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Abstract

We review the use of complex physiological traits, of tolerance and performance, as biomarkers of the toxicological effects of contaminants in subtropical and tropical freshwater fishes. Such traits are growing in relevance due to climate change, as exposure to contaminants may influence the capacity of fishes to tolerate and perform in an increasingly stressful environment. We review the evidence that the critical oxygen level, a measure of hypoxia tolerance, provides a valuable biomarker of impacts of diverse classes of contaminants. When coupled with measures of cardiorespiratory variables, it can provide insight into mechanisms of toxicity. The critical thermal maximum, a simple measure of tolerance of acute warming, also provides a valuable biomarker despite a lack of understanding of its mechanistic basis. Its relative ease of application renders it useful in rapid evaluation of multiple species, and in understanding how the severity of contaminant impacts depends upon prevailing environmental temperature. The critical swimming speed is a measure of exercise performance that is widely used as a biomarker in temperate species but very few studies have been performed on subtropical or tropical fishes. Overall, the review serves to highlight a critical lack of knowledge for subtropical and tropical freshwater fishes. There is a real need to expand the knowledge base and to use physiological biomarkers in support of decision making to manage tropical freshwater fish populations and their habitats, which sustain rich biodiversity but are under relentless anthropogenic pressure.

Key-words:

Biomarker, critical oxygen level, critical swimming speed, critical thermal maximum, ecotoxicology, hypoxia, warming.

Introduction

Freshwater ecosystems, and their resident fish populations, are particularly at risk from negative impacts of anthropogenic global change (Mason, 2002; Costa and Barletta, 2016; Higgins et al., 2021). Freshwater ecosystems are suffering relentless contamination with a bewildering diversity of chemical hazards and toxic substances such as agricultural chemicals; industrial solvents, surfactants and flame-retardants; metals, and pharmaceutical products, among many other pollutants (Stauffer, 1998; Mason, 2002; Rantin et al., 2020). These enter waterways directly from treated and untreated industrial, agricultural and domestic wastewaters, from run-off and atmospheric deposition (including spray drift) and indirectly from leaching (Holt, 2000). The diversity of contaminants and their potential toxic effects on freshwater fishes grow incessantly (e.g. Jobling and Tyler, 2003; Pérez-Parada et al., 2018; Wagner and Lambert, 2018).

In aquatic organisms, biomarkers are used to provide early warning signals of exposure or effects of specific pollutants or pollutant classes (Peakall, 1994; van der Oost et al., 2003). They are defined, in a broad sense, as any biological response by an organism to exposure to environmental chemicals, or to their toxic effects. The term is most commonly used to refer to measurements in body fluids, cells or tissues, which are indicative of bioaccumulation of toxic chemicals, biochemical and cellular modifications provoked by specific toxicants (van der Oost et al., 2003; Kroon et al., 2017) but would also be pertinent for other responses, for example behavioural alterations (Little and Finger, 1990; Saaristo et al., 2018).

For fishes it has long been recognized that complex traits of whole animal physiology, especially traits of functional integrity and energetics, are valuable indicators of sublethal toxicological impacts of contaminants (Brett, 1958; Cairns, 1966; Sprague, 1971; McKenzie et al., 2007). They have direct ecological relevance because the survival of an organism under stressful conditions depends on its ability to balance energy demand and energy supply (Calow and Forbes, 1998), and complex traits of functional integrity and energetics will influence how effectively a fish can achieve this, and cope with the conditions and the demands of life in their habitat (Cairns, 1966; Sprague, 1971; McKenzie et al., 2007). Physiological traits are particularly useful as components of an approach combining multiple biomarkers, which can capture different response kinetics and toxicological impacts of contaminants, especially in natural environments where contaminants occur as complex mixtures (Hook et al., 2014; Dalzochio et al., 2016).

Traits of tolerance of environmental stressors, such as hypoxia or warming, were first proposed as biomarkers 50 years ago (Cairns, 1971; Sprague, 1971). They

are now gaining increased ecological relevance because of ongoing climate change, which is predicted to make freshwater environments increasingly physiologically challenging for fishes (Diaz and Breitburg, 2009; Noyes et al., 2009; Stillman, 2019). Aquatic hypoxia is expected to be ever more common as a stressor, including an increased frequency of severe hypoxic episodes (Diaz and Breitburg, 2009; Costa and Barletta, 2016). The same is true of warming where, beyond gradual rises in seasonal mean temperatures, an increased frequency, intensity and duration of seasonal heatwaves is projected to be a major source of environmental stress for fauna globally (Williams et al., 2016; Stillman, 2019). The extent to which contaminants can influence tolerance of hypoxia or warming may, therefore, be determinants of the capacity of fish populations to persist in certain habitats (Noyes et al., 2009; Noyes and Lema, 2015; Khursigara et al., 2019; Isaza, 2020). The capacity to perform exercise is also a valuable and ecologically relevant biomarker of sub-lethal toxic effects of pollutants on fishes, because activities such as foraging, escaping predators, protecting territory, and migration, all depend upon swimming (Brett, 1958; Cairns, 1966; Beamish, 1978; McKenzie et al., 2007; Domenici, 2010; Domenici and Hale, 2019). One particular advantage, of complex physiological traits of tolerance and performance as biomarkers, is that they integrate the function of multiple underlying physiological systems, and can provide insight into why fish fail to colonize some polluted habitats (McKenzie et al., 2007). It is therefore possible to investigate what causes impairment of tolerance or performance, to gain valuable understanding of the impacts of contaminants on fish health and populations (e.g. Beaumont et al., 1995, 2000; Tierney et al., 2007; Thomaz et al., 2009; Gomez Isaza et al., 2020), which is particularly useful within programmes of ecological risk assessment.

We review knowledge on the application of physiological biomarkers in subtropical and tropical fish species, which we take to mean those that can occupy habitats where average monthly temperatures do not fall below 18 °C. Many subtropical and tropical freshwater ecosystems suffer from pollution and limited environmental regulation, so exposure to contaminants represents a serious hazard to population health of resident fishes (Langiano et al., 2008; Cazenave et al., 2009; Braz-Mota et al., 2015; Beltrão et al., 2019; Barreto et al., 2020). At the same time, tropical ecosystems are relatively poorly studied in terms of ecotoxicology, despite harbouring important resources in terms of biodiversity (Lacher and Goldstein, 1997; Daam and Van Den Brink, 2010; Wang et al., 2019). They can also be naturally subject to stressful hypoxic and warming events, especially because many have seasonal variations in rainfall that severely modify the volume and connectivity of habitats. We consider studies that have used the critical oxygen level (O_{2crit}) as a biomarker. For

these studies, we pay particular attention to how changes in O_{2crit} caused by contaminant exposure are linked to elements of cardiorespiratory physiology and performance, as these are core components of whole animal tolerance (Driedzic and Gesser, 1994; Gamperl and Driedzic, 2009). We then consider studies that have used traits of thermal tolerance as biomarkers, in particular the critical thermal maximum (CT_{max}). Finally, we review the few studies that have used critical swimming speed (U_{crit}) as a biomarker.

Effects of contaminants on tolerance of hypoxia

Hypoxic events can be very frequent in subtropical and tropical freshwater ecosystems, with dissolved oxygen (DO) fluctuations of different duration and magnitude, diurnally, spatially, and seasonally (Almeida-Val et al., 2006). Although fluctuating levels of DO can be a natural phenomenon, hypoxia caused by eutrophication and pollution is one of the most pressing and critical water problems in the world (Pollock et al., 2007). Hypoxia and contaminant exposure often co-occur in aquatic environments but their interactions remain poorly studied (Monteiro et al., 2020). There is evidence that co-exposures to hypoxia and polycyclic aromatic hydrocarbon mixtures caused several disorders in fishes, such as altered growth and sexual differentiation, reduced reproductive capacity, and developmental toxicity (Matson et al. 2008; Di Giulio and Clark, 2015; Mu et al., 2017). Rahman et al. (2018) demonstrated that co-exposure to hypoxia and polychlorinated biphenyl reduced biotransformation of organic xenobiotics by monooxygenase enzyme cytochrome P450-1A (CYP1A), but markedly augmented hepatic endothelial nitric oxide synthase (eNOS) and interleukin-1β (IL-1β) mRNA levels, and protein carbonyl (PC) contents.

Hypoxia survival requires a well-coordinated response to either secure more O_2 from the depleted environment or to protect against the metabolic consequences of low O_2 levels at the mitochondria, which limit aerobic ATP production (Regan et al., 2017; Richards, 2019). The critical oxygen level (O_{2crit}) is widely used as a quantifiable trait of hypoxia tolerance in fishes. It represents the minimum water oxygen level required to sustain standard metabolic rate (SMR, the basal metabolic rate of an ectotherm at their acclimation temperature) (Claireaux and Chabot, 2016; Rogers et al., 2016). It is determined by plotting rates of oxygen uptake (MO_2) against water O_2 tension (kPa), to identify the point at which MO_2 falls below SMR, and becomes directly dependent upon water O_2 tension (Rogers et al., 2016). Once below their O_{2crit}, fishes must depend on anaerobic energy metabolism and basal functions can be compromised (Pörtner, 2010). If the hypoxic conditions worsen and persist, the combination of hypoxic severity and accumulation of metabolic products can threaten survival (Claireaux and Chabot,

2016). Although the ecological significance of the O_{2crit} threshold has been debated (Wood, 2018; Regan et al., 2019; Ultsch and Regan, 2019), it varies among species in a manner that is consistent with their overall hypoxia sensitivity, being lower in more tolerant species (Rogers et al., 2016). A low O_{2crit} value indicates a higher capacity for O_2 extraction by the gills and O_2 delivery to the tissues as O_2 availability declines, indicating a greater hypoxia tolerance (Mandic et al., 2009).

Figure 1 shows data for one species, matrinxã *Brycon amazonicus*, where sublethal exposures to various contaminants all increased O_{2crit} values. The degree of physiological plasticity for hypoxia tolerance is a key determinant of species performance (Rogers et al., 2016). This was associated with various impacts of the contaminants on MO_2 during graded hypoxia (Table 1). This is shown in more detail for the matrinxã, where contaminants had various effects on O_2 uptake such as increases and decreases in MO_2 during normoxia (for Roundup Original® and Roundup Transorb®, respectively) and/or early decline in MO_2 during graded hypoxia for all pollutants (Figure 2). All these effects had the consequence of impairing the capacity of the species to oxyregulate. According to McRae et al. (2018) metabolic rate is, itself, a useful integrated measurement of toxicological impact because it is a measure of energy resource associated with multiple sublethal processes and ecologically important endpoints such as reproduction, growth and survival.

The exposure to various contaminants markedly increased O_{2crit}, and therefore reduced tolerance of hypoxia, in four species of tropical fishes, including hypoxia-tolerant fish species such as Nile tilapia *Oreochromis niloticus* and trahira *Hoplias malabaricus* (Table 1). These data clearly indicate that exposure to contaminants may reduce survival chances of many species of fishes in tropical hypoxic environments. Table 1 also summarises how different pollutants can have a diverse array of effects on both ventilatory and cardiac activity in normoxia, and how such activity can be affected at given levels of hypoxia.

Oxyregulation is achieved by reflex cardiorespiratory responses that aim to sustain oxygen uptake and delivery, and possibly protect vital organs such as the heart. There is a profound hyperventilation, involving increases in gill ventilation volume ($\dot{V}G$) achieved through increased respiratory frequency (f_R) and amplitude, and a bradycardia (Farrell, 2007; Perry et al., 2009; Joyce et al., 2016; Rantin et al., 2020). The hyperventilation increases O_2 transport to the site of gas exchange (Bergsson et al., 2019). Enhancements in $\dot{V}G$ are required to maintain a constant MO_2, which implies that fish survival in hypoxic environments can be compromised if the ability to increase $\dot{V}G$ is affected (Martins et al., 2011). Pollutants can, indeed, impair the ability to

increase f_R and $\dot{V}G$ in response to hypoxia, impairing the regulation of metabolic rate. For example, the lower f_R induced by acute exposure to the organophosphate trichlorfon in Nile tilapia *Oreochromis niloticus*, both in normoxia and hypoxia, impaired gill ventilation and compromised the hyperventilatory response in severe hypoxia (Thomaz et al., 2009). The same response pattern was observed in trahira after subchronic and trophic exposure to mercury chloride (Monteiro et al., 2013). In matrinxã, increases of f_R and $\dot{V}G$ in response to hypoxia were significantly reduced after acute exposure to the emerging contaminant triclosan, an antimicrobial agent widely used in personal care products. In normoxia, triclosan had caused an increased f_R with concomitant decreased $\dot{V}G$ (Martins, 2018). Under hypoxia, $\dot{V}G$ values were also considerably lower in Nile tilapia 48 h after intraperitoneal injection of mycrocystin-LR (Martins et al., 2011) and in matrinxã acutely exposed to glyphosate-based herbicides Roundup WG® and Roundup Transorb® (Anelli Jr., 2010), probably due to alterations of homeostatic reflexes that impaired respiratory responses to hypoxia, due to unknown toxic mechanisms. All these effects described above and presented in Table 1 can reduce the ability of tropical fish to maintain a constant O_2 uptake when they encounter hypoxia in contaminated environments

Organophosphate (OP) pesticides, mercury chloride, triclosan, and glyphosate-based herbicides inhibit acetylcholinesterase activity resulting in paralysis, mainly of the fins and respiratory muscles, uncoordinated movements, seizures, and even central depression of respiration (Suresh et al., 1992; Sancho et al., 1998; Antonijevic and Stojiljkovic, 2007; Thomaz et al., 2009; Modesto and Martinez, 2010; Menéndez-Helman et al., 2012; Pullaguri et al., 2021). Furthermore, contaminants such as microcystins and glyphosate-based herbicides cause oxidative stress and alterations in gill structure and respiratory epithelium (Gupta and Guha, 2006; Shiogiri et al., 2012; Braz-Mota et al., 2015; Martins et al., 2017) which could result in disturbances in respiratory function. Specifically for matrinxã exposed to Roundup Original®, Roundup WG® and Roundup Transorb®, $\dot{V}G$ values were already high in normoxia (Table 1), indicating increased respiratory drive to maintain O_2 extraction.

On the other hand, contaminants can also induce rises in respiratory frequency and conspicuous increases in gill ventilation, including in hypoxia, which may indicate a desperate attempt to meet tissue oxygen demand. Increased gill ventilation was detected in matrinxã after methyl parathion and Roundup Original® exposure, mainly during hypoxia (Table 1, Olle 2007; Anelli Jr., 2010). Wang et al. (2013) evaluated the ventilatory responses of 3 tropical species, zebrafish D*anio rerio*, rare minnow *Gobiocypris rarus* and grass carp *Ctenopharyngodon idellus* to acute exposure to 4 heavy metal ions (Hg^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}). Ventilatory frequency and amplitude

increased after exposure to Hg^{2+} and Cu^{2+} but markedly decreased with increasing concentrations of Cd^{2+} and Zn^{2+}. This shows that respiratory activity of fishes is highly sensitive to heavy metal pollution but in complex ways (Wang et al., 2013). Differences in ventilatory responses to hypoxia could also be related to changes in blood O_2-carrying capacity (Pereira et al., 2013; Islam et al., 2019), according to the type and concentration of contaminants and the physiology of the target species. In the silver perch *Bidyanus bidyanus* from Australia, exposure to nitrate caused animals to perform aquatic surface respiration (ASR) at a higher oxygen level during progressive hypoxia (from 19 to 1 kPa), coupled with increased ventilation frequency. This was linked to lower levels of hemoglobin and hematocrit, indicating reduced blood oxygen carrying capacity (Gomez Isaza et al., 2021a). Fish exposed to pollutants often show hyperplasia and hypertrophy of gill epithelia cells with fusing and thickening of gill lamellae and mucus hypersecretion, which increases the water/blood diffusion distance and impairs O_2 uptake, leading to hypoxemia (Schjolden et al., 2007; Sokolova and Lannig, 2008; Bilberg et al., 2010). In traíra, exposure to a single dose of microcystin-LR (100 μg Kg^{-1} of body weight, i.p.) caused hyperventilation coupled with significantly reduced O_2 extraction from the ventilatory current under normoxic and hypoxic conditions, which compromised O_{2crit} and was attributed to compromised gill gas transfer (Martins et al., 2019).

Many pollutants (methyl parathion, tricholorfon, Roundup WG®, Roundup Transorb®, mercury chloride, microcystin) also impaired cardiac activity, causing a bradycardia that then lead to very low heart rates under hypoxic conditions (Table 1). This may indicate direct toxic effects on cardiac myocytes. Several studies have reported decreased myocardial isometric twitch force development in tropical fishes exposed to pollutants (Incardona et al., 2004; Thomaz et al., 2009; de Andrade Waldemarin et al., 2012; Monteiro et al., 2017). Reduced cardiac performance can increase the likelihood of starvation, predation vulnerability, and disease susceptibility (Incardona and Scholz, 2017). Exposure to 1 mg L^{-1} of copper sulfate for 96 h caused significant changes in Nile tilapia cardiomyocytes, including decreased myocardial contractility, cellular swelling, mitochondrial swelling, and alterations in mitochondrial inner membranes, which may impair ATP supply (de Andrade Waldemarin et al., 2012). Monteiro et al. (2020) found that cardiac responses to hypoxia were impaired in both matrinxã and traira after exposure to inorganic mercury by different routes (water and diet) and durations (acute and sub-chronic). There were alterations in the rhythm of the cardiac pacemaker such as first-degree atrioventricular block, lengthening of the action potential (AP) plateau, and the development of cardiac arrhythmias and abnormalities (episodes of missing QRS complexes with sequential P waves,

extrasystoles, negative QRS complex, T wave inversion) (Monteiro et al., 2020). These disruptions of electrical conduction could underly the myocardial dysfunction and increase hypoxia sensitivity by impairing cardiac output (Rantin et al., 1995).

Overall, these findings demonstrate that O_{2crit} is a valuable biomarker of impacts of contaminants in tropical freshwater fishes, irrespective of the ecology and life history characteristics of a given species. In addition, analyses of effects on MO_2 and cardiorespiratory variables can improve understanding of the mode of toxic action of some contaminants, which can aid in the environmental risk assessment of aquatic systems. The fact that contaminants can impair hypoxia tolerance may have significant ecological consequences, not just directly influencing survival but potentially also reducing fitness, by impairing the capacity to forage, capture and digest food, with consequences for growth and/or reproductive rates. Nonetheless, the knowledge base is critically limited and should be extended to further species and contaminants.

Effects of contaminants on tolerance of warming

There are complex interactions between temperature and the toxicity of contaminants in fishes, which depend upon multiple factors that are too extensive to review here (Noyes et al., 2009; Patra et al., 2015). We focus exclusively upon the use of thermal tolerance as a biomarker. In fishes, limits of thermal tolerance are measured using acute thermal ramping protocols, especially the critical thermal (CT) methodology that reveals the threshold temperature which causes loss of equilibrium (LOE). The protocol is simple and defines the temperature where survival is threatened because at LOE the fish cannot escape the conditions (Beitinger and Lutterschmidt, 2011). There are, however, quite major limitations to the critical thermal protocol as an experimental tool (Rezende et al., 2014; Blasco et al., 2020). Notably, the idea that the complexity of thermal tolerance limits can be captured by a single measure is a major oversimplification (Rezende et al., 2014; Rezende and Bozinovic, 2019; Lefevre et al., 2021). There are also methodological issues; particularly that the measured CT_{max} depends upon heating rate (Lutterschmidt and Hutchison, 1997). Furthermore, although the mechanism(s) that causes LOE are presumed to involve impaired function of critical organs such as the heart and brain (Wang et al., 2014; Brijs et al., 2015; Jutfelt et al., 2019; Lefevre et al., 2021), they are not known and may differ among species and with warming rate (Currie and Schulte, 2014; Healy et al., 2018; Blasco et al., 2020). What is well-established, however, is that a given increase in acclimation (or acclimatization) temperature (T_{acc}) is not linked to an equivalent increase in CT_{max}, both among and within fish species (Cossins and Bowler, 1987; Currie and Schulte, 2014; McKenzie et al., 2020). As a consequence, when comparing among species, tropical

fishes have a lower thermal safety margin (TSM) than temperate, where TSM is calculated as the net difference between T_{acc} and CT_{max} (Comte and Olden, 2017a; b). Within a species, TSM also becomes smaller as a fish is acclimated to progressively warmer temperatures across its thermal range (e.g. McDonnell et al., 2019; McKenzie et al., 2020).

The notion that CT_{max} could be used as a biomarker of sublethal toxic effects of contaminants dates from the 1970s (Cairns, 1971; Johnson, 1978; Dale Becker and Wolford, 1980) although the trait has only been used in this way on 11 species of subtropical or tropical fish (Table 2). As for all biomarkers that are complex physiological traits, CT_{max} can provide a sensitive indicator of functional impairment caused by a contaminant. The lack of mechanistic understanding does, however, hinder comprehension of essential questions such as why CT_{max} is modified by exposure to contaminants. Nonetheless, the CT_{max} is interesting from a toxicological viewpoint because it is an incipient lethal threshold, therefore if it is reduced by contaminants this could have clear consequences for persistence of fish populations that suffer heat stress in polluted waters. The low TSM of subtropical and tropical species acclimated (or acclimatized) to warm waters may render them particularly vulnerable to heatwaves if they are in contaminated environments, especially if they are at temperatures close to the top of their thermal range.

There have been various investigations of effects on CT_{max} of organic contaminants, notably pesticides, herbicides and common pollutants from industry such as phenol. Many of these molecules act as neurotoxins in vertebrates, hence they might be expected to influence CT_{max} since LOE almost certainly has some neurological component (Lutterschmidt and Hutchison, 1997; Jutfelt et al., 2019). In many cases, the studies involved first establishing lethal concentrations (LC50) for the focal species, and then evaluating effects of sublethal concentrations on CT_{max}. Johnson (1978) studied the effects of four widely-used OP insecticides on CT_{max} in the western mosquitofish *Gambusia affinis*, when acclimated to 20 °C (Table 2). *Gambusia affinis* is a subtropical North American species that is highly eurythermal. As acetylcholinesterase inhibitors, OP insecticides have neurotoxic effects on fishes. Taking concentrations that were proposed as doses for field applications, the pesticides had no significant effects on mosquitofish CT_{max} aside from chlorpyrifos, which caused a modest but significant decline in tolerance (Table 2). Takle et al. (1983) reported that sublethal concentrations of the biocide endothall, which is a protein phosphatase A_2 inhibitor, had no significant effect on CT_{max} in another subtropical eurythermal north American species, the red shiner *Cyprinella lutrensis*, acclimated to 20 °C. In the same species, Messaad et al. (2000) investigated effects on CT_{max} of

sublethal concentrations of the triazine herbicide, atrazine, of an OP pesticide, terbufos, plus a cocktail of these, comparing effects on fish acclimated to 23° or 30 °C. Toxic actions of atrazine in animals are supposedly related only to endocrine disruption. CT_{max} of control fish increased with acclimation temperature but the contaminants caused more marked discrete declines in CT_{max} at a T_{acc} of 30 °C compared to 23 °C (Table 2). In particular, the cocktail of the two pesticides caused a very profound decline in CT_{max} at 30 °C (Table 2). The mechanisms underlying the increased impact upon CT_{max} at the higher temperatures may relate to increased metabolic rates and ventilatory water flows, which would cause increased uptake of contaminants from the water (Yang et al., 2000). Such results indicate that, as T_{acc} increases, fishes may be more vulnerable to heat stress when they are also exposed to certain environmental contaminants (Messaad et al., 2000).

Patra et al. (2007) studied the effects on CT_{max} of sublethal concentrations of three contaminants, endosulfan, an organochlorine pesticide; the OP chlorpyrifos, and an organic contaminant, phenol, in three subtropical Australian species, silver perch, eastern rainbowfish *Melanotaenia duboulayi* and western carp gudgeon *Hypseleotris klunzingeri*. Endosulfan is neurotoxic, a GABA-gated chloride channel antagonist, and a Ca^{2+}, Mg^{2+} ATPase inhibitor. It is used to control insect pests but is acutely toxic to animals and is now banned in many countries. Phenol is one of the most common molecules in the chemical industry globally with a vast array of uses, it can have toxic effects on the central nervous system and heart of vertebrates. Although both endosulfan and chlorpyrifos caused significant declines in CT_{max} in all species, while phenol was without effect. Kumar et al. (2016) reported that, in a euryhaline tropical Asian species the milkfish *Chanos chanos* acclimated to 28 °C, sublethal concentrations of endosulfan reduced CT_{max} compared to a control (Table 2), an effect that was abolished by dietary supplements of vitamin B_6, pyroxidine. Pyroxidine supplementation was linked to changes in tissue oxidative status and expression of the heat shock protein HSP70, which may have been related to the improved thermal tolerance (Kumar et al., 2016).

Zebral et al. (2018) investigated effects of the glyphosate-based herbicide, Roundup®, on thermal tolerance of embryos of an endangered annual neotropical killifish, *Austrolebias nigrofasciatus*, hatched at 20 °C. This annual species leaves eggs in diapause in dried water courses during the dry season, which hatch when rains arrive. Glyphosate is not considered to have acute toxic effects on vertebrates. Glyphosate caused, however, a significant decline in CT_{max} in hatched embryos held at 21 °C, when measured as cardiac arrest followed by mortality despite being immediately returned to acclimation temperature (Table 2). Philippe et al. (2019)

374 investigated the effects, on CT_{max} of adults of the turquoise killifish *Notobranchus*
375 *furzeri*, an annual species from Africa, of a highly persistent compound released from
376 chlorinated aniline herbicides, 3,4-dichloroaniline (3,4-DCA). Fish were acclimated to
377 either 24 or 28 °C and exposed to two concentrations of 3,4-DCA, CT_{max} increased with
378 T_{acc} and the contaminant only caused a significant decline in CT_{max} at the highest
379 concentration at 28 °C (Table 2).
380 There has been some investigation of effects of sublethal concentrations of
381 toxic heavy metals on CT_{max} in subtropical or tropical fish species. Cadmium and lead
382 are both highly toxic to fishes, they attack various organs systems including gills,
383 intestine and kidney. Notably, at sublethal concentrations they are neurotoxic (Green
384 and Planchart, 2018) and, therefore, could influence thresholds for LOE and CT_{max}.
385 Carrier and Beitinger (1988) found that sublethal concentrations of cadmium decreased
386 CT_{max} in the red shiner acclimated to 20 °C, with a severity that increased with
387 exposure dose and duration (Table 2). By contrast, Philippe et al. (2018) found that
388 two sublethal concentrations of cadmium had no effect on CT_{max} in the turquoise
389 killifish acclimated to either 24 or 28 °C, although CT_{max} increased with acclimation
390 temperature (Table 2). In the Mekong striped catfish *Pangasianodon hypophthalmus*
391 acclimated to 34 °C, exposure to a sublethal concentration of lead caused a significant
392 decline in CT_{max}, which was ameliorated by dietary supplementation with selenium or
393 zinc (Kumar et al., 2017, 2018). This amelioration was concomitant with changes in
394 redox balance of the tissues and in expression of HSPs, further circumstantial evidence
395 that these elements of cell physiology may be involved in thermal tolerance in fishes.
396 There has also been some investigation of effects of nutrient contaminants on
397 tolerance of warming. Nitrite is a pervasive form of nutrient pollution that enters aquatic
398 habitats from diverse sources such as nitrogen-based fertilisers, livestock manure,
399 sewage wastewater, and atmospheric deposits. The major toxic effect of nitrite is that it
400 compromises blood oxygen transport in fishes, by causing methemoglobin formation
401 (Lewis and Morris, 1986; Jensen, 2003). Watenpaugh et al. (1985) found that
402 increasing sublethal concentrations of nitrite caused a progressive decline of CT_{max} in
403 the subtropical eurythermal channel catfish *Ictalurus punctatus* acclimated to 20 °C.
404 The CT_{max} was negatively correlated with blood methaemoglobin concentrations, which
405 would seem to indicate that oxygen transport capacity is a determinant of CT_{max} in this
406 species. Rodgers and De Boeck (2019) reported that a sub-lethal nitrite concentration
407 caused a significant decline in CT_{max} of in a globally extremely widespread subtropical
408 eurytherm, the common carp *Cyprinus carpio*, when acclimated to 23°C (Table 2).
409 Within the extreme boundaries delineated by CT_{max}, thermal tolerance in fishes
410 is typically evaluated with a thermal performance curve (TPC) that measures a trait of

organismal performance over a range of temperatures to identify features such as a thermal optimum for performance (Schulte et al., 2011; Currie and Schulte, 2014). The most common TPC is for aerobic metabolic scope (AS), the net capacity to supply oxygen for aerobic metabolic activities beyond basal maintenance (Fry, 1971; Schulte et al., 2011; Schulte, 2015). Very few studies have investigated how contaminants might influence elements of a TPC in subtropical or tropical fishes (Rodgers and De Boeck, 2019; Gomez Isaza et al., 2020a), also because this requires very extensive experimental work.

The study by Rodgers and De Boeck (2019) on common carp exposed to nitrite is a rare example where changes in CT_{max} were related to aerobic performance and aerobic scope. Although nitrite reduced CT_{max} this was not related to any decline in AS, indicating that CT_{max} in the carp is not affected by capacity to provide oxygen for metabolism when warmed. The most comprehensive evaluations of how a contaminant might influence thermal tolerance and thermal sensitivity of performance in a tropical freshwater fish are studies on effects of nitrate on the silver perch (Gomez Isaza et al., 2020a, 2021b). Nitrate is the most abundant nutrient contaminant globally; although less toxic than nitrite it nonetheless shows persistently high concentrations in freshwaters due to agricultural runoff from nitrogen-based fertilisers, fossil fuel combustion and wastewater treatment plants. Nitrate also causes the formation of methaemoglobin, compromising blood oxygen carrying capacity (Gomez Isaza et al., 2020b). Gomez Isaza et al. (2020a) investigated whether nitrate exposure would increase susceptibility to elevated temperatures, measured as CT_{max}, and limit aerobic performance at warmer temperatures, measured as AS by swimming respirometry. In silver perch acclimated to 28 °C, exposure to either 50 or 100 mg l^{-1} nitrate caused a decline in CT_{max} that was not, however, observed in fish acclimated to a warmer temperature of 32 °C, despite these latter having higher overall CT_{max} (Table 2). Furthermore, when swum at three temperatures (28, 32 or 36 °C), nitrate-exposed fish with a T_{acc} of 28 °C showed a decline in AS (and swimming performance, see below) at 36 °C, whereas those with a T_{acc} of 32 °C displayed a thermally-insensitive phenotype, with no change in AS or performance across temperatures. It was subsequently demonstrated that the silver perch with a T_{acc} of 32 °C exhibit thermally-related plastic compensation of the cardiorespiratory system, including increased gill surface area and greater ventricular thickness (Gomez Isaza et al., 2021b). This demonstrates how the thermal physiology of fishes may have complex interactions with their relative sensitivity to contaminants (Gomez Isaza et al., 2020a, 2021b).

One major conclusion to be drawn from Table 2 is that the knowledge base for effects of contaminants on thermal tolerance in subtropical and tropical freshwater

448 fishes is extremely limited, with a majority of information for subtropical species. Given
449 that the mechanisms that cause LOE in CT_{max} are not known, it is not possible to
450 speculate about why some contaminants cause a decline in thermal tolerance while
451 others do not. Some patterns can, however, be demonstrated in the limited dataset, as
452 a basis for stimulating further research. When simply considering all CT_{max} data in
453 Table 2, there is a very clear and highly significant decline in TSM of control animals as
454 T_{acc} increases (Fig 3). This analysis makes no effort to correct for warming rate,
455 developmental stage (one species was studied as an embryo), but the results confirm
456 that is known about effects of T_{acc} on TSM in fishes (Comte and Olden, 2017a; b). The
457 question that then arises is whether exposure to contaminants will exacerbate this
458 effect, rendering tropical species even more vulnerable to warming events when they
459 are in contaminated habitats. For those species in Table 2 where a contaminant
460 caused a significant decline in CT_{max}, there was also a highly significant decline in TSM
461 with T_{acc} (Figure 3). The intercept and slope of the relationships did not, however, differ
462 significantly from those of control animals (Fig 3). Fig 4 shows, however, the
463 proportional extent (as a percentage) to which contaminants reduced TSM compared
464 to control conditions, as a function of T_{acc}. There was a significant effect whereby the
465 higher T_{acc}, the greater the extent to which a contaminant would compromise TSM (Fig
466 4). We show this figure precisely to highlight why it is unsatisfactory, namely that there
467 is a critical lack of information for effects of contaminants on TSMs of fishes at warm
468 tropical temperatures. There are major reasons for caution in interpreting this figure.
469 Firstly, the four points at 30 °C and above are from two species, red shiner and striped
470 catfish; the three points at 30°C all come the from the study on red shiner that
471 demonstrated how two pesticides and their cocktail caused much more profound
472 declines in CT_{max} in fish acclimated to 30 ° compared to 23 ° C by (Messaad et al.
473 2000). Indeed, at 30 °C the pesticide cocktail reduced the TSM by over 80% compared
474 to uncontaminated animals (Fig 4). Secondly, as mentioned above, warmer
475 temperatures did not increase susceptibility to nitrate in silver perch, because of plastic
476 physiological responses by this eurythermal subtropical species (Gomez Isaza et al.,
477 2020a, 2021b), so this data is not carried on the figure. Therefore, it is very important to
478 extend the knowledge base on how contaminants affect thermal tolerance of tropical
479 species, to investigate whether they are particularly vulnerable to impacts of heatwaves
480 when exposed to common pollutants. This is highly relevant to the life cycle of many
481 tropical species that, during dry seasons, may find themselves confined to increasingly
482 smaller water bodies, which will tend to concentrate any pollutants as water evaporates
483 and also to be submitted to extremes of warmth due to solar radiation (Patra et al.,
484 2007, 2015).

Critical swimming speed as a biomarker

Swimming performance has clear ecological relevance for many fish species, so measures of performance can provide valuable biomarkers of toxic effects of aquatic contaminants (Cairns, 1966; Beamish, 1978; Tudorache et al., 2008; McKenzie et al. 2007, 2008; Domenici, 2010). In particular, the critical swimming speed protocol (U_{crit}, Brett, 1964) was first proposed as a sublethal physiological biomarker more than 50 years ago (Cairns, 1966; Beamish, 1978). The U_{crit} protocol allows investigation of proximate mechanisms that underly impaired performance following exposure to contaminants, so gaining insight into mechanisms of toxic effects in target organs such as skeletal muscle (Beaumont et al., 1995, 2000; Tierney et al., 2007) or the heart (Khursigara et al., 2019).

The U_{crit} protocol exposes fish to stepwise increases in speed in a swimming respirometer, to establish the speed at which it fatigues. The knowledge base is dominated by work on temperate species and especially salmonids, while the Deepwater Horizon disaster led to extensive research on subtropical marine species. Studies have demonstrated impacts of many classes of contaminant under controlled laboratory conditions, including metals (e.g. Beaumont et al., 1995), xenobiotic organics (Wood et al., 1996; Tierney et al., 2007), crude oil itself (Stieglitz et al., 2016), waste products such as ammonia (Shingles et al., 2001), and other consequences of anthropogenic pressures, such as harmful algae (Corriere et al., 2020). Critical swimming speed has also been used to show impacts of exposure to water containing complex contaminant mixtures (e.g. Goertzen et al., 2012; Folkerts et al., 2020) including by exposing fishes in cages (McKenzie et al., 2007). The zebrafish is an important model vertebrate species for laboratory studies to demonstrate impacts of contaminants on swimming performance, to demonstrate toxic effects and their mechanisms (e.g. Thomas and Janz, 2011; Gerger et al., 2015; Lucas et al., 2016; Folkerts et al., 2017). Such studies on zebrafish have never, however, been related to conditions in their natural environment in tropical south Asia, so we will not review them here.

There are so few studies that, to our knowledge, have used U_{crit} as a biomarker of effects of contaminants on subtropical and tropical freshwater species (other than zebrafish), that it is almost trivial to review them. We do this, however, to stimulate further work. One necessity for such studies is, of course, access to a swim tunnels or swim flume. These are now commercially available, which opens up possibilities of using swimming performance as a valuable biomarker to support decision-making for water quality control in tropical countries. This is especially relevant for the protection of

economically valuable species that perform remarkable migrations through tropical river systems that are at increasing risk of contamination by multiple pollutants (Hogan et al., 2007; Duponchelle et al., 2016; Speranza et al., 2016). Other traits of performance such as the escape response (Tudorache et al., 2008; McKenzie et al., 2009) or aspects of swimming behavior (Little and Finger, 1990), which do not require a swim tunnel, can also be used as biomarkers.

Two studies have investigated effects of organic pollutants on U_{crit} in freshwater subtropical/tropical species. Perfluorooctane sulfonic acid (PFOS) is a fluorosurfactant with a wide range of commercial and industrial applications, which has become a pervasive and persistent pollutant of freshwaters globally (Augustsson et al., 2021). Xia et al. (2015) studied effects of a range of sublethal concentrations of PFOS on U_{crit} in qingbo *Spinibarbus sinensis*, a subtropical Asian species acclimated to two temperatures, 18 ° and 28 °C. Increasing concentrations of PFOS caused progressive declines in U_{crit}, while T_{acc} had no interaction with these impairments. Swimming respirometry showed that the decline in performance was linked to reduced swimming efficiency, in that higher rates of oxygen uptake were needed to achieve a given a swimming speed. The author suggested that this might be due to sensorimotor or muscle tissue impairments, due to toxic effects of PFOS. McKenzie et al. (2017) used U_{crit} to evaluate individual variation in sub-lethal sensitivity to the OP pesticide trichlorfon in a globally widespread tropical species, the Nile tilapia. At a T_{acc} of 27 °C, trichlorfon reduced U_{crit} significantly and fish only exhibited a partial recovery 96h after return to control freshwater. The decline in U_{crit} was due to reduced swimming efficiency, which was attributed to the fact that OPs are acetylcholinesterase inhibitors that interfere with cholinergic nerve function at motor endplates and in the central nervous system of vertebrates. This would impair neuromuscular coordination in fishes, reducing their swimming efficiency (Tierney et al., 2007; McKenzie et al., 2017). This study is a rare investigation of how individuals in populations differ in their sensitivity to a pollutant, finding that intrinsically efficient swimmers were the least affected by the OP (McKenzie et al., 2017).

Gomez Isaza et al. (2020a) investigated effects of nitrate, the most prevalent nutrient contaminant globally, on thermal sensivity of swimming performance in silver perch, acclimated to either 28 or 32 °C. Two sublethal exposure concentrations were evaluated at three temperatures (28, 32 and 36 °C), to reveal complex effects whereby fish acclimated to 28 °C had better overall U_{crit} performance than those acclimated to 32 °C. Exposure to nitrate, however, impaired U_{crit} of the 28 °C fish, in a manner proportional to nutrient concentration, but was without effect on performance of the 32 °C fish. This absence of effects of nitrate in fish acclimated to 32 °C was unexpected

but paralleled the findings for CT_{max} and aerobic performance that are reported above. In a study on the subtropical highly eurythermal spangled perch *Leiopotherapon unicolor*, Gomez Isaza et al. (2020b) investigated effects on U_{crit} of combinations of sublethal nitrate and low pH (acidification). The U_{crit} was impaired by both nitrate and acid pH, with most severe impairment being in fish at the highest nitrate concentration and low pH, although the combined effect was additive rather than synergistic. These impairments in performance were linked to a reduced capacity to raise oxygen uptake during swimming, leading to lower AAS. These proximate effects could, in turn, be attributed to measured negative effects of nitrate and low pH on blood oxygen carrying capacity (Gomez Isaza et al., 2020b). Such multifactorial studies provide excellent but challenging templates for future investigations of effects of contaminants on the environmental tolerance and physiological performance of freshwater tropical fishes.

Conclusions

This review demonstrates that O_{2crit}, CT_{max} and U_{crit} can all be useful physiological biomarkers of the toxicological effects of water contaminants on tropical and subtropical freshwater fishes. Understanding the interactive effects on fish physiology of sublethal exposures to contaminants, hypoxia and heatwaves will be crucial for monitoring the health of populations and projecting their biological consequences before serious changes in population size occur. This review very clearly demonstrates the need to extend the knowledge base to more species and contaminants, to better evaluate subtropical and tropical freshwater fish vulnerability in light of ongoing climate change. Complex physiological traits should be used to support decision-making in formulating water quality criteria for fishes and conservation strategies for their habitats, to protect their rich biodiversity. They may be particularly useful in procedures to identify adverse outcome pathways (van der Oost et al., 2020). Despite the technical challenges involved, future studies should consider interactions among pollutants, hypoxia and warming in tropical fishes, as the stresses all co-occur in nature.

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Conflict of Interest

The authors declare that there are no conflicts of interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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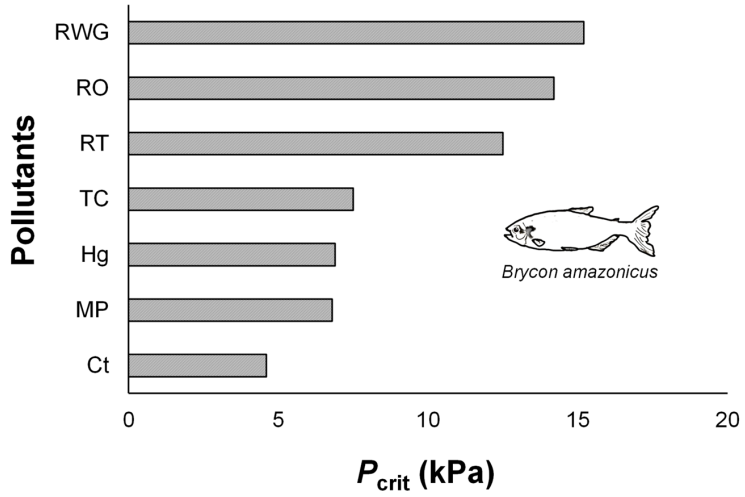
Figure Legends

Figure 1. Reduced tolerance of hypoxia, as measured by critical oxygen level (O_{2crit}), in matrinxã *Brycon amazonicus*, after exposure to various different pollutants. Ct = control condition, MP = methyl parathion, Hg = $HgCl_2$, TC = triclosan, RT = Roundup Transorb, RO = Roundup Original, RWG = Roundup WG. (Adapted from: Olle, 2007; Anelli Jr., 2010; Monteiro et al., 2013; Martins, 2018).

Figure 2. Influence of ambient oxygenation on oxygen uptake rate ($\dot{M}O_2$) of matrinxã *Brycon amazonicus*, after exposure to various different pollutants. Ct = control, MP = methyl parathion, Hg = $HgCl_2$, TC = triclosan, RT = Roundup Transorb, RO = Roundup Original, RWG = Roundup WG. Closed symbols denote a significant difference in relation to the normoxic values ($P < 0.05$). (Adapted from: Olle, 2007; Anelli Jr., 2010; Monteiro et al., 2013; Martins, 2018).

Figure 3. The relationship of acclimation temperature to thermal safety margin (TSM) in subtropical and tropical freshwater fishes, where TSM is the difference, in °C, between acclimation T and CT_{max}. Open symbols, control conditions for all data carried in Table 2. Grey symbols, conditions where contaminants caused a significant decline in CT_{max}. Based upon data from 14 studies on 12 species exposed to 15 contaminants (Table 2). Control data, dashed line, $R^2 = 0.828$, $P < 1 \times 10^{-8}$, exposed data, dotted line, $R^2 = 0.716$, $P < 1 \times 10^{-5}$. Slopes are not significantly different ($P = 0.129$, two-way T-Test).

Figure 4. Relationship between acclimation temperature (T_{acc}) and the extent, as a percentage of control conditions, to which contaminants decrease thermal safety margin (TSM) in sub-tropical and tropical freshwater fishes. Data are taken from Table 2, for all those situations where contamination caused a significant decline in CT_{max} ($n = 17$, from 11 studies of 11 contaminants on 10 species). Line describes a linear regression, dotted lines are 95% confidence intervals, $R^2 = 0.43$, $P = 0.0012$. This figure is based upon a very limited dataset and should, therefore, be interpreted with caution until the knowledge base is extended (see main text for details)


RWG
RO
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P_{crit} (kPa)
Brycon amazonicus

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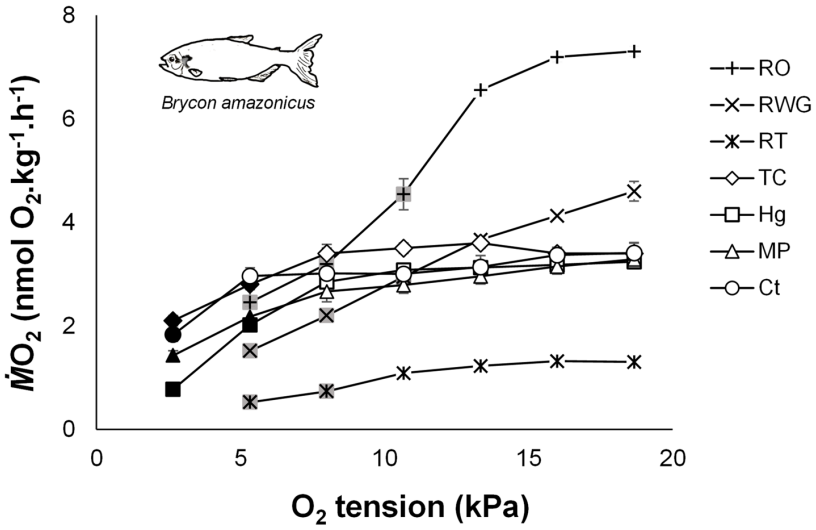
Brycon amazonicus
$\dot{M}O_2$ (nmol O_2.kg^{-1}.h^{-1})
O_2 tension (kPa)
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2
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RO
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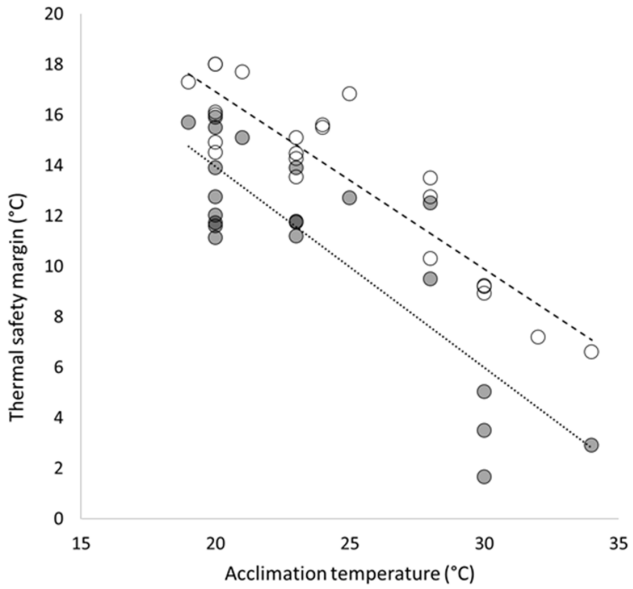
Thermal safety margin (°C)
Acclimation temperature (°C)
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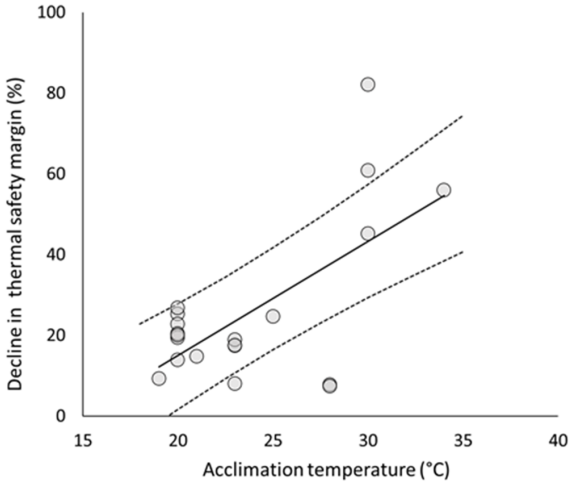
100
80
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0
Decline in thermal safety margin (%)
15
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25
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Acclimation temperature (°C)

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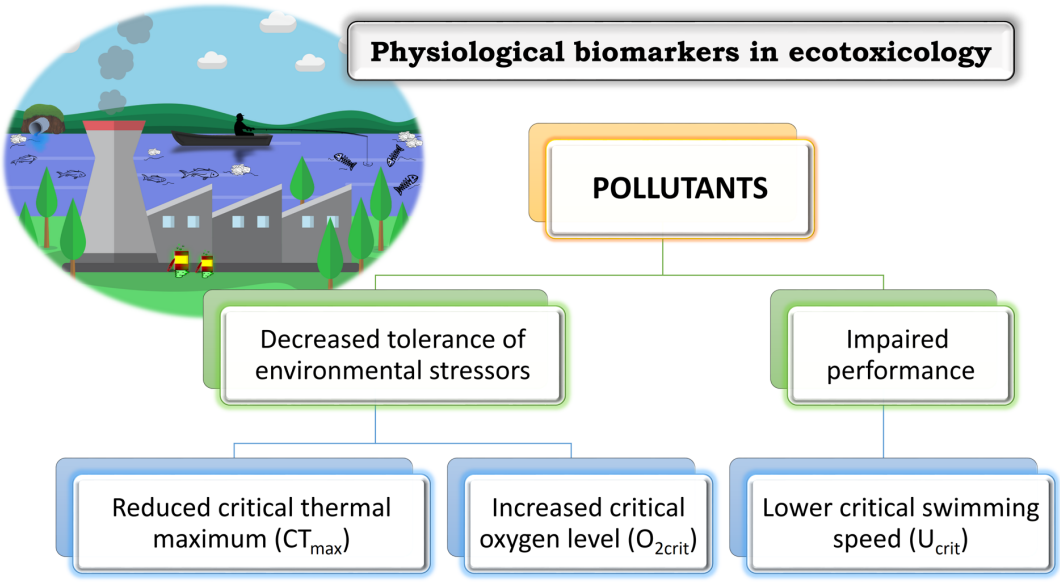
1143 Graphical Abstract.



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