilibrium with the atmosphere. Current atmospheric CO_2 averages about 413 µatm (CO2now.org), having risen since the industrial revolution from a previously stable value of ~280 µatm and is predicted to continue this exponential rise reaching ~1,000 µatm by 2100 (Meinshausen *et al.*, 2011), infamously leading to 'ocean acidification'. However, it is more accurate to refer to this phenomenon as 'aquatic acidification' because all surface waters (fresh water and saline) are affected. However, whilst many papers have investigated how increased CO_2 may affect marine organisms, relatively few have focussed on freshwater organisms, and even fewer have considered any interactions between dissolved CO_2 and toxicants.

It is worth pointing out that levels of dissolved CO_2 in fresh water are often much higher than in the atmosphere or in oceans, even when compared to climate change predictions for the year 2100 (see Fig. 2C). For example, the average pCO_2 for over 6,700 streams and rivers was >2,300 µatm according to Raymond *et al.* (2013). Furthermore, the natural variability in freshwater CO_2 concentrations far exceeds oceans, ranging from effectively zero to >2,400 µatm on both diel and seasonal

timescales; driven by extremes in the rate of photosynthesis in response to day/night and summer/winter cycles (Maberly, 1996; Hartmann *et al.*, 2019; Xu, Xu & Yang, 2019).

Dissolved CO_2 in freshwater environments is important because it can affect the toxicity of chemicals by affecting the acid-base chemistry of both the external water and the internal fluids of the organism. An increase in carbon dioxide causes acidification by reacting with water to form carbonic acid which dissociates to form protons (H^+) and bicarbonate ions (HCO_3^-). The chemical speciation of many toxicants shifts towards greater bioavailability for uptake in more acidic waters (see Section II.1). However, elevated environmental CO_2 simultaneously causes increased internal CO_2 (hypercapnia) in aquatic organisms, due to the permeability of their gas exchange surfaces. When this occurs rapidly it initially causes internal acidosis (Brauner *et al.*, 2019), i.e. reduced pH in the blood of fish or haemolymph of invertebrates, and in the intracellular fluids of all organisms, including algae. The structure and functions of all proteins are sensitive to pH, and most organisms have evolved mechanisms that eventually restore internal pH in response to prolonged exposure to high environmental CO_2. For fish and crustaceans at least, this is typically achieved by active ion-transporting cells in the gills and/or skin (Hwang & Lee, 2007; Evans, 2008). The net effect is an accumulation of HCO_3^- in the internal fluids, which can restore internal pH despite internal CO_2 remaining elevated. Although the internal pH may be completely restored, the new acid-base chemistry is very different, often with internal HCO_3^- levels elevated several-fold. This may influence the chemical speciation and therefore the toxicity of chemicals once internalised. Metals tend to become less toxic when complexed with HCO_3^-, although the CO_2-induced external acidification will increase their likelihood of uptake into the organism. However, the potential for internal complexation of metals (and so enhanced 'protection') by elevated plasma HCO_3^- will

496 also depend on the relative affinity of the metal for plasma proteins, which may limit
497 such beneficial effects. This concept of external *versus* internal acid-base impacts of
498 CO_2 has been poorly studied in freshwater animals, but there is evidence that it is
499 important to ecotoxicology in marine organisms. For example, DNA damage caused by
500 copper exposure was four times lower in sea urchins (good acid-base regulators)
501 compared to mussels (poor acid-base regulators) when simultaneously exposed to
502 elevated CO_2 (Lewis *et al.*, 2016). It is worth noting that the ability to regulate the acid-
503 base balance is variable, and any protective effects of CO_2 against toxicants are likely to
504 be specific to both life stage and species (Brauner *et al.*, 2019; Melzner *et al.*, 2009).
505 Although very few studies have explicitly investigated the potential role of
506 elevated internal HCO_3^- in moderating chemical toxicity during simultaneous exposure
507 to high CO_2, there are some additional examples. For example, elevated environmental
508 CO_2 provided some protection against the physiological impacts of waterborne copper
509 in fish, both in fresh water (rainbow trout; Wang *et al.*, 1998) and in sea water (cod
510 *Gadus morhua*; Larsen, Portner & Jensen, 1997). Exposure of saltwater medaka
511 (*Oryzias melastigma*) embryos to ~1,000 µatm CO_2 combined with the water-soluble
512 fraction of crude oil resulted in greater histological damage to eyes, kidney, pancreas
513 and liver compared to larvae from embyros treated with each stressor separately (Sun *et*
514 *al.*, 2019).
515 Currently, chemical test guidelines do not contain recommendations for CO_2,
516 but it seems clear that: (*i*) freshwater ecosystems are highly variable in terms of
517 dissolved CO_2, and (*ii*) there is evidence for some chemicals that CO_2 affects both the
518 external water chemistry and the internal physiology of aquatic animals in ways that can
519 dramatically influence their toxic impacts. It is also worth highlighting that toxicologists
520 use various methods to adjust and maintain target pH levels during toxicity tests. These

methods can include the manipulation of CO_2, but also addition of mineral acid or base or organic buffers. However, each approach has implications for the physiology of the fish (e.g. for internal acid-base regulation when CO_2 is manipulated) and/or the chemistry of the toxicant under study. Therefore, replicating the natural ambient conditions as far as possible is recommended, for example to avoid overestimating the impact of pH on metal toxicity when using CO_2 or an organic buffer (Esbaugh *et al*., 2013).

(5) Divalent cations

Water hardness reflects the quantity of divalent cations, mainly calcium and magnesium, dissolved in the aquatic environment (Wurts, 1993). It is well known that water 'hardness'can have protective effects against chemical toxicity and allowable toxicant limits are adjusted for water hardness in many environmental guidelines. Where the BLM is used to estimate the toxicity of metals, calcium and magnesium are important input parameters. The presence of divalent cations varies greatly among inland aquatic environments (Fig. 2H, I) depending on the surrounding geological characteristics and erosion processes, as well as anthropogenic factors including agriculture, mining and the dumping of industrial and domestic waste (Wurts, 1993). Concentrations of Ca^{2+} and Mg^{2+} are usually reported in µM units, but hardness is often reported in milligrams per litre as calcium carbonate (mg l^{-1} $CaCO_3$) or general hardness (°dH; 1 ºdH = 17.9 mg l^{-1} $CaCO_3$) which assumes that all divalent cations are derived from dissolution of solid calcium carbonate. Thus, while hardness indicates the total quantity of divalent cations dissolved in the water, it does not provide information on the separate contributions of calcium and magnesium. For example, in theory, it is possible to have water with a high hardness that contains no calcium (i.e. all hardness is

546 derived from magnesium; Wurts, 1993). The freshwater environment is often classified
547 in terms of hardness; water with low hardness is referred to as 'soft' water and high
548 hardness as 'hard' water.

549 Various studies have shown that hardness can influence the toxicity of chemicals
550 to freshwater organisms (Soucek *et al.*, 2011; Marchand *et al.*, 2013; Baldisserotto *et*
551 *al.*, 2014; Hundt *et al.*, 2016). For example, chloride toxicity is markedly reduced by
552 elevated water hardness in *Simulium simile*, *Gyraulus parvus*, and *Tubifex tubifex*
553 (Soucek *et al.*, 2011). Elevated Ca^{2+} and, to a lesser extent Mg^{2+}, concentrations, are
554 thought to tighten cellular junctions in the gills and skin, reducing the paracellular
555 permeability of the epithelium to the diffusion of chloride ions, and hence saving energy
556 required for ion regulation (Soucek *et al.*, 2011).

557 Water hardness has also been shown to affect the toxicity of disinfectants and
558 antibiotics to fish (Marchand *et al.*, 2013; Hundt *et al.*, 2016). For peracetic acid, the
559 toxicity to zebrafish embryos was negatively correlated with water hardness (25, 250
560 and 2,500 mg l^{-1} $CaCO_3$; Marchand *et al.*, 2013), and oxytetracycline (OTC) was more
561 toxic to zebrafish at extremes of water hardness (5.5 and 32.5 °dH), compared to
562 intermediate hardness levels (15.5 and 25.5 °dH; Hundt *et al.*, 2016). For OTC, it
563 appeared that low levels of cations increased the quantity of the free, more toxic form.
564 Its greater toxicity may result from higher levels of OTC–metal precipitate inhibiting
565 respiration (Hundt *et al.*, 2016). Water hardness can also attenuate the toxicity of
566 nitrogenous compounds such as ammonia (NH_3) in freshwater fish, for example
567 protecting against its neurotoxic effects and improving growth in juvenile silver catfish
568 *Rhamdia quelen* (Carneiro *et al.*, 2009; Ferreira, Cunha & Baldisseroto, 2013;
569 Baldisserotto *et al.*, 2014).

570 Calcium is well known to mitigate directly against the toxicity of several metals
571 (Zn, Cd, Co, Pb and Sr) primarily *via* direct competition with these metals for
572 physiologically important Ca^{2+}-uptake pathways in the gills (Wood, 2011). However,
573 calcium also plays a less well-known role in regulating processes that can influence the
574 uptake of chemicals, and ionized chemicals in particular. Firstly, calcium is potent at
575 controlling the permeability properties of fish gills and skin, and indeed of epithelia
576 more generally, by binding to tight-junction proteins and decreasing paracellular
577 permeability to all molecules (Lauren & McDonald, 1985; Wood, 2011). Specifically
578 for charged molecules, external calcium (like water pH, see Section II.1) within the
579 naturally occurring range (Fig. 2H) strongly regulates the TEP (Potts, 1984; Eddy,
580 1975; McWilliams & Potts, 1978; Wood *et al.*, 1998; Fig. 3B), with a magnitude that is
581 sufficient to influence the uptake of charged molecules *via* the electrochemical gradient
582 across the gills and skin. However, as for the effect of pH on TEP (see Fig. 3A and
583 Section II.1), this has yet to be considered in ecotoxicological studies and risk
584 assessments, but should be taken into account when designing media for testing
585 toxicity.
586 There are currently no recommendations for water hardness levels in guidelines
587 for tests with algae. For *D. magna* [OECD Test Nos 202 and 211 (OECD, 2004,
588 2012*a*)] suggested levels are 140–250 mg l^{-1} $CaCO_3$, and for fish recommendations
589 vary among different tests. For Test No. 203 [fish acute toxicity test (OECD, 2019)]
590 recommended levels are 40–250 mg l^{-1} $CaCO_3$ but preferably <180 mg l^{-1} $CaCO_3$, for
591 Test No. 212 [fish short-term toxicity test on embryo and sac-fry stages (OECD, 1998)]
592 they are 250 mg l^{-1} $CaCO_3$, for Test No. 215 [fish juvenile growth test (OECD, 2000)]
593 they are >140 mg l^{-1} $CaCO_3$, and for Test No. 236 [fish embryo acute toxicity FET test
594 (OECD, 2013*b*)] they are 30–300 mg l^{-1} $CaCO_3$. However, given the potential for water

595 hardness to influence the toxicity of many chemicals, guidelines should perhaps include
596 testing a range of hardness levels to allow a fuller understanding of potential biological
597 impacts.
598
599 **(6) Anions**
600 A very specific and well-studied example of a naturally abundant freshwater anion
601 influencing the toxicity of anthropogenic chemicals is that of Cl^- reducing the toxicity
602 of nitrite (NO_2^-). High levels of nitrite can kill fish very quickly by rapid uptake into the
603 blood followed by inhibition of methaemoglobin reductase in red blood cells. This
604 enzyme repairs haemoglobin (Fe^{2+}) that has been oxidised to methaemoglobin (Fe^{3+})
605 (Freeman, Beitinger & Huey, 1983); as methaemoglobin is unable to bind oxygen, its
606 formation compromises tissue oxygen delivery (Jensen, 2003). The initial uptake of
607 nitrite ions from the external water is *via* the same molecular pathways that transport
608 chloride ions in freshwater fish and invertebrates. Thus, higher environmental
609 concentrations of chloride can be extremely effective at reducing nitrite uptake and
610 toxicity, in accordance with the predictions of a competitive inhibition model (Jensen,
611 2003). This has been demonstrated in multiple freshwater fish including rainbow trout,
612 perch *Perca fluviatilis*, pike *Esox lucius,* eel *Anguilla anguilla,* carp *Cyprinus carpio,*
613 tench *Tinca tinca*, killifish *Fundulus heteroclitus*, channel catfish *Ictalurus punctatus*
614 and bluegill *Lepomis macrochirus* (Williams & Eddy, 1986; Tomasso & Grosell, 2005).
615 A similar relationship has been shown in some freshwater invertebrates, including
616 crayfish (*Astacus astacus*), an amphipod (*Eulimnogammarus toletanus*) and a planarian
617 (*Polycelis felina*) (Jensen, 1996; Alonso & Camargo, 2008). For both fish and
618 invertebrates, the mitigating impact of chloride on nitrite toxicity is proportional to the
619 animal's capacity for chloride transport *via* the gills or skin. Species that have low

capacity for chloride uptake (e.g. eel, carp, tench, bluegill, killifish, and *P. felina*) are both less sensitive to nitrite *per se*, and benefit less from the protective effects of environmental chloride (Jensen, 2003; Tomasso & Grosell, 2005; Alonso & Camargo, 2008).

Another example is protection against fluoride toxicity by chloride ions. In the freshwater invertebrate *H. azteca*, fluoride toxicity decreases (lethal median concentration [LC50] increases from 8.1 to 24.8 mg l^{-1} fluoride) as freshwater chloride increases (from 3 to 25 mg l^{-1}; Pearcy, Elphick & Burnett-Seidel, 2015). The same effect was observed in soft water conditions in rainbow trout: when chloride increased from 2 to 30 mg l^{-1}, fluoride toxicity decreased from an LC50 of 27.7 to 90.9 mg l^{-1} fluoride. However, in hard water conditions, chloride had no effect. This can be explained by the effect of calcium on fluoride solubility (i.e. CaF_2 precipitation in hard water; Pearcy *et al.*, 2015). Similar effects have been reported for other species of aquatic organisms including *H. azteca*, and *P. promelas* (Pearcy *et al.*, 2015). At very high concentrations of chloride in both soft or hard water chloride itself can be toxic through osmotic stress caused by disruption to cellular processes associated with acid-base regulation (De Boek *et al.*, 2000).

(7) Carbonate alkalinity

Bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, collectively known as carbonate alkalinity, are also anions. However, as bicarbonate is the second most abundant anion in fresh waters (as a global average, equating to 58% of the chloride concentration; Hartmann *et al.*, 2019), and because these ions have such a major influence on the impact of many toxicants, we consider carbonate alkalinity separately from the other inorganic anions.

645 The major source of carbonate alkalinity is an underlying geology consisting of
646 minerals of calcium carbonate (e.g. chalk, limestone) or calcium magnesium carbonate
647 (dolomite). This results in fresh waters with high concentrations of both bicarbonate and
648 carbonate, and typically a high pH (see Section II.1). However, carbonate alkalinity has
649 an influence on the speciation of many toxicants that is separate to the effects of pH.
650 Furthermore, although a high pH and high alkalinity typically are found together, the
651 relationship is only consistent if pCO_2 is constant, which is rarely the case for inland
652 aquatic ecosystems. As an example of the complexity of this relationship, for a given
653 pH value, such as the global average of 7.6, given the common fivefold range in pCO_2
654 (see Section II.4), then alkalinity will also vary by fivefold.
655 Carbonate alkalinity refers to the combined effect of the concentrations of
656 bicarbonate and carbonate ions in terms of their ability to neutralise acid. Each
657 carbonate ion (CO_3^{2-}) can neutralise two protons compared to only one by each
658 bicarbonate ion (HCO_3^-), hence carbonate alkalinity is numerically equal to $[HCO_3^-] +$
659 $2[CO_3^{2-}]$, with units most usefully reported as µM or µEq. However, units of milligrams
660 per litre as calcium carbonate (mg l^{-1} $CaCO_3$) are often used which theoretically
661 assumes all the alkalinity is derived from dissolution of solid calcium carbonate. The
662 use of these different units can cause confusion. For clarity, the molecular mass of
663 calcium carbonate is 100, so a carbonate alkalinity of 10 mg l^{-1} as $CaCO_3$ equates to
664 100 µM (0.1 mM) as $CaCO_3$, but because each carbonate ion can neutralise two protons
665 this also equates to 200 µEq (which would have the same acid-neutralising capacity as
666 200 µM of HCO_3^-).
667 The consideration of alkalinity as being entirely derived from calcium (and/or
668 magnesium) carbonate minerals can create further confusion because it is often assumed
669 that hardness (i.e. calcium and magnesium concentrations) changes in parallel with

alkalinity. Although this is commonly true due to the prevalence of calcium carbonate-based geology, it is not always the case, and alkalinity can be derived from sodium rather than calcium (or magnesium)-based carbonate minerals, and/or calcium/magnesium can be precipitated in high-pH waters, yielding low hardness but high alkalinity (Witherow & Lyons, 2011). Thus hardness and carbonate alkalinity should be considered separately, even though they are often well correlated in nature.

When considering its protective effects against chemical toxicity, adjustments are made for alkalinity in many environmental guidelines, as they are for water hardness (see Section II.5). For example, alkalinity is a key input parameter when using the BLM to evaluate metal toxicity and ambient water quality criteria (AWQC). Furthermore, carbonate alkalinity varies enormously (Fig. 2E) from essentially zero (in more acidic waters) to 4,000 μEq (200 mg l^{-1} as $CaCO_3$) in chalk streams, to 23,000 μEq (1,150 mg l^{-1} as $CaCO_3$) in freshwater alkaline lakes, reaching an extreme of 450,000 μEq (22,500 mg l^{-1} as $CaCO_3$) in some saline soda lakes (Wilkie & Wood, 1996).

The mitigating influence of carbonate alkalinity is particularly well understood for toxic metals; alkalinity (together with pH for the inorganic components of fresh water) has a dominating effect on the speciation of Cu, Cd, Pb and Zn, with the proportion of free metal ion, its uptake and ultimately toxicity, all decreasing as alkalinity increases, due to the complexation of, and therefore reduction in, free metal ion concentrations (Wood, 2011).

Water alkalinity will also interact with environmental CO_2: higher alkalinity increases the speed at which blood pH is regulated in fish in response to increased CO_2 levels (Larsen & Jensen, 1997). Thus water alkalinity could also affect the toxicity of compounds once they have been taken up into the blood (see Section II.4).

(8) Salinity

The total concentration of all ions (salinity) in fresh waters will reflect the local geochemistry together with the cumulative contact time. However, it will also be influenced by a range of anthropogenic activities, including mining, irrigation practices, vegetation removal for agriculture, fertilizers, pesticides, industrial waste, aquaculture, road de-icing salts, and alterations to natural buffering margins between salt and freshwater systems (Mimura, 2013; Hossain & Hasan, 2017; Canedo-Arguelles, Kefford & Schafer, 2019; Schuler *et al.*, 2019). The total salt content is normally quantified by the weight in grams of the inorganic matter dissolved in one kilogram of water (Stumm & Morgan, 1996) and salinity is expressed in S ‰ [parts per thousand (ppt)]. It can also be measured by the electrical conductivity of the water, or specific conductance (measured in Siemens cm^{-1} or mhos cm^{-1}; Harris, 2009), which shows enormous variation globally (Fig. 2G).

It is well established that the quantity of salts present in the aquatic environment, as well as their variation, can affect the toxicity of pollutants to freshwater organisms (Hooper *et al.*, 2013; Borecka *et al.*, 2016; Bosker, Santoro & Melvin, 2017; Saranjampour, Vebrosky & Armbrust, 2017; Hasenbein *et al.*, 2018; Canedo-Arguelles *et al.*, 2019). This effect may occur due to changes in the solubility of the pollutant (e.g. pesticides), alterations to its chemical fate and transport, as well as *via* changes in the physiological responses of organisms (i.e. osmoregulation, detoxification processes and toxicant sensitivity). Furthermore, high-salinity conditions also inevitably mean there are greater amounts of cations present, which can attach to plasma membrane binding sites and decrease the toxicity of xenobiotic chemicals like pharmaceuticals and insecticides (Hooper *et al.*, 2013; Borecka *et al.*, 2016; Bosker *et al.*, 2017; Saranjampour *et al.*, 2017; Hasenbein *et al.*, 2018).

Following exposure of *Chlorella vulgaris* to the drugs sulfapyridine, sulfamethoxazole, sulfadimethoxine, and trimethoprim, toxicity was reduced at higher salinities due specifically to higher levels of Na^+ binding with available hydroxyl functional groups on the algal surface, reducing cell wall permeability to the drugs (Kulacki & Lamberti, 2008; Latała, Nedzi & Stepnowski, 2010; Borecka *et al.*, 2016). By contrast, the pyrethroid insecticide bifenthrin had greater impacts on survival and swimming performance in *H. azteca* at higher salinity (Hasenbein *et al.*, 2018) due to its influence on contaminant bioavailability (Saranjampour *et al.*, 2017), interference with ion regulation, and heightened organismal sensitivity to the contaminant (Hooper *et al.*, 2013; Bosker *et al.*, 2017). For the pyrethroid insecticide deltamethrin, salinity/conductivity had no effect on toxicity in the invertebrates *Ceriodaphnia* cf. *dubia* and *Paratya australiensis*, or in eastern rainbow fish (*Melanotaenia duboulayi*) larvae (Thomas *et al.*, 2008).

Salinity can also affect the route of uptake for chemicals. Teleost fish, and many invertebrates, are osmoregulators, meaning they maintain a relatively constant osmolality of their internal fluids in the face of large variations in external salinity (Marshall & Grosell, 2005). A key osmoregulatory mechanism as environmental salinity rises is a switch from low drinking rates in dilute fresh water, to very high rates in sea water (e.g. >10% of body mass per day in some marine fish), with the most dramatic increase above the iso-osmotic point (when internal and external osmolality are equal), which is around 10–12 ppt for most teleosts (Marshall & Grosell, 2005). As drinking rate increases so the gut becomes an increasingly important site of exposure to waterborne chemicals, together with the gills and skin. That this can influence the toxicity of chemicals is illustrated by studies on the uptake and impacts of various

744 metals in fish and crustaceans (Wilson & Taylor, 1993*a*,*b*; Wood *et al.*, 2004; Gerdes *et*
745 *al.*, 2005; Blanchard & Grosell, 2006; Capparelli, McNamara & Grosell, 2017).
746 Salinity is another variable that can influence TEP in fish (Potts, 1984; Potts &
747 Eddy, 1973; Wood *et al.*, 2020), thereby affecting the electrochemical gradient across
748 the gills and the uptake of charged molecules. Interestingly the effect of rising salinity
749 on TEP is not due to the simultaneous change in external osmolality, but is rather
750 related to the change in concentrations of major ions, in particular Na^+ and Cl^- (Potts &
751 Eddy, 1973; Wood & Grosell, 2008; Wood *et al.*, 2020). Specifically, for Na^+ both the
752 body fluid concentration and the gill permeability are higher than for Cl^-. Thus a
753 slightly greater outward diffusion of Na^+ compared to Cl^- creates the negative TEP
754 (inside relative to outside) typically seen in freshwater fish. However, as external
755 salinity increases, the diffusional loss of Na^+ is slowed down (or reversed where
756 external concentrations exceeds blood concentrations), making the TEP become less
757 negative or even positive (e.g. in European flounder *Platichthys flesus* TEP changes
758 from –78 mV in fresh water to +19 mV in sea water; Potts & Eddy, 1973). How these
759 effects of salinity affect the uptake and toxicity of charged chemicals has not been
760 investigated. It is also noting that increased salination of freshwater environments is a
761 growing concern (Herbert *et al.*, 2015; Cañedo-Argüelles *et al.*, 2016). One emerging
762 multi-ion toxicity (MIT) model (EPRI, 2018) for assessing salt pollution assumes that
763 the mechanism of toxicity is the disturbance of gill TEP as salinity increases (Wood *et*
764 *al.*, 2020). While the major inorganic ions have very different toxicities (a 28-fold range
765 in LC50), the degree of disturbance of the TEP was extremely consistent for each ion
766 when expressed as a percentage of its LC50 concentration (Wood *et al.*, 2020).
767
768 **(9) Dissolved organic matter**

769 Organic matter in freshwater bodies originates from natural sources and from
770 human, farm animal and agricultural wastes. Natural organic matter (NOM) represents a
771 complex mixture of ill-defined biogenic organic molecules derived from the
772 decomposition mainly of dead plant material, but also from animals and microbes and
773 quantitatively (by mass concentration) often surpasses the inorganic components of
774 natural fresh waters (Thurman, 1985; Tipping, 2002; Hartmann *et al.*, 2019). Organic
775 matter can bind toxic chemicals and thus influence their bioavailability, and
776 concentrated discharges of organic matter can also affect chemical toxicity indirectly by
777 reducing oxygen availability *via* their fertilising effect on microbial respiration. NOM
778 can be divided into 'humic acids' (the high-molecular-mass colloidal component
779 susceptible to flocculation) and 'fulvic acids' (the acid-soluble, low-molecular-mass
780 non-colloidal component that resists flocculation); organic matter that cannot be
781 extracted is called humin (Bleam, 2017). Humic and fulvic acids make up the majority
782 of aquatic NOM with other bio-macromolecules such as carbohydrates, proteins and
783 amino acids making up smaller portions (Thurman, 1985; Al-Reasi, Wood & Smith,
784 2011).

785 The dissolved fraction of NOM (dissolved organic matter, DOM), usually
786 defined as the material able to pass through a 0.45 μm sieve, can interact with chemicals
787 *via* a wide range of mechanisms including ion exchange, hydrogen bonding, charge
788 transfer, covalent bonding, hydrophobic adsorption and partitioning, all of which can
789 affect the distribution of different pollutants, and their bioconcentration as well as
790 toxicity in water (Haitzer *et al.*, 1998; Qiao & Farrell, 2002; Zhang *et al.*, 2014; Chang
791 & Bouchard, 2016; Ding *et al.*, 2018). Approximately half of DOM by mass is carbon,
792 referred to as dissolved organic carbon (DOC; Tipping, 2002) and DOC is often the
793 variable measured and reported. DOC varies greatly in natural waters (see Fig. 2D).

794 Humic acid has been shown to reduce the toxicity of triclosan in the freshwater
795 alga *Cymbella* sp. (Ding *et al.*, 2018): complexation or adsorption of triclosan causing
796 reduced bioavailability. Zhang *et al.* (2014) found a variety of functional groups, such
797 as –OH, –$CONH_2$, –CONH, alcohol, and carboxylic and carbonyl groups, on the surface
798 of humic acids, which enabled interaction with hydroxyl groups in the triclosan
799 molecule. Humic acids can also accumulate on the surface of algae to cause electrostatic
800 repulsion between xenobiotics and the algal surface, thus inhibiting uptake (Tang *et al.*,
801 2015). DOM has also been shown to reduce the toxicity of triclosan in the crustacean *G.*
802 *pulex*, again due to complexation between triclosan and humic acid present in sewage
803 effluent (Rowett *et al.*, 2016). Similar effects have been reported in invertebrates for
804 various pesticides. For example, toxicity of the insecticide deltamethrin was reduced for
805 *P. australiensis* and *Ceriodaphnia* cf. *dubia* when exposed in river water (with organic
806 matter) compared to laboratory water without organic matter (Thomas *et al.*, 2008). In
807 addition to sorptive processes being involved, increased degradation of deltamethrin by
808 humus-mediated photosensitisation has been implicated for other pyrethroid insecticides
809 (Jensen-Korte, Anderson & Spiteller, 1987), and/or by bacterial degradation of the
810 compound (Das & Mukherjee, 1999).
811 The only study we found evaluating the effect of organic carbon on organic
812 chemical toxicity in any aquatic vertebrate was for deltamethrin on larvae of the eastern
813 rainbow fish (*M. duboulayi*), where there were no conclusive findings (Thomas *et al.*,
814 2008). However, there is substantial evidence of DOM moderating the uptake as well as
815 toxicity of many metals (e.g. Ag, Al, Cd, Co, Cu, Hg, Ni, Pb, Zn; Wood, 2011).
816 Although the underlying basis for this protective effect of DOM is becoming more
817 apparent, the precise molecular mechanisms involved are still less well-studied than for
818 the natural inorganic components of fresh water. Nevertheless, both the quantity and

quality (i.e. molecular composition) of DOM are important in its influence on toxicants. The optical properties of DOM are often used as a surrogate to characterise its molecular composition (e.g. using absorbance and fluorescence spectroscopy), with darker coloured DOM having a higher aromatic carbon content than lighter coloured DOM (Al-Reasi, Wood & Smith, 2013). Darker DOM has a higher proton-binding index (PBI) which is linked to greater protection against metal toxicity (Al-Reasi *et al.*, 2013). Given that the chemical properties of the molecular groups within DOM vary, and these properties differentially influence the binding and toxicity of metals, it seems likely that such surrogate measures will prove useful in investigations of the influence of DOM on the toxicity of organic chemicals. Interestingly, the chemical signatures of the components of DOM may vary naturally in a reasonably predictable manner, for example as they do between naturally acidic, circumneutral and groundwater-fed freshwater systems in Australia (Holland *et al.*, 2018). This could prove useful in targeting ecotoxicological risk assessments based on the environments that receive a particular toxic chemical.

Finally, DOC is another component of freshwater chemistry that is known to influence TEP in fish, and so also should affect the uptake of charged molecules by altering the electrochemical gradient between water and the blood. The effect on TEP is proportional to the aromaticity (= darkness in absorbance/spectrophotometric measurements) of the DOC at a given total concentration of organic carbon, both *in vivo* and *in vitro* using cultured gill cells (Wood, Al-Reasi & Smith, 2011; see Fig. 3C). However, as for pH and calcium, the effect of DOC on TEP has not yet been considered in an ecotoxicology context, and this remains an intriguing knowledge gap to fill in the future.

OECD test guidelines [Test Nos 202, 203, 210, 211, 212, 215, 229, 234, 235, 236 and 240 (OECD, 1998, 2000, 2004, 2011*b*,*c*, 2012*a*,*b*, 2013*a*,*b*, 2015, 2019)] recommend that total organic carbon concentration should be below 2 mg l^{-1}, however, measured concentrations have been shown to range between 1 and 10 mg l^{-1} (Chapman, 1996) in the freshwater environment, but in municipal wastewaters range from 10 to > 100 mg l^{-1} (Chapman, 1996).

III. CONCLUSIONS

(1) The interrelationship between the physico-chemistry of fresh waters and chemical toxicity relates both to (*a*) effects on the chemical to affect its form, fate and bioavailability in the water column/sediment, and (*b*) effects on the organism's internal chemistry, physiology or behaviour. There are thus strong arguments for regulatory testing of chemicals to include conditions relevant to the natural environments occupied by the organisms we seek to protect. Water physico-chemistry may in turn affect the dynamics of pollutant transfer through trophic food webs with potentially more pervasive, or different outcomes, but there is almost no information available with which to assess this.

(2) Although various studies have demonstrated the potential for pollutants to alter the capacity of aquatic organisms to adapt to current and future physico-chemical environmental changes, fewer have addressed how altered climatic conditions in the future may affect the responses of freshwater organisms to pollutants. This will depend on the magnitude of changes in physico-chemical conditions, pollutant concentrations, and the capacity of organisms to acclimate. In all cases, organisms are less likely to be able to adapt where there are rapid and severe changes to water physico-chemistry.

867 (3) Adaptations to altered water physico-chemistry may also affect other fitness traits
868 and the ability of an organism to adapt to other changes in their environment. It is
869 possible that direct effects of physico-chemical changes in the environment may
870 enhance the ability of some organisms to acclimate to pollutant exposures, although
871 there are limited examples of such facilitating or stimulatory effects. Understanding the
872 capacity to adapt to future physico-chemical conditions in the context of susceptibility
873 to pollution exposure is an important, and much needed area for future research for the
874 protection of ecosystem health.
875 (4) It is now possible to combine maps of global hotspots for individual chemicals, or
876 classes of chemicals, using concentration data (exposure concentrations) collated from
877 regional or national data repositories (e.g. EU WFD databases) with physico-chemical
878 data sets for freshwater environments (e.g. global freshwater environmental variables;
879 Domisch, Amatulli & Jetz, 2015; Hartmann *et al.*, 2014, 2019). This could allow us to
880 identify areas of greatest concern regarding chemical toxicity in freshwater
881 environments globally, and future predicted climatic conditions.
882 (5) Using such data sets it will be possible to develop risk profiles for chemicals across
883 global freshwater ecosystems accounting for the influence of water physico-chemistry.
884 Such risk profiles for specific chemicals could be derived from measured environmental
885 concentrations collated from existing databases (e.g. GEMStat), toxicity assessments
886 from laboratory-based studies using standardised tests conditions (UNEP, 2019), and
887 other suitable data sources (e.g. ECOTOX Knowledgebase; US EPA, 2020). Realistic
888 estimates of risk could be achieved through combining a range of different physico-
889 chemical variables and obtaining more accurate information on how they alter toxicity.
890 Such risk profiles could be extended to cocktails of chemicals. These analyses will
891 require more complex methods, for example, modelling the physico-chemical variables

as coefficients that influence the relative toxicity of a given pollutant, for which we would require additional information from detailed studies. The development of such global risk assessments could help us to rationalise the risk posed by pollution in the face of global environmental change.

(6) We show herein that physico-chemical characteristics of freshwater ecosystems can have a strong influence on the toxicity of divergent chemicals, including pharmaceuticals, pesticides, metals and inorganic nitrogenous compounds. These influences result from effects on compound solubility, radical and complex formation, and on sensitivity of an organism to toxicants, which can vary with ontogenetic stage and among species. Chemical hazard assessment therefore should be performed with greater consideration of how features of water physico-chemistry affect chemical toxicity in aquatic organisms and with more relevance to the natural water conditions in which these organisms live.

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Figure Legends

Fig. 1. The main water physico-chemistry variables that can alter the bioavailability and toxicity of chemicals to aquatic organisms, and the known mechanisms by which they exert their influence. Organisms images from PhyloPic (http://www.phylopic.org/). All other images from IAN/UMCES symbol and image libraries (https://ian.umces.edu/imagelibrary/).

Fig. 2. Global variation in freshwater physico-chemical variables for (A) pH, (B) temperature, (C) CO_2, (D) dissolved organic carbon (DOC), (E) alkalinity, (F) dissolved oxygen (DO), (G) specific conductivity, (H) dissolved calcium (1 µM of calcium is 0.040 mg l^{-1} calcium), (I) dissolved magnesium (1 µM of magnesium is 0.0243 mg l^{-1}

magnesium), and (J) dissolved sodium (1 µM of sodium is 0.023 mg l^{-1} sodium). Data for each variable were extracted from the GLORICH database (Hartmann *et al.*, 2014, 2019) and plotted as the number of records (*n*) for each variable (i.e. count per bin on the histogram).

Fig. 3. The influence of A) water pH, B) calcium concentration, and C) dissolved organic carbon (DOC), on the transepithelial electrical potential (TEP) in various species of fish. Relationships were redrawn (excluding raw data points) using data from McWilliams & Potts (1978) for brown trout (*Salmo trutta*) at 10 °C, and Wood *et al.* (1998) for tambaqui (*Colossoma macropomum*) at 28 °C in A, and from the same papers plus Eddy (1975) for goldfish (*Carassius auratus* – temperature not specified) in B. In C the relationships were redrawn from Wood *et al.* (2011). In A and B the TEP represents absolute measurements made *in vivo* relative to the external (water-side) potential. A negative TEP therefore means the blood side is negative relative to the water side. The same principle applies to C, except that the *y*-axis represents the change in TEP measured following transfer from control water with low DOC (Lake Ontario water) and low aromaticity (2.2 cm^2/mg C) to different waters containing consistently high DOC (all at 10 mg/l) with a range of aromaticity values from 2.7 to 53.5 cm^2/mg C. SAC refers to the specific absorption coefficient (at 340 nm). The two lines in C represent TEP changes measured *in vivo* (dashed line) using cannulated rainbow trout, and *in vitro* (solid line) using cultured rainbow trout gill epithelia. Note that increasing H^+ and Ca^{2+} concentrations cause TEP to become more positive, whereas increasing aromaticity of DOC causes TEP to become more negative.

1569 **Figures**

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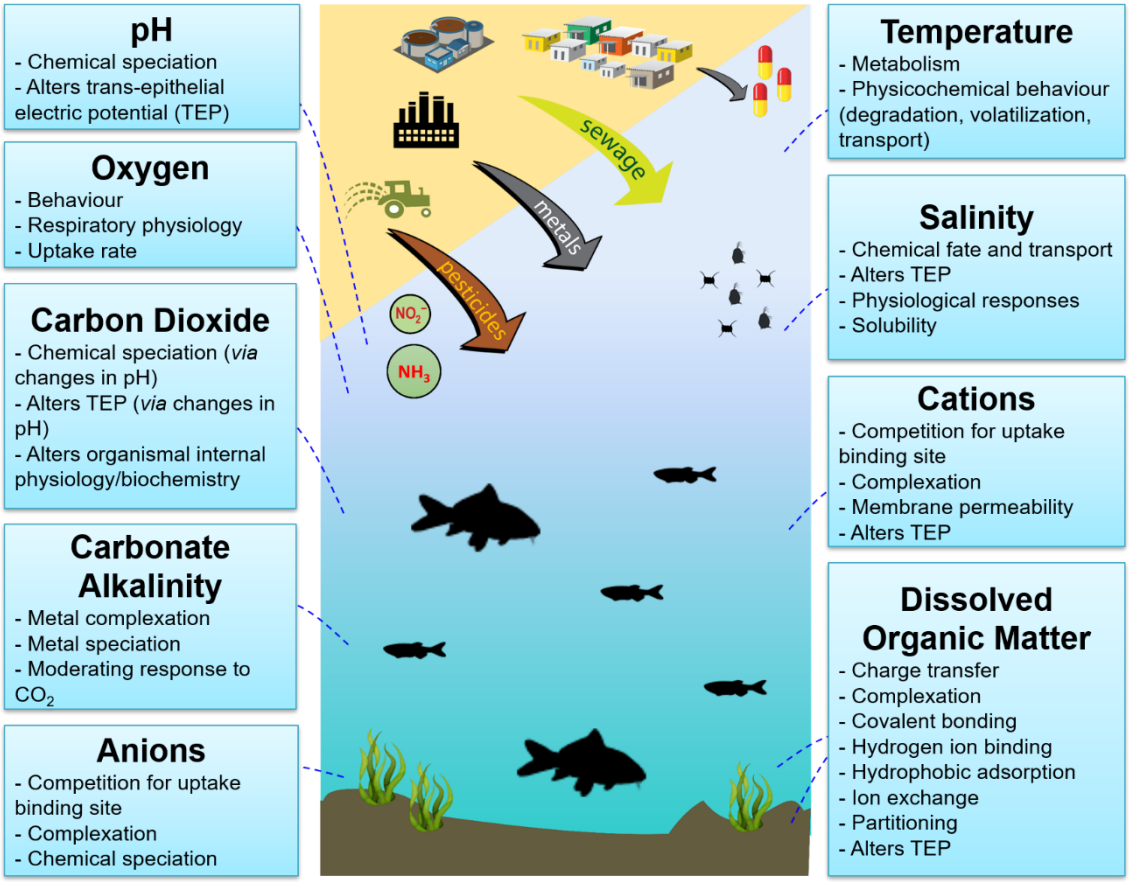

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1572 **Fig 1.**

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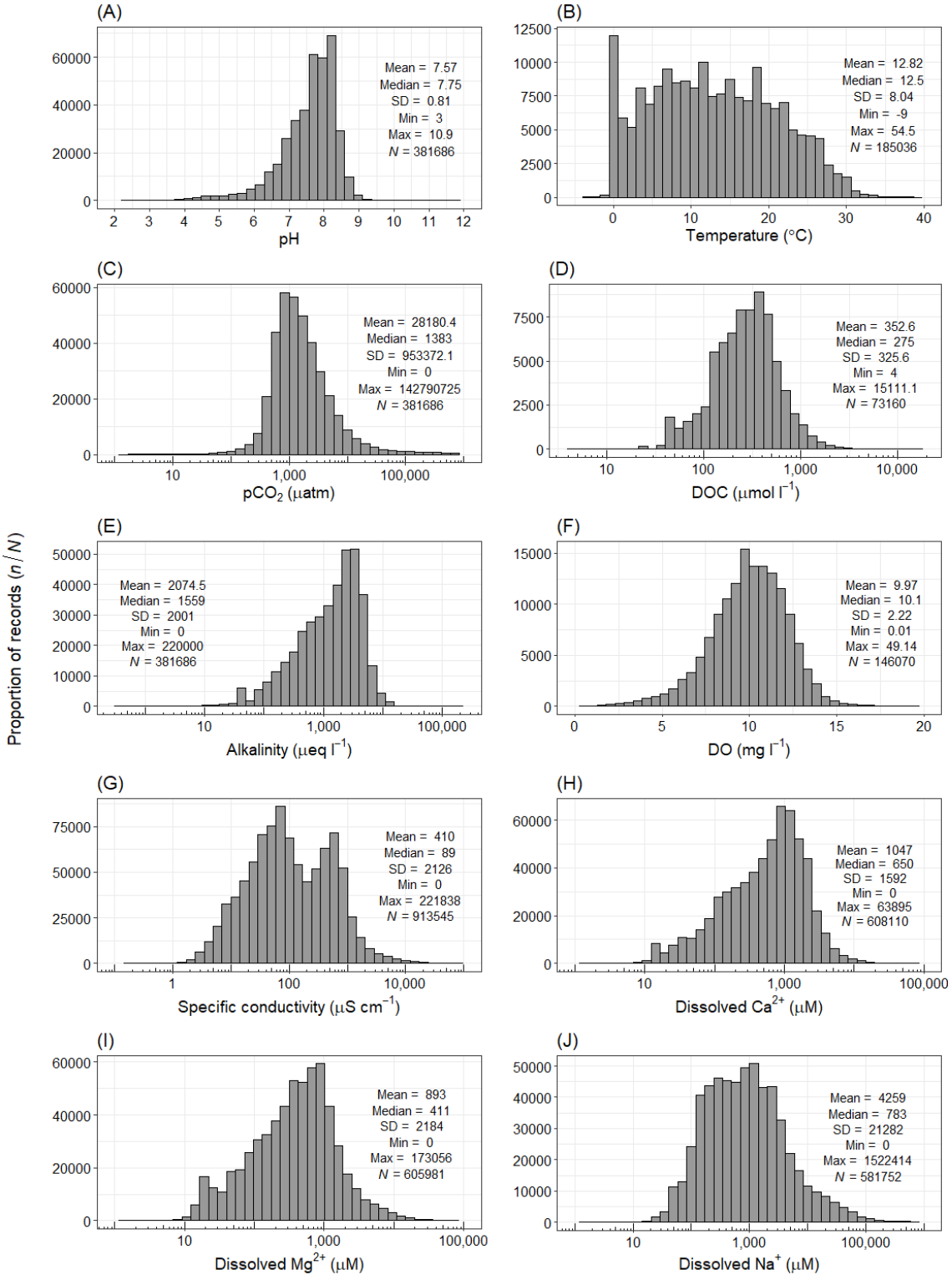

1574 **Fig 2.**

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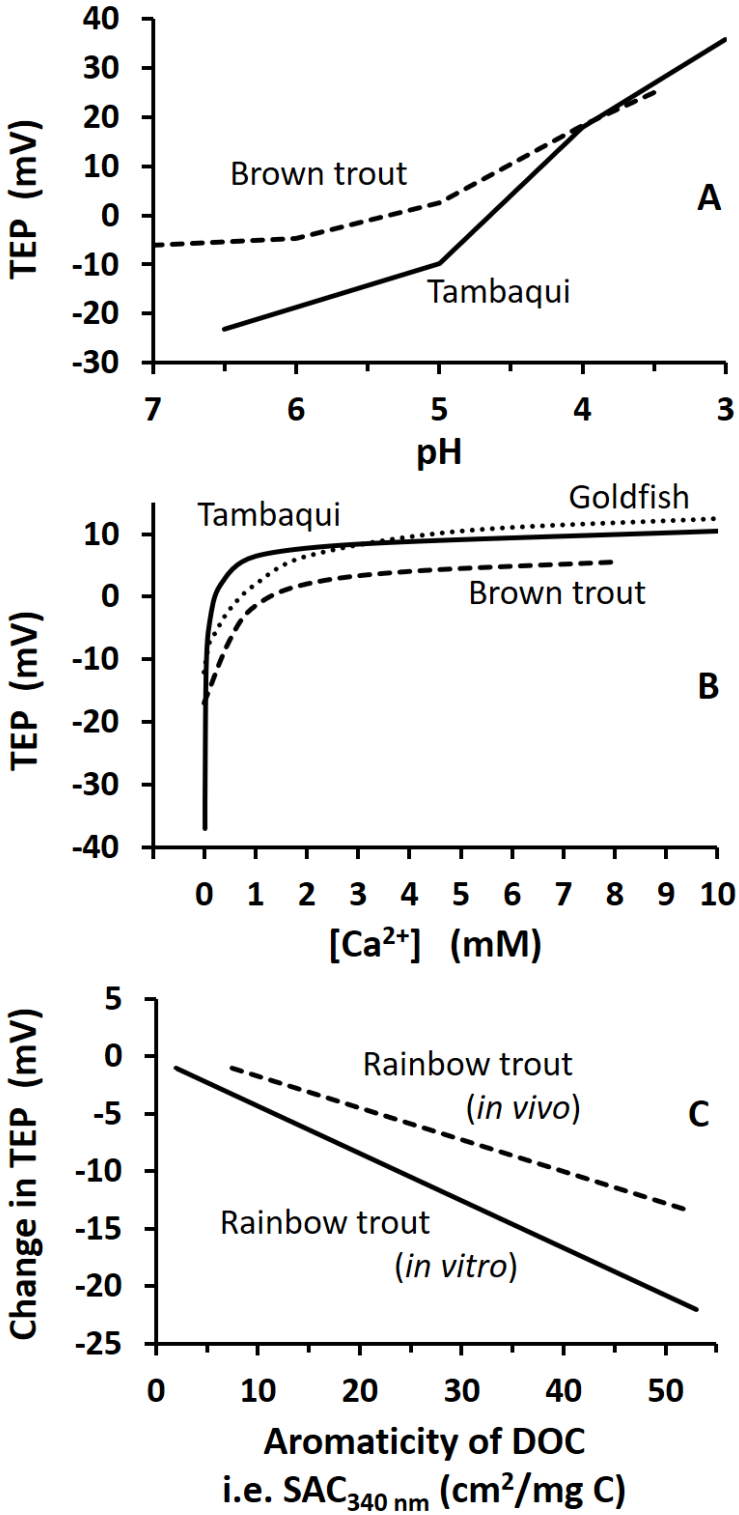

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1577 **Fig 3.**

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