



# Cardiac and neurobehavioral impairments in three phylogenetically distant aquatic model organisms exposed to environmentally relevant concentrations of boscalid<sup>☆</sup>

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## ABSTRACT

Boscalid (2-Chloro-N-(4'-chlorobiphenyl-2-yl) nicotinamide), a pyridine carboxamide fungicide, is an inhibitor of the complex II of the respiration chain in fungal mitochondria. As boscalid is only moderately toxic for aquatic organisms (LC<sub>50</sub> > 1–10 mg/L), current environmental levels of this compound in aquatic ecosystems, in the range of ng/L–µg/L, are considered safe for aquatic organisms. In this study, we have exposed zebrafish (*Danio rerio*), Japanese medaka (*Oryzias latipes*) and *Daphnia magna* to a range of concentrations of boscalid (1–1000 µg/L) for 24 h, and the effects on heart rate (HR), basal locomotor activity (BLA), visual motor response (VMR), startle response (SR), and habituation (HB) to a series of vibrational or light stimuli have been evaluated. Moreover, changes in the profile of the main neurotransmitters have been determined. Boscalid altered HR in a concentration-dependent manner, leading to a positive or negative chronotropic effect in fish and *D. magna*, respectively. While boscalid decreased BLA and increased VMR in *Daphnia*, these behaviors were not altered in fish. For SR and HB, the response was more species- and concentration-specific, with *Daphnia* exhibiting the highest sensitivity. At the neurotransmission level, boscalid exposure decreased the levels of L-aspartic acid in fish larvae and increased the levels of dopaminergic metabolites in *D. magna*. Our study demonstrates that exposure to environmental levels of boscalid alters cardiac activity, impairs ecologically relevant behaviors, and leads to changes in different neurotransmitter systems in phylogenetically distinct vertebrate and invertebrate models. Thus, the results presented emphasize the need to review the current regulation of this fungicide.

## 1. Introduction

Fungicides (Zubrod et al., 2019) have been used in agriculture to control fungal infections in plants, such as stem rusts caused by pathogenic basidiomycetes, since the 1960s. Over the years, broad-spectrum compounds have been developed, including fluopyram, penthiopyrad (Qian et al., 2019a) and boscalid (Wang et al., 2020). Nevertheless, some fungi with agricultural importance are out of reach of these new-generation fungicides, hence the need to combine different treatments to protect the crops and ensure the best production (Van den Bosch et al., 2011). French wine production receives a "cocktail" of more

than ten types of broad-spectrum pesticides (Knäbel et al., 2014), not counting specific treatments to combat occasional infections. Among these vineyard-specific products, the most widely used are undoubtedly fungicides (Lefrancq et al., 2014; Manjarres-López et al., 2021), the most common synthetic one being boscalid. Boscalid (2-Chloro-N-(4'-chlorobiphenyl-2-yl) nicotinamide) is a pyridine carboxamide fungicide inhibiting fungal mitochondrial complex II, the succinate dehydrogenase (SDH) (Bouillaud, 2023; Epa and of Pesticide Programs, 2003). Its toxicological mode of action (Yanicostas and Soussi-yanicostas, 2021) is the inhibition of mitochondrial respiration (Bénil et al., 2019a; Riepe et al., 1996) through binding to the ubiquinone reduction site of SDH

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(Coles and Singer, 1978), an enzyme involved in both the citric acid cycle and the electron transport chain. This enzyme, highly evolutionarily conserved, is expressed in many prokaryote cells and in the inner mitochondrial membrane of eukaryotes, playing a critical role in living organisms (Bénil et al., 2019a, 2014).

In recent years, there has been some evidence that SDH inhibitors may not be selective for fungi (Bénil et al., 2019a; Brenet et al., 2021; D'hose et al., 2021). Widely used fungicides, such as bixafen and boscalid, inhibit SDH activity in target fungi, but can also block this enzyme function in honeybee, earthworm, zebrafish, and *in vitro* human cell models, at mg/L ranges (Bénil et al., 2019a; D'hose et al., 2021; Qian et al., 2019b). Furthermore, pre-existent mitochondrial abnormalities, such as partial dysfunction of SDH in the organism, increased the susceptibility to suffer mitochondrial damage after sub-chronic exposure to SDH inhibitor (SDHI) fungicides (D'hose et al., 2021). Alterations in the neurological development and neurobehavioral, metabolic, and reproductive functions are some of the adverse outcomes reported in zebrafish models exposed to carboxamides (Li et al., 2020; Lin et al., 2021; Qian et al., 2021, 2020, 2019b, 2018; Yang et al., 2017; Yuan et al., n. d.). Genotoxic effects have also been observed in human cell cultures exposed to SDHI fungicides (Bénil et al., 2019b, 2019a; D'hose et al., 2021). Therefore, the lack of specificity of this class of fungicides is potentially dangerous for most exposed organisms.

The results of toxicological studies (Aubee and Lieu, n.d.) on aquatic species have led to the classification of boscalid (Cayman Chemicals, 2018; Epa and of Pesticide Programs, 2003) as moderately toxic to freshwater invertebrates (EC<sub>50</sub>: 5.33 mg/L; NOAEC: 0.79 mg/L) (Epa and of Pesticide Programs, 2003; INERIS, 2014) and vertebrates (LC<sub>50</sub>: 2.7 mg/L; NOAEC: 0.116 mg/L) (Epa and of Pesticide Programs, 2003; INERIS, 2014). Regarding the presence of this fungicide in surface waters, boscalid stands out from the other SDH inhibitors in its class, being found in 50–60% of the measurement analyses carried out each year on French territory (ANSES, 2019). Since boscalid is usually detected in the range of 0.003–36.0 µg/L (Reilly et al., 2012; INERIS, 2014; Zubrod et al., 2019; Manjarres-López et al., 2021) in European and North American surface waters, environmental levels are considered safe for aquatic organisms. However, these environmental detection levels are not necessarily informative of the highest concentrations that can be found in agricultural areas after the spraying campaigns of this pesticide, in concentration peaks due to a critical meteorological situation or to a human accident (Zubrod et al., 2019; Manjarres-López et al., 2021). On the other hand, recent studies have demonstrated that the presence of extremely low concentrations of mixture of different pesticides (Damásio et al., 2010) in the aquatic environment can result in unfavorable biological responses (Costa et al., 2008). For instance, we recently reported increased heart frequency and altered behavior in different freshwater species after a 24 h-exposure to environmental concentrations of carbaryl and fenitrothion, two broad-spectrum insecticides (Bedrossiantz et al., 2023; Faria et al., 2022; Faria et al., 2021b). Hormesis is a fundamental strategy of adaptation, conserved throughout the evolution by living organisms, characteristic of many biological processes (Calabrese, 2021; Calabrese and Blain, 2011). Since hormesis is related to homeostatic mechanisms that attempt to adapt the organism to mild stressors, there is a misconception that hormetic effects are always beneficial (Calabrese, 2011, 2009; Calabrese and Baldwin, 2010). Hormesis is characterized by an inverted U-shaped dose-response, with the curve corresponding to the beneficial response, whereas the arms of the "U" represent toxic concentrations (Calabrese, 2009). It is important to know the biological context to predict whether the effects are beneficial or detrimental to the organism (Calabrese, 2006; Calabrese and Baldwin, 2010).

The main objective of our study has been to determine the potential physiological and behavioral effects of the short-term exposure to environmental concentrations of boscalid in different model organisms commonly used in aquatic toxicology considering potential hormetic effects. With this aim, we performed a comparative study between three

model organisms, two vertebrates [zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) larvae] and one invertebrate (*Daphnia magna*). Fish larvae and water fleas were exposed for 24h to a broad range of environmentally relevant concentrations of boscalid in the µg/L range. First, effects on heart rate (HR), basal locomotor activity (BLA), visual motor response (VMR), vibrationally evoked startle response (SR) and its habituation were determined. Secondly, the effects on the neurotransmitter profile of each organism were analyzed. The results obtained in these three phylogenetically distant species suggest that the presence of the fungicide in the environment could be detrimental to a wide range of living beings. The overall responses to boscalid in fish larvae were quite similar, although with differences in sensitivity with the species. The data presented demonstrate the importance to studying several models to make accurate predictions for risk assessment. In conclusions, we firmly believe that the regulation and uses of this compound must be reviewed.

## 2. Material and methods

### 2.1. Experimental organisms and culture conditions

Adult zebrafish were supplied by Pisciber (Terrassa, Spain). Embryos, obtained by natural mating of adults, were grown in fish water (FW) under standard conditions at the Research and Development Centre of the Spanish Research Council (CID-CSIC) facilities (License n° 11,336).

We received embryos of Japanese medaka larvae (CAB line) at early gastrula stage, 24 h post-fertilization (hpF), from Gis-Amagen (INRA, Jouy-en-Josas, France). All the experiments conducted with medaka species were performed at the EPOC laboratory (University of Bordeaux, France) under the authorization number APAFIS#28934.

Zebrafish and medaka larvae were grown in fish water (FW) until the beginning of experiments, (7 days post-fertilization (dpf) and 9 dpf for zebrafish and medaka larvae, respectively) as previously described (Bedrossiantz et al., 2023; Le Bihanic et al., 2014). Detailed information on growing conditions can be found in the Supplementary Methods.

Several larger parthenogenic cultures of 100 individuals *Daphnia magna* with third- to sixth-brood neonates (<24 h old) were started from bulk cultures and maintained in 1.5 L hard synthetic water (ASTM) plus algae ( $5 \times 10^5$  cells/mL of *Chlorella vulgaris*, renewed three times a week), in a thermostatic chamber, at  $20 \pm 1$  °C with 16L:8D photoperiod cycle, for 7 days until use (Bedrossiantz et al., 2021, 2020).

### 2.2. Determination of real concentrations

Boscalid (CAS, 188425-85-6, purity  $\geq$  98%, Tokyo Chemical Industry ©TCI Europe N.V) was provided by ©CymitQuímica, Barcelona. Acetonitrile (ACN) LC-MS grade was purchased from VWR Chemicals Prolabo (Leuven, Belgium) and Formic acid (FA) by Fischer Scientific (Loughborough, UK), while Sigma-Aldrich (St. Louis, MO, United States) supplied ammonium formate. Ultra-pure water was daily obtained through Millipore Milli-Q purification system (Millipore, Bedford, MA, USA).

As the stability of boscalid under similar experimental conditions has been demonstrated several times (Aksakal, 2020; Qian et al., 2020, 2019b; 2018), using reliable methodology (Faria et al., 2022; M. Faria et al., 2021b; Qian et al., 2021), we concentrated the analytical work on the determination of actual boscalid concentrations in FW and ASTM using the ultrahigh-performance liquid chromatography coupled to a triple quadrupole detector (UPLC–MS/MS) analysis.

### 2.3. Boscalid exposures

Four boscalid concentrations have been evaluated in fish larvae and *D. magna*, 1 µg/L, 10 µg/L, 100 µg/L, and 1000 µg/L. The selected concentrations cover the range of levels reported in aquatic ecosystems

(1 and 10 µg/L) (INERIS, 2014; Reilly et al., 2012; Zubrod et al., 2019), the accepted NOAEC for aquatic organisms (100 µg/L) (Epa and of Pesticide Programs, 2003; INERIS, 2014), and a worst-scenery concentration (1000 µg/L; potentially after an accidental event or during the treatment period of vineyard crops). Stock concentrations of boscalid were prepared in DMSO, with final solvent concentration of 0.1% across all conditions, control, and treatment groups. The use of solvent controls with 0.1% DMSO has been reported to be safe and is widely used to screen libraries of small chemicals in aquatic species (Maes et al., 2012; Vliet et al., 2017). None of the selected concentrations resulted in systemic toxicity (impaired gross morphology or induced lethality) in the three species.

Exposures of fish models were conducted in multi-well microplates, using 48-well plates for behavioral assays (one larva per well in 1 mL of working solution), and 6-well plates for cardiac activity assessments (20 larvae per well in 10 mL of working solution). Fish larvae were maintained overnight in the climatic chamber in optimal conditions before starting the experiments. The following day we proceeded directly to behavioral and cardiac activity assays in fish species (zebrafish larvae from 7 to 8 dpf and medaka larvae from 9 to 10 dpf).

*Daphnia* were not fed during the 24h-exposure in 120 mL borosilicate glass bottles (25–50 individuals per 100 mL of working solution). For behavior studies (Bedrossiantz et al., 2020), *Daphnia* 7–8 to 8–9 days old juveniles were first placed individually in 24-well plates with 1 mL of working solution. We repeated 2–3 exposures with different batches of animals to evaluate the biological responses of our model organisms considering inter-batch variability.

#### 2.4. Cardiac activity analysis

Cardiac activity analyses were conducted in an isolated thermo-regulated behavioral room, under optimal temperature conditions, i.e., adapted to each organism (20 or 28 °C for daphnias and fish larvae, respectively). To obtain quality videos of cardiac movement, we had to briefly anesthetize the fish larvae with MS222 (tricaine, 170 mg/L), a procedure that was not necessary with *Daphnia*, to properly place them in a drop of 4% methylcellulose in lateral or ventral view, before recording as we have formerly reported (Bedrossiantz et al., 2023; Faria et al., 2022). The procedure is fully described in the Supplementary Methods.

#### 2.5. Behavioral analysis

Behavioral assessments were conducted using a high throughput tracking system to obtain easily comparable results between species (Faria et al., 2019b; Ford et al., 2021; Green et al., 2012; Kane et al., 2005). For this, we performed all the behavior tests using a DanioVision system, which has a Temperature Control Unit (TCU) that maintains the DanioVision Observation Chamber (DVOC) at 28 °C for zebrafish and medaka or 20 °C for *daphnia*. The EthoVision XT 14 software (Noldus, Wageningen, The Netherlands) allows us to control both the action of the near-infrared camera and the stimuli sent to the plate in a fully automated way. The trajectory of each animal within a holder that allowed complete freedom of movement (*fish larvae*: 48-well plates, *daphnia*: 24-well plates) was recorded at 30 frames per second (fps) and subsequently analyzed using the multiple tracking module of the EthoVision software.

The behavioral battery that we applied in our behavioral studies (Bedrossiantz et al., 2023) looks similar for vertebrate and invertebrate models. In fact, this protocol was developed from the Vibrational Startle Response Assay (VSRA) designed for zebrafish larvae (Faria et al., 2022, 2020; 2019a, 2019b; Faria et al., 2021a,b) and the *Daphnia* Photomotor Response Assay (DPRA) (Bedrossiantz et al., 2020; Bellot et al., 2021b).

The behavior routine begins with an acclimation time in the dark (*fish larvae*: 20 min, *Daphnia*: 5 min) followed by a series of repeated stimuli at very short intervals (*fish larvae*: tapping stimuli [intensity: 8;

repetitions: 51; interstimulus interval (ISI): 1 s], *Daphnia*: 1 s light flashes [intensity: 290 lux; repetitions: 30; ISI: 4 s]. A 10 min recovery in dark allows the animals to recover sensitivity before moving on to the light/dark cycling phase (*fish larvae*: light/dark cycle [intensity: 100-0%; duration: 10 + 10 min], *Daphnia*: long light signal [intensity: 50-0%; duration: 5 + 1 min]).

*D. magna* and fish larvae are sensitive to unexpected changes in their environment; this is an innate survival response. In the first part of the behavioral routine, we can evaluate two aspects of the escape response: (1) startle, corresponding to the innate escape response, and (2) habituation, corresponding to the adaptation to the stimulus identified as irrelevant.

The startle response (SR), evoked in fish larvae by the vibrational stimulus and in *Daphnia* by the light-flash stimulus, is assessed by measuring the distance moved (mm) after the stimulus (*fish larvae*: maximum over the 1 s stimulus, *daphnia*: maximum over the 5 s stimulus) and its habituation is calculated as the area under the response curve (AUC). The second part of the behavioral routine assesses the basal locomotor activity (BLA) of the animals, defined as the distance traveled (mm) by the larvae or *daphnia* during a 10-min period without stimulation, and the visual motor response (VMR), which corresponds to the period of hyperactivity in reaction to a sudden change of light in the environment. VMR is calculated in fish as the distance moved during the first 2 min of the second dark period minus the distance moved during the last 2 min of the light period, whereas in *Daphnia* is calculated as the distance moved during the light period (5 min) minus the distance moved during the first dark period (5 min).

#### 2.6. Neurotransmitter analysis

In this study, we chose to analyze a list of 16 neurotransmitters including: L-aspartic acid (Asp), acetylcholine chloride (ACh), L-tryptophan (Trp), 5-hydroxy-L-tryptophan (5-HTP), serotonin hydrochloride (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), L-tyrosine (Tyr), L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine hydrochloride (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), (–) epinephrine (Eppy), L-glutamic acid (Glu),  $\gamma$ -aminobutyric acid (GABA) and histamine (HSM) by liquid chromatography coupled to a triple quadrupole detector (LC-MS/MS) (Xevo TQD, Waters, USA), as previously described (Bellot et al., 2021b, 2021a; Gómez-Canela et al., 2019). Reference standards for these neurotransmitters and the corresponding labeled internal standards were supplied by Sigma-Aldrich (Steinheim, Germany) and Toronto Research Chemicals (TRC, Toronto, Canada). The protocols for analysis, quantification and quality evaluation of neurotransmitters are fully described in the Supplementary Methods.

#### 2.7. Risk assessment

The risk of chemicals is commonly estimated using the Risk Quotient (RQ) in Health and Ecological Risk Assessments.

$$RQ = \text{Exposure level/No Effect Level.}$$

Exposure level refers to predicted or measured environmental concentration (PEC, MEC).

No effect level refers to predicted no-effect concentration (PNEC), usually estimated from the most sensitive no-observed effect concentration (NOEC) across endpoints and species, divided by an assessment factor going from 1 to 1000 (European Chemical Bureau, 2003).

Whereas compounds with  $RQ > 1$  are potentially at risk and those with  $RQ \leq 1$  values are considered as slightly or negligible (Epa and Opp, 2007).

#### 2.8. Statistical analysis

Data of the three species were processed using IBM® SPSS® Statistics (Version 28.0.1.0, Chicago, IL). First, each replicate was normalized by

its respective control. Thus, we could pool the data corresponding to 2–3 independent experiments. Data was tested for normality with the Shapiro-Wilk test and variance the homoscedasticity with the Levene's test. When data met normality and variance homoscedasticity treatments were compared by, one-way ANOVA followed by Dunnett's multiple comparison test, otherwise non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test (Zar, 1996), were used. Significance was set at  $p < 0.05$ . For each experimental condition, the mean values, presented as percentage of the controls  $\pm$  standard error (SE) or median and interquartile range, were plotted using GraphPad Prism 9 for Windows (GraphPad Software Inc, La Jolla, CA).

Multivariate Principal Component Analysis (PCA) was used to explore the relationships between the aquatic species studied, exposure conditions and their biological responses. Since the variables were very different and were not measured using the same scale units, normalized data were used, expressed as a percentage of their respective control.

### 3. Results

#### 3.1. Actual concentrations of boscalid in fish and daphnia waters

Supplementary Table S2 summarizes the nominal concentrations (1, 10, 100, and 1000  $\mu\text{g/L}$ ), actual concentrations in ASTM and FW and the percentage of decrease of boscalid in the experimental solutions over 24 h. By staying in the expected order of values, we decided to express the results using nominal boscalid concentrations, for additional information, see Supplementary Material.

#### 3.2. Zebrafish, medaka larvae, and *Daphnia magna* showed changes in heart rate after 24 h exposure to environmental concentrations of boscalid

The heart rate in control zebrafish and medaka larvae was similar,  $148.81 \pm 16.97$  beats per minute (bpm) ( $n = 30$ ) and  $142.14 \pm 12.51$  bpm ( $n = 50$ ) respectively, and about 2.5 times lower than the corresponding rate in *D. magna*,  $391.61 \pm 8.01$  bpm ( $n = 18$ ). Given the large difference between the basal heart rate of the vertebrate models with *Daphnia*, we have plotted the effects of boscalid on relative heart rate (Fig. S1; raw data are available in Supplementary Material). A chronotropic effect of boscalid on heart rate was observed after 24h-exposure, with differences in the direction and intensity of the response depending on concentration and model organism. Exposure to 10–1000  $\mu\text{g/L}$  boscalid led to a positive chronotropic effect on heart rate in both fish models [zebrafish:  $F(4, 86) = 15.609, P = 1.23\text{E-}09$ ; medaka:  $F(4, 107) = 67.623, P = 2.00\text{E-}28$ ], with 10–23% and 9–11% increase in the heart rate of zebrafish and medaka larvae, respectively. In contrast, *D. magna* exposed to 1000  $\mu\text{g/L}$  boscalid exhibited a significant decrease in the heart rate, with values approximately 5% lower than controls [ $F(5, 93) = 55.590, P = 1.87\text{E-}26$ ]. Curiously, a similar 2% significant decrease was found with the lowest boscalid concentration in medaka larvae, as can be seen in Fig. 1 and more especially in the heatmap (Supplementary Fig. 1), with the help of p-value coloring.

#### 3.3. Zebrafish, medaka larvae and *Daphnia magna* exhibited altered behaviors after only 24h of exposure to environmental concentrations of boscalid

Figs. 2–4 show the effect of boscalid on different behaviors in zebrafish, medaka, and *D. magna*, respectively. In addition, Supplementary Figs. S2–4 show the distance traveled (mm) over time and/or in response to perceived stimuli, which helps to understand how each model reacts to the test and how the exposure conditions influence this basal response.

The results of startle response (SR) assay in fish species (Figs. S2A–S3A) show that exposure to 10  $\mu\text{g/L}$  boscalid leads to different responses in medaka and zebrafish larvae, with a decrease in the escape response elicited by a vibrational stimulus in the former [ $F(4,$

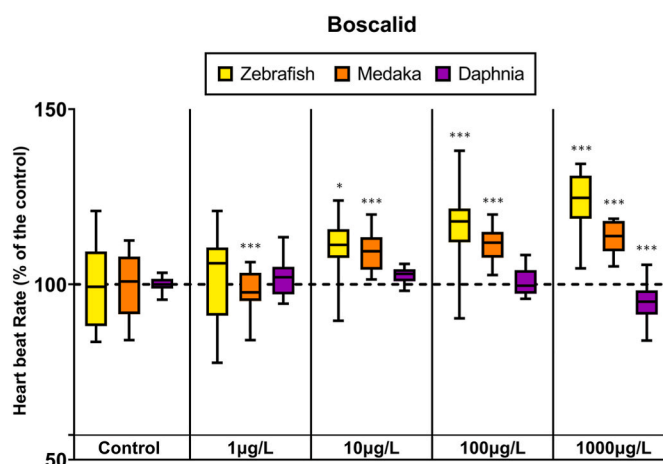


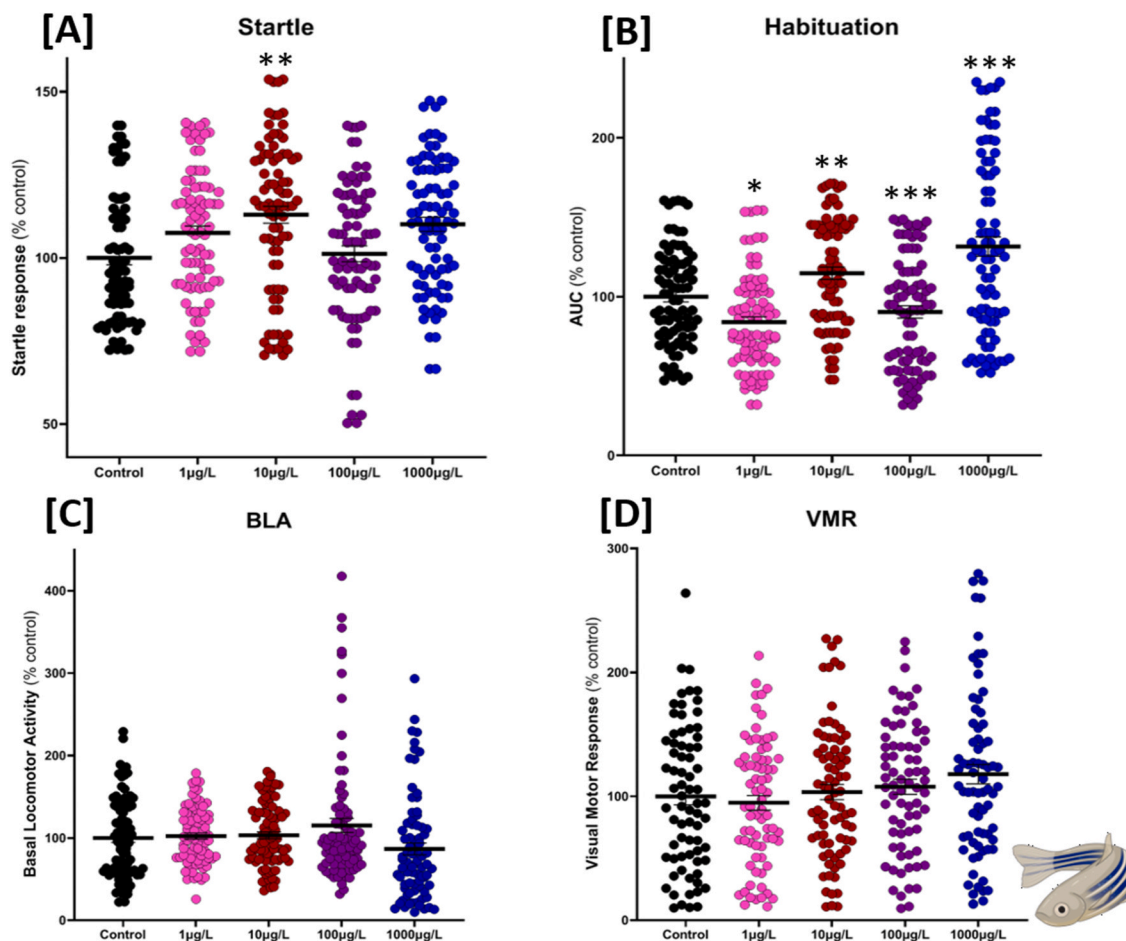
Fig. 1. Effect of a 24 h-exposure to environmental concentrations of boscalid (1mg/L-1000 mg/L) on the heart rate in zebrafish larvae, Japanese medaka larvae and *D. magna*. Boxplot representation, with the box indicating the 25th and 75th percentiles, the whiskers indicating the maximum and minimum values, and the thin line within the box indicating the median (zebrafish:  $n = 15-30$ ; Japanese medaka:  $n = 24-50$ ; *D. magna*:  $n = 13-21$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; one-way ANOVA with Dunnett's multiple comparison test. Data from 2 to 3 independent experiments.

273) = 2.077,  $P = 0.084$ ], and an increase in this response in the latter [ $H(4) = 11.559, P = 0.021$ ]. In *Daphnia* (Fig. S4A), exposure to 1000  $\mu\text{g/L}$  boscalid resulted in a strong decrease of SR [ $F(5, 108) = 11.011, P = 1.39\text{E-}08$ ].

Habituation, a non-associative learning process necessary to filter out irrelevant stimuli, can be evaluated by determining the area under the curve of the distance moved in response to a series of vibrational or visual stimuli. Boscalid affected the habituation of zebrafish larvae in a non-monotonic concentration manner (Fig. 2, Supplementary Fig. S2 [A, B]) with a 16% decrease in the habituation time at the lowest concentration and a 32% increase at the highest concentration [ $H(4) = 29.292, P = 6.82\text{E-}06$ ]. Medaka larvae showed no effect of boscalid on habituation (Fig. 3A, Supplementary Fig. S3 [A, B]). Hong and Zha (2019) also reported the limited or no response of medaka fish to repetitive light-dark or acoustic stimulations, suggesting that this behavioral metric is poorly suitable for its use in assessing behavioral impairments in medaka. *D. magna* was clearly the most sensitive organism to the effect of boscalid on habituation (Fig. 4A, Supplementary Fig. S4 [A, B]), exhibiting a significant increase in the time required to filter the series of visual stimuli [ $F(5, 110) = 7.265, P = 6.50\text{E-}06$ ]. The response of habituation to boscalid in *D. magna* followed an inverted-U pattern, reaching its maximum after 24 h of exposure to 100  $\mu\text{g/L}$  of boscalid, taking up to 30% more time to filter the visual stimulus as irrelevant.

Boscalid exposure did not lead to any significant effect on BLA or VMR in the two fish species. In *Daphnia*, however, 24 h exposure to 10–100  $\mu\text{g/L}$  boscalid resulted in a 20% decrease in BLA, whereas exposure to 10  $\mu\text{g/L}$  resulted in a 38% increase in VMR [Fig. S4 [C, D];  $F(5, 110) = 5,684, P = 1.06\text{E-}04$  for BLA,  $F(5, 100) = 3,474, P = 0.006$  for VMR]. In addition, we were able to identify that the increase of the VMR in *Daphnia* exposed to boscalid is related to the increase of motor activity in the light phase that followed the dark one [Supplementary Fig. S4 [C, D];  $F(5, 98) = 7.989, P = 2.38\text{E-}06$  for distance in light,  $F(5, 98) = 8.169, P = 1.77\text{E-}06$  for distance in dark].

To make easy the comparison, the biological effects of boscalid on the three model organisms were plotted on a heat map (Supplementary Fig. 1). The gradient of the heat map varies according to the level of significance, the p-values used for this representation are shown in Supplementary Table S3. The heat map not only summarizes, but also highlights similarities or differences in the response of each species, which will be addressed in the discussion section.



**Fig. 2.** Behavioral changes observed in zebrafish larvae after 24 h of exposure to environmental concentrations of boscalid (1mg/L-1000 mg/L). Data reported as scatter plot with the median  $\pm$  SE for [A] Startle Response (SR), [B] habituation (calculated as the area under the response curve (AUC)), [C] Basal Locomotor Activity (BLA) and [D] Visual Motor Response (VMR). Results were normalized as percentage of their respective controls [n = 80–84 for SR, n = 82–84 for habituation; n = 78–84 for BLA, and n = 72–78 for VMR], \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 one-way ANOVA with Dunnett's multiple comparison test or Kruskal Wallis test with Bonferroni correction. Data from 2 to 3 independent experiments.

### 3.4. Neurotransmitter expression profiles of zebrafish, medaka larvae and *Daphnia magna* are changed after 24h of exposure to environmental concentrations of boscalid

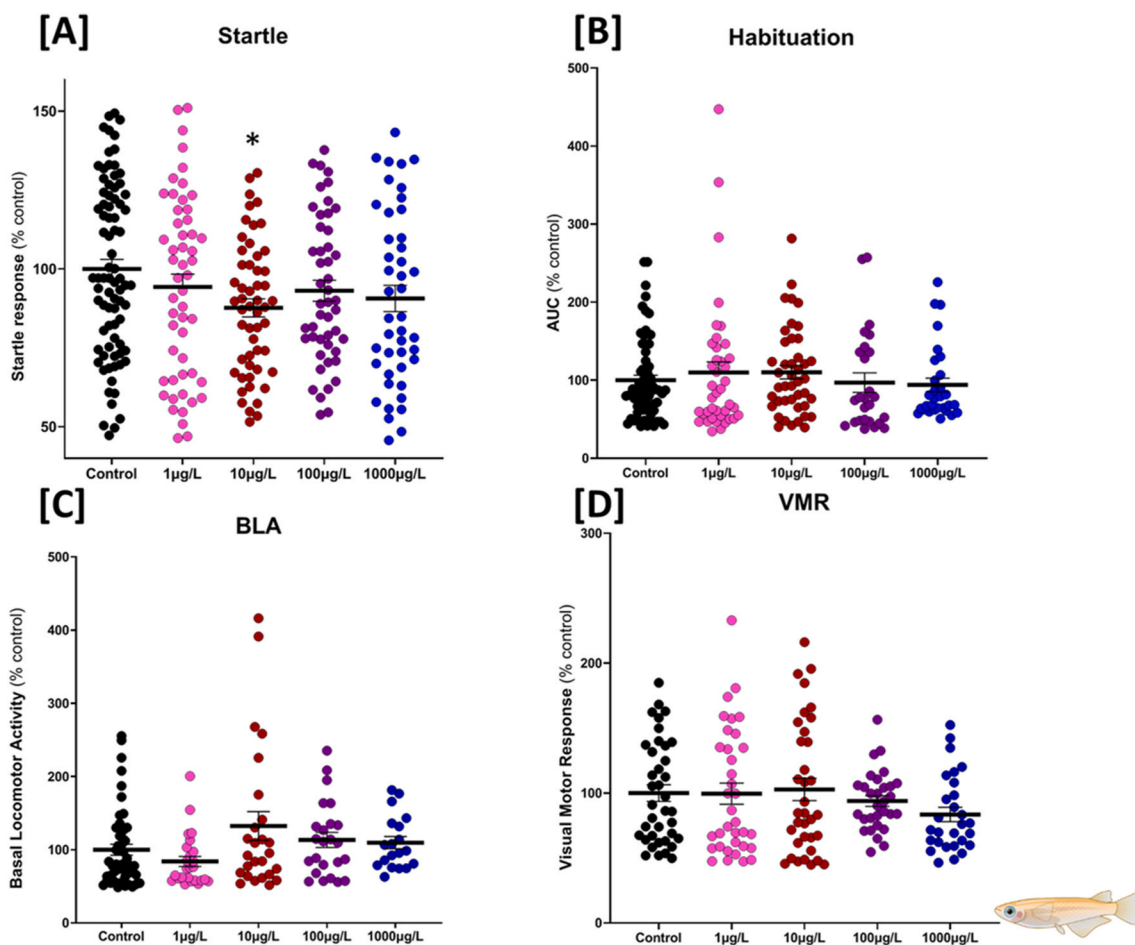
From the analytical method initially developed for 20 neurotransmitters, precursors, and degradation products, we focused the study on 15 of them for which we obtained satisfactory quality control results. The recovery values of the quality controls, calculated as the average of 3 replicates for each neurotransmitter, are reported in [Supplementary Table S4](#). Recoveries in pools of five zebrafish larvae ranged from 76% to 130.9%, from 73.6 to 157.0% for pools of five medaka larvae and between 57.7 and 133.4% for pools of four daphnias. L-aspartic acid (Asp) was not recovered in *D. magna*, and L-glutamic acid (Glu) was only recovered in *D. rerio*.

[Supplementary Figs. 5–7](#) show the neurotransmitter profiles of zebrafish larvae, medaka and *Daphnia* after 24 h exposure to 1–1000  $\mu$ g/L boscalid. We found a 50% decrease in the concentrations of the excitatory amino acid Asp, and approximately 20% decrease in the serotonin precursor Trp in zebrafish larvae exposed to the highest concentration of boscalid [ $F(4, 24) = 10,346, P = 1.04E-04$  for Asp and  $H(4) = 9,304, P = 0.054$  for Trp]. A 30% decrease in Asp levels was also observed in medaka larvae exposed to the two highest concentrations of boscalid [ $F(4, 20) = 10,346, P = 1.98E-04$  for Asp]. On the other hand, the increasing trend observed for the serotonin degradation metabolite, 5-HIAA, in zebrafish larvae, was confirmed in medaka, specifically for

the highest concentration of boscalid [ $F(4, 22) = 5.647, P = 0.003$  for 5-HIAA]. In fact, we observed that the 5-HIAA/5-HT ratio doubled in medaka larvae exposed to 1000  $\mu$ g/L boscalid [[Supplementary Table S5](#); 5-HIAA/5-HT = 2.113,  $P = 0.018$ ], although we did not observe any decrease in 5-HT levels. For the rest of the metabolites analyzed in larvae exposed to boscalid, we could only appreciate trends of changes, such as an increase in 5-HIAA levels and a decrease in ACh, Trp, and HVA levels in both fish species.

In contrast, in the invertebrate model, we found significant changes in the levels of metabolites from the dopaminergic, GABAergic, and histaminergic systems. After exposure of *Daphnia* to boscalid, we observed a strong increase in the precursors of dopamine [ $F(4, 19) = 13.841, p = 1.90E-05$  for Tyr and  $H(4) = 14.838, P = 0.005$  for L-DOPA] and degradation product [ $F(4, 21) = 9.304, p = 1.73E-04$  for HVA], with values between 1.7 and 2.2 times higher than those of the control, at all concentrations tested, while the DA's own levels are maintained. Therefore, the HVA/DA ratio was almost doubled for daphnias exposed to boscalid [[Supplementary Table S5](#); HVA/DA = 2.339,  $P = 0.009$ , for 10  $\mu$ g/L and HVA/DA = 1.883,  $P = 0.066$ , for 100  $\mu$ g/L]. A more moderate increase in GABA [ $F(4, 22) = 3.489, P = 0.024$  for GABA] and histamine [ $F(4, 22) = 4.389, P = 0.009$  for HSM] levels was found in daphnia exposed to 1–10  $\mu$ g/L boscalid.

To highlight the effects of boscalid on the different neurotransmitter systems in the three model organisms, they were plotted on a heatmap ([Fig. 5](#)). As in the previous one, the gradient of this second heat map



**Fig. 3.** Behavioral changes observed in Japanese medaka larvae after 24 h of exposure to environmental concentrations of boscalid (1mg/L-1000 mg/L). Data reported as scatter plot with the median  $\pm$  SE for [A] Startle Response (SR), [B] habituation (calculated as the area under the response curve (AUC)), [C] Basal Locomotor Activity (BLA) and [D] Visual Motor Response (VMR). Results were normalized as percentage of their respective controls [n = 44–81 for SR, n = 26–67 for habituation; n = 19–49 for BLA, and n = 28–38 for VMR], \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  one-way ANOVA with Dunnett's multiple comparison test or Kruskal Wallis test with Bonferroni correction. Data from 2 to 3 independent experiments.

varies according to the level of significance. The p-values used for the representation are shown in [Supplementary Table S6](#).

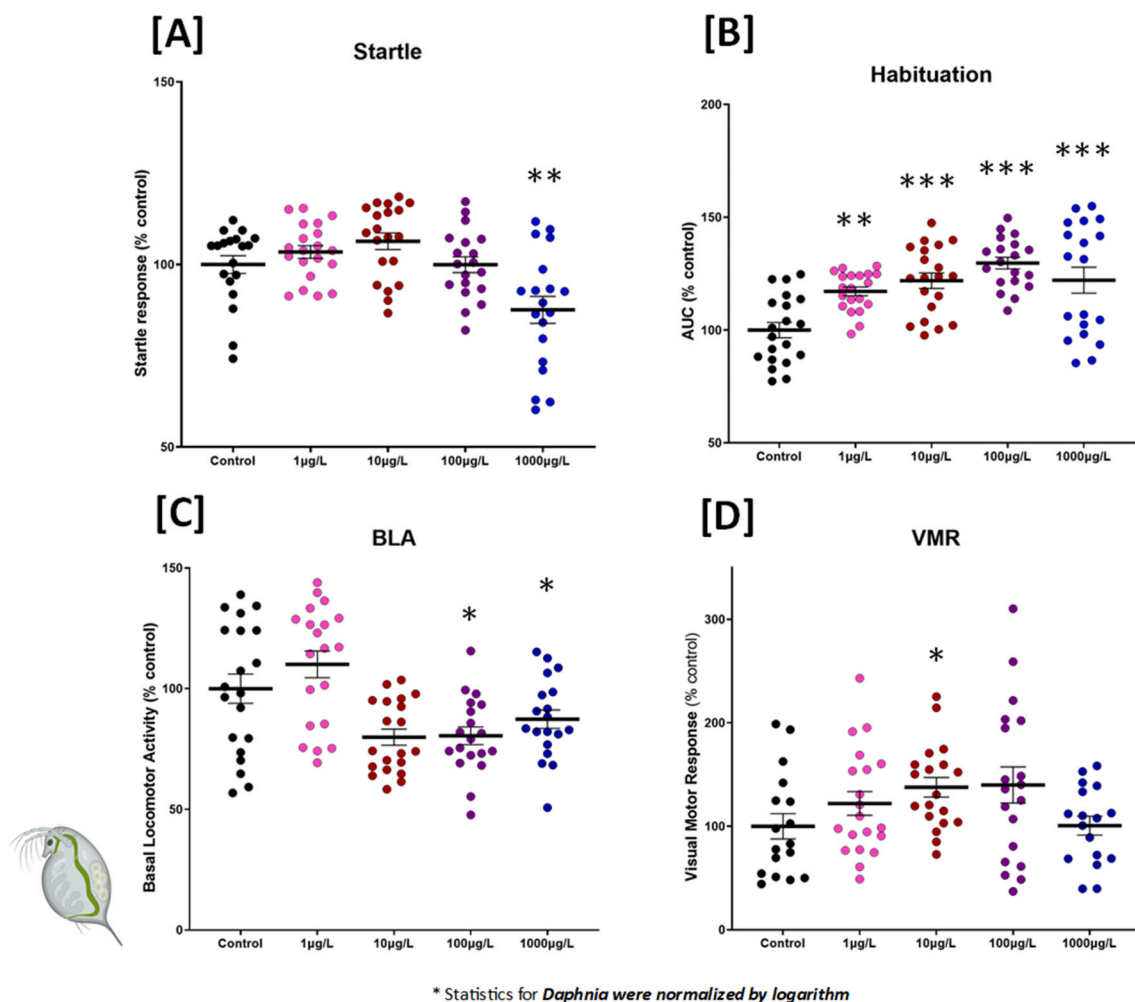
### 3.5. Revisiting the risk assessment for boscalid

The finding that environmental concentrations of boscalid can alter cardiac activity, essential behaviors, and neurotransmission in zebrafish and medaka larvae and *D. magna* strongly suggests that the environmental risk of this fungicide (Environmental Quality Standard (EQS) for Boscalid: 11.6  $\mu\text{g/L}$  in surface fresh water) should be reviewed (Carvalho et al., 2016). The predicted no-effect concentration (PNEC) of boscalid in aquatic organisms, invertebrates, and vertebrates, can be determined using the no observed effect concentration (NOEC) on the most sensitive behavioral or physiological endpoint, across the three studied species. The most sensitive endpoint for medaka was the cardiac activity at the lowest concentration tested of 1  $\mu\text{g/L}$  boscalid. For zebrafish and *D. magna*, the most sensitive endpoint was the habituation at the lowest concentration tested of 1  $\mu\text{g/L}$  boscalid. Accordingly, we could set up a tentative NOEC at 0.5  $\mu\text{g/L}$  boscalid (lowest concentration where an effect of less than 20% effect has been observed, LOEC/2). Since we used three different species from two different tropic levels, we applied an assessment factor of 50 to our estimated NOEC (European Chemical Bureau, 2003; Syberg and Hansen, 2016). Therefore, the environmental risk will be high for organisms populating the aquatic ecosystems with boscalid levels above the PNEC of 0.01  $\mu\text{g/L}$ . Based on boscalid levels

measured in European and North American surface waters (INERIS, 2014; Reilly et al., 2012; Zubrod et al., 2019), the risk quotient (RQ) values are between [0.3–3600], considering that more than 99.9% of the concentrations belonging to this range present risk quotient higher than 1,  $\text{RQ} > 1$ .

### 3.6. Possible relationships between the biological responses of zebrafish, medaka larvae and *Daphnia magna* exposed to environmental concentrations of boscalid

To better understand the main factors explaining the biological effects reported in this manuscript, a multivariate test was performed with the loads of the multiple variables, namely behavior endpoints, cardiac activity, neurotransmitters, and the sample scores, represented in a biplot. As we can see on [Fig. 6](#), PCA component 1 could explain 49% of the variability while PCA component 2 explained 13% of it. In total, more than 62% of the variability of the data was explained by the first two PCA components. The bi-pot of the variable loadings and sample scores of the first two PCA components clearly separated the three species irrespectively of the concentrations tested. *D. magna* samples exposed to boscalid were associated with high levels of VMR, decreased habituation (High loadings of HB) and increased concentrations of Tyr, L-DOPA, HVA, GABA, Ach and Trp. Alternatively Medaka samples were negatively related to most of the above-mentioned variables and positively related with the levels of 5-HIAA. Zebrafish samples were



**Fig. 4.** Behavioral changes observed in *D. magna* after 24 h of exposure to environmental concentrations of boscalid (1mg/L-1000 mg/L). Data reported as scatter plot with the median  $\pm$  SE for [A] Startle Response (SR), [B] habituation (calculated as the area under the response curve (AUC)), [C] Basal Locomotor Activity (BLA) and [D] Visual Motor Response (VMR). Results were normalized as percentage of their respective controls [n = 17–20 for SR, n = 18–20 for habituation; n = 18–20 for BLA, and n = 14–20 for VMR], \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  one-way ANOVA with Dunnett's multiple comparison test or Kruskal Wallis test with Bonferroni correction. Data from 2 to 3 independent experiments.

associated with intermediate levels of most variables and with high heartbeats loadings.

#### 4. Discussion

The mode of action (MoA) of boscalid as a fungicide is inhibition of fungal SDH (Brenet et al., 2021; Yang et al., 2020), a key enzyme in cellular energy metabolism (Bouillaud, 2023; Mowery et al., 1976). Several recent studies have provided evidence of the low specificity of the mode of action of SDH inhibitors (Bénit et al., 2019a; Brenet et al., 2021; D'hose et al., 2021). A large part of the SDH sequence has been evolutionarily conserved (Bénit et al., 2019a), which would explain why boscalid, among other fungicides of this class, not only inhibits the function of the fungal enzyme but also of this same enzyme in other species (D'hose et al., 2021). During SDH inhibition, ubiquinone is not reduced and consequently, cannot reach the second enzyme complex of the respiratory chain. The second part of the exergonic reaction that catalyzes SDH, the oxidation of succinate into fumarate and reduction of flavin adenine dinucleotide (FAD), also cannot occur if the enzyme is inhibited, which has a direct consequence on the functioning of the tricarboxylic acid cycle (TCA cycle). Therefore, the toxic effect of boscalid is mainly due to the alteration of essential energy processes and the induction of cellular hypoxia in fungi (Coles and Singer, 1978;

Mowery et al., 1976). In mammals, it has been shown that SDH can also perform other reactions, for example, forming enol-oxaloacetate from the malate molecule (Panchenko and Vinogradov, 1991). Oxaloacetate is an important metabolic intermediate involved in multiple pathways apart from the TCA cycle, such as in the oxidation and biosynthesis of amino acids. There are several ways to synthesize oxaloacetate that do not require the functioning of SDH, such as the carboxylation of pyruvate that could be the main route of synthesis, or the reversible transamination of aspartate. In this study, we observed a decrease in L-acid aspartic (Asp) levels in the neurotransmitter profile of zebrafish and medaka larvae, exposed for 24 h to 100 and 1000  $\mu\text{g/L}$  of boscalid. These concentrations are well below the  $\text{LC}_{50}$  reported in aquatic species after acute exposure, but above the NOAEC for SDH inhibition reported in chronic toxicity studies (Epa and of Pesticide Programs, 2003). It is assumed that the 24 h-exposure to low concentrations of boscalid should not inhibit the SDH of our aquatic models. However, the decrease in free Asp could be explained by its transamination into oxaloacetate and its use in the glycogenolysis as an alternative energy source to face the decrease in the activity of the respiratory chain and the TCA cycle. Previous studies have shown that Asp plays a central role in the mitochondrial and cytosolic biochemical processes of myocardial muscle (Sivakumar et al., 2008). It is postulated that in oxygen-deprived cardiac muscle, Asp and Glu levels decrease, since free, these amino acids can

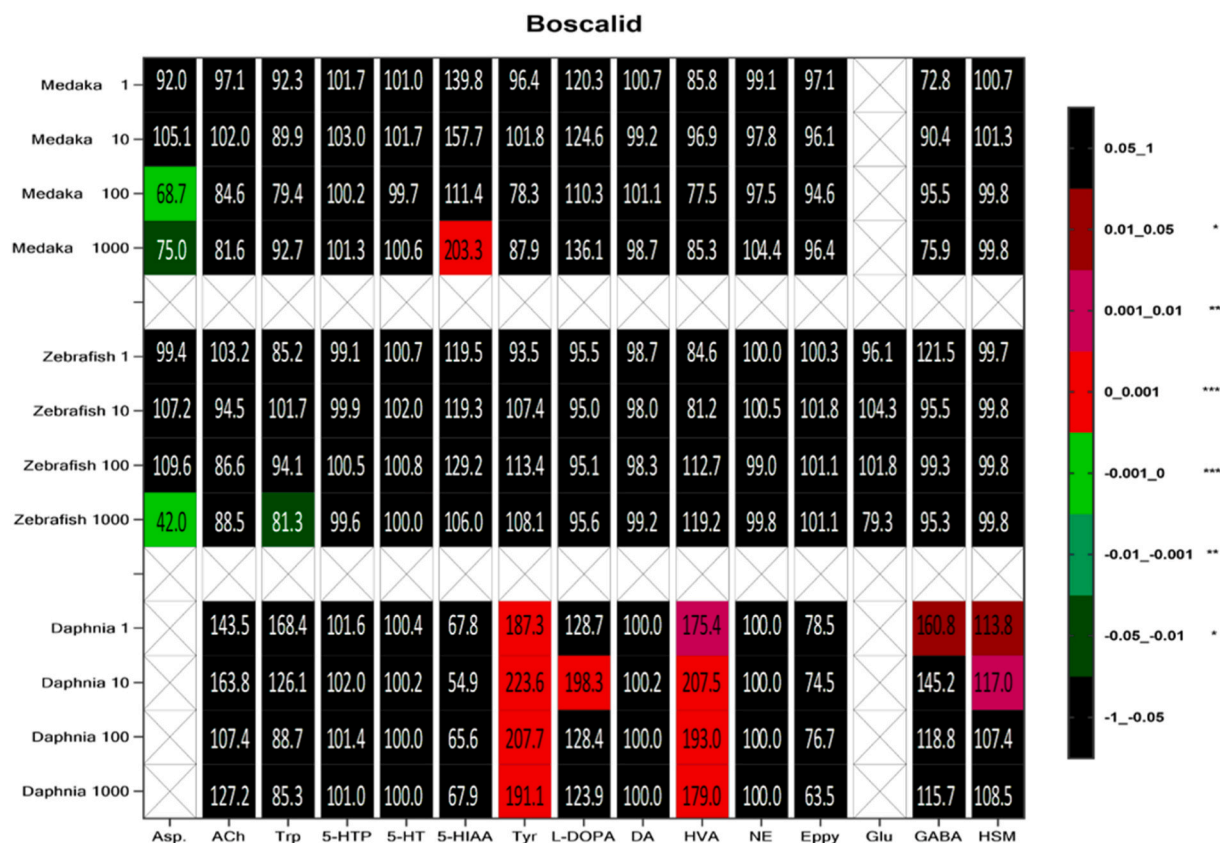


Fig. 5. Heat map diagram summing the changes in the neurotransmitter's profiles of zebrafish larvae, Japanese medaka larvae and *D. magna* after 24 h treatment boscalid (1 µg/L-1000 µg/L). Colors in the heat map represent the deviation from the control larvae (black color), with a gradient of green or red for values below or above the controls, respectively. The number inside each cell corresponds to the concentration averages normalized as percentage of their respective controls. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001; one-way ANOVA with Dunnett's multiple comparison test or Kruskal Wallis test with Bonferroni correction; Data from 4 to 6 replicates, four or five animals per replicate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

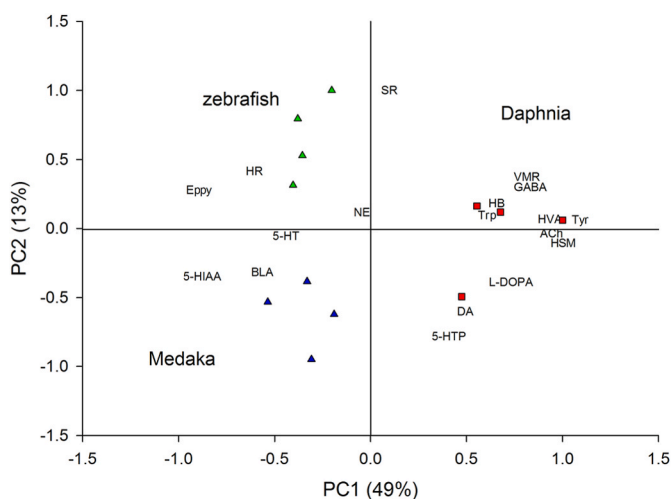


Fig. 6. Bi-plots the first two PCA sample scores of the three tested species exposed to boscalid and loadings of behavioral, physiological, and related metabolites. Percentages of variance of the data explained for each of the two PCA components are plotted. Abbreviations are explained in the text.

become an intermediate of the TCA cycle and be used as energy substrates in the metabolic response to cardiac hypoxia. Blocking ATP production and increasing mitochondrial oxidative stress can lead to arrhythmias such as ventricular tachycardia (McGee and

Abdel-Rahman, 2016). In fish larvae exposed for 24 h to concentrations of boscalid between 1 and 1000 µg/L, one of the results observed was precisely the increase in heart rate. Therefore, the tachycardic effect of boscalid on the heart of fish larvae could reflect an alteration of mitochondrial energy metabolism due to inhibition of SDH. The intermediates of the TCA cycle have been shown to have antioxidant properties (Sivakumar et al., 2008), so the decrease of Asp in both fish species and Glu in zebrafish could indicate a protective and compensatory response to the lack of cellular energy in organisms poisoned by the fungicide. Asp is also one of the main excitatory neurotransmitters of the CNS, it stimulates NMDA receptors (Kubrusly et al., 1998), although to a lesser degree than Glu. In previous studies (Best et al., 2008; Faria et al., 2017), it has been shown that the administration of NMDA receptor antagonists such as memantine, could modulate behavioral responses in zebrafish larvae, increasing the escape response and decreasing habituation. Thus, behavioral alterations observed in fish larvae exposed to boscalid could be related to the observed changes in Asp levels, as this neurotransmitter is a potent selective agonist of NMDA receptor (Pittaluga and Raiteri, 1992).

Although the physiological and behavioral responses of *D. magna* were quite different from those of vertebrates, it would be interesting to adjust the method of neurotransmitter analysis to obtain data on Asp and Glu levels in this matrix.

Studies on the cerebral serotonergic system demonstrate its great conservation in the vertebrate phylum (Araujo et al., 2013; Faria et al., 2021a). Serotonergic activity plays a critical role in mediating behavioral effects. In gregarious species with an established hierarchy, subordinate animals have decreased brain levels of Trp and higher 5-HIAA



(Maximino et al., 2013; Stewart et al., 2013; Winberg and Thörnqvist, 2016). In contrast, brain concentrations of 5-HT remain generally unchanged, so there must be increased tryptophan hydroxylase (TPH) activity. Mammals under chronic stress have also been reported to have a similar serotonergic profile. Social subordination produces dramatic effects on fish behavior, namely a general inhibition of behavior, not only aggressive, but also feeding and locomotion (Herculano and Maximino, 2014; Winberg and Thörnqvist, 2016). In this study, the 5-HIAA/5-HT ratio doubled in medaka larvae exposed to the highest boscalid concentration, although we did not observe any decrease in 5-HT. This 5-HIAA/5-HT ratio is an indicator of 5-HT turnover. Its increase suggests an upregulation of serotonergic activity, precisely of the enzymes TPH and monoamine oxidase (MAO). Both, the increased serotonergic activity, and the generation of free radicals by MAO over-activation could explain the observed inhibition in the startle response of medaka larvae.

MAO also catalyzes the dopamine degradation reaction in HVA in the catecholamine synthesis pathway (Daubner et al., 2011). Analysis of the neurotransmission profile of daphnias exposed 24h to different concentrations of the fungicide showed an impressive increase in the levels of dopamine precursors and degradation products. The HVA/DA ratio is almost doubled for daphnias exposed to 10 and 100 µg/L boscalid, indicating an increase in dopaminergic activity. Moreover, DA levels did not show any alteration, which could be justified by a turnover maintenance. MAO, tyrosine hydroxylase, and phenylalanine hydroxylase activities could be upregulated, resulting in increased levels of Tyr, L-DOPA and HVA while the levels of DA are maintained. Data on Phe levels would be helpful in confirming this hypothesis. Aromatic amino acids such as Phe and Tyr are key precursors of biogenic amines such as dopamine and octopamine that play multiple roles in the endocrine and neurotransmission systems of invertebrates (Bownik et al., 2018). A previous study in *Daphnia* exposed to environmental levels of organophosphates reported a possible correlation between increased levels of amino acids, including Phe and Tyr, and cessation of energy metabolism (Nagato et al., 2016). Apart from the increase in catecholamines, we found in this study that daphnias exposed for 24 h to 10–1000 µg/L boscalid also presented a bradycardic effect, as well as a decrease of BLA and SR, and an increase of HB. The response to cellular damage involves an additional energy cost to maintain the basal metabolism. In stressful situations, aquatic invertebrates can produce energy from the process of anaerobic respiration (Hochachka and Mustafa, 1972). Despite being less efficient than aerobic respiration, the induction of anaerobic metabolism could be an adaptive strategy of invertebrates to respond to the increased energy demand resulting from the damage repair, turnover of damaged cells and detoxification processes (Aksakal, 2020; Nagato et al., 2016). Another alternative source of energy in a situation of stress would be the use of amino acids as keto acids to produce energy through the TCA cycle, as we have proposed as a possible explanation for the results observed in fish models. However, a sign of the excessive use of amino acids in energy metabolism would be the decrease in their concentrations and not the increase as we observed here. Increased levels of free amino acids in response to exposure to a fungicide could mean that the available energy is lower and that the body is saving energy on processes such as protein synthesis. That is, in response to the acute toxicity of boscalid, *Daphnia* would reduce its overall metabolism as a first survival strategy (Aksakal, 2020; Jeong et al., 2018). One way to reduce the metabolic cost could be by decreasing BLA and cardiac activity as we observed in this study. Amino acids with altered levels in daphnia play also a particularly key role in cell signaling. In fact, their release in response to acute stress could precede the release of hormones to initiate a metabolic response, or their possible reorientation as ketoacids to fuel the TCA cycle (Lari et al., 2018).

In the neurotransmitter profile of *Daphnia*, we could also observe an elevation of GABA levels and slight elevation of HSM levels after exposure to boscalid concentrations below the NOAEC for the inhibition of SDH. The histaminergic system is crucial in *Daphnia* species and more

generally in the visual system of arthropods (Ismail et al., 2021; McCoolle et al., 2011). Exposure of daphnids to histaminergic receptor antagonists, such as cimetidine, demonstrated the role of this amine in generating the animal's negative phototactic response. The alteration of VMR could be explained by the increase in the levels of available HSM and the inability of the neurotransmitter to bind adequately to its receptor. Changes in the natural negative phototaxis response may pose a risk to the survival of the animal. The antipredator strategies of *Daphnia* are neurophysiologically encoded by different neurotransmission systems, including cholinergic and GABAergic systems (Bedrossiantz et al., 2021; Weiss et al., 2012). A previous study allowed us to identify GABA as key metabolite in modulating the phototactic response. Daphnias exposed to Diazepam presented higher levels of GABA but did not present the geotaxis response to fish kairomones (Bedrossiantz et al., 2021). We observed a remarkably similar increase in the profile of this neurotransmitter after exposure to boscalid, so the involvement of this GABAergic pathway could also be the cause of the observed changes in behavior.

Finally, this study reflects the differences in responses that may exist between one species and another. These differences in sensitivity may be due to phylogenetic distance, as suggested by the results of the multivariate study. Even so, some metabolic processes, very well evolutionarily conserved in eukaryotic cells, may be the targets of certain substances. It is necessary to improve our knowledge of these adverse outcome pathways (AOPs) since they affect most species. Moreover, the inclusion of multiple taxa provides a more complete understanding and more accurate determination of the toxicity of the substance and the susceptibility of various ecosystems.

The NOAEC represents the highest concentration of a substance at which non-adverse effects are observed under controlled experimental conditions. Nevertheless, a non-adverse effect in laboratory conditions could turn adverse in real-life scenarios, where environmental pressure and risk of predation are higher. Current environmental regulations consider survival, growth, and reproduction as the main endpoints approved for assessing the toxicity of chemicals at very low exposure levels. To ensure the safety of living organisms and ecosystems, it is necessary to consider a wider range of toxicity endpoints, as is already currently done in human toxicology studies (Krewski et al., 2010; von Hellfeld et al., 2023). Endpoints that delve into molecular, cellular, physiological, or behavioral effects have proven to be valuable tools for estimating the concentration at which non-adverse effects occur in the ecosystems (Barascou et al., 2021; Roberts et al., 2020; Soose et al., 2023). For the recalculation of risk quotient of boscalid, we considered the reported levels in surface waters (0.003–36 µg/L) and the lowest concentration of boscalid (1 µg/L) that produced behavioral or cardiac impairments in *D. magna*, *D. rerio* and *O. latipes* after 24h-exposure. The new risk quotient was between [0.3–3600], more than 300 times higher than the values established using the endpoints of mortality and reduction of young [ $<0.001$ – $0.006$ ] (Epa and of Pesticide Programs, 2003). These differences in the risk estimation highlighted that the current endpoints used in environmental risk assessment does not provide enough protection to the exposed organisms.

## 5. Conclusions

In this study, changes in heart rate, behavior, and neurotransmitter profile of three phylogenetically very distant aquatic organisms were reported after only a 24h-exposure to environmental concentrations of boscalid. The observed effects of the fungicide on heart rate, neuronal signaling and some behaviors turned out to be remarkably similar between the fish species, *D. rerio* and *O. latipes*. Despite responding differently to boscalid, the results obtained in *D. magna* also suggest the disruption of energy mechanisms very well conserved throughout evolution by all eukaryotic organisms. Therefore, the present results demonstrate the importance of reviewing toxicity thresholds and environmental quality standards for certain chemicals such as SDH

inhibitors. They also emphasize the need to integrate new models and sublethal endpoints into toxicological studies to improve the predictability of results in other exposed organisms. Hormetic effects can be both harmful and beneficial, therefore, toxicological studies should consider the possible adverse effects of high and low doses of this kind of pesticides.

### CRedit authorship contribution statement

**Juliette Bedrossiantz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Júlia Goyenechea:** Methodology, Investigation, Formal analysis, Data curation. **Eva Prats:** Writing – review & editing, Investigation. **Cristián Gómez-Canela:** Supervision, Formal analysis, Data curation. **Carlos Barata:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Demetrio Raldúa:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation. **Jérôme Cachot:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

### Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.123685>.

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