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Toxicity evaluation of single and combined exposures to polyhydroxybutyrate nanoparticles and caffeine using *Xenopus laevis in vivo* and *in vitro* models

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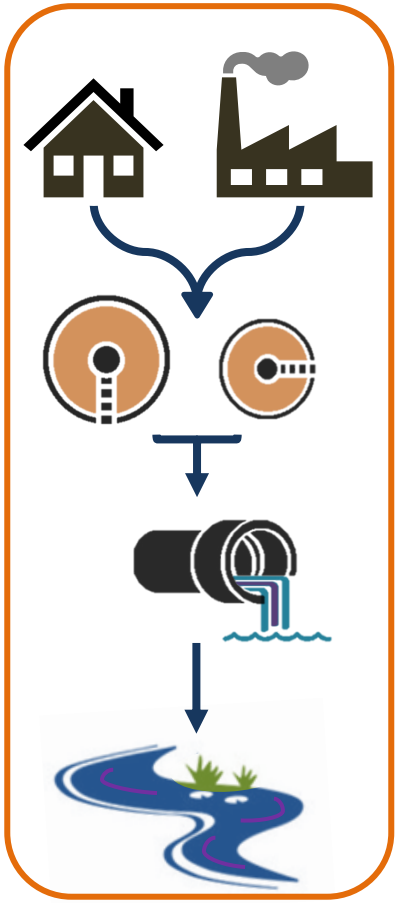
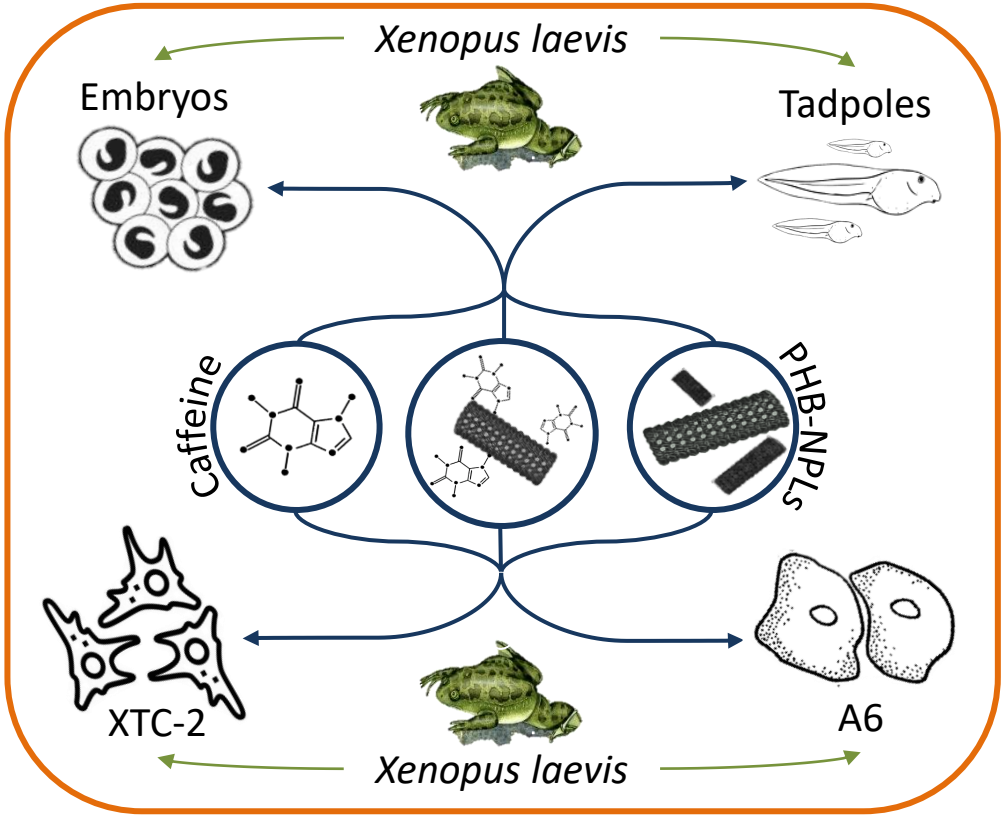
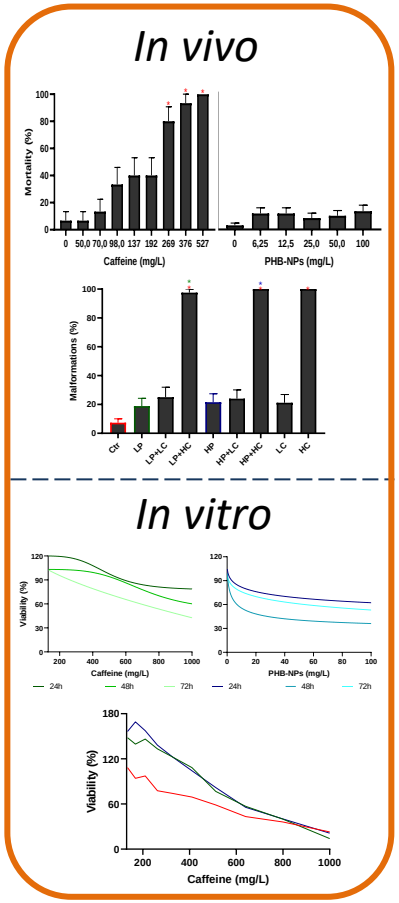
applEE applied Ecology and Ecotoxicology Research Group

universidade de aveiro departamento de biologia dbio

Toxicity evaluation of single and combined exposures to polyhydroxybutyrate nanoparticles and caffeine using *Xenopus laevis in vivo* and *in vitro* models







Abstract: Pollution is regarded as a relevant driver of both Human and Environmental Health. Plastic contaminants have been a target of extensive risk assessment research, mainly because of their persistence and fragmentation to nano sizes in the environment, potentiating its uptake to biota, including humans. Polyhydroxybutyrate (PHB) is a bio-based and biodegradable polyester, praised in the medical field due to its biocompatibility and non-toxicity to humans. Notwithstanding, little is known regarding its toxicity when used as nanoparticles (PHB-NPs). In addition to its individual exposure threat, NPs may serve as vectors for chemicals, promoting its incorporation by Humans (e.g., inhalation of NPs loaded with other chemicals) and other biota. Caffeine is the world's most widely consumed psychoactive drug and a relevant representative of pharmaceutically active pollutants. On mammals, caffeine can induce teratogenic and embryotoxic effects. This work aimed to assess the lethal and sublethal toxicity of these two xenobiotics, in single and mixed exposures, by using in vivo (embryos and tadpoles of *Xenopus laevis*) and in vitro (two cell lines of *X. laevis*) biological models. Caffeine was toxic to both life stages of *X. laevis*. Embryos were more sensitive than tadpoles with LC50 of 196 and 226 mg/L, and EC50, malformations of 124 and 241 mg/L, respectively. PHB-NPs showed no effect when exposed alone and in mixture. Cytotoxicity assays revealed LC50s of 864 (XTC-2), 587 (A6) and 131 (XTC-2) mg/L after caffeine and PHB-NPs exposure, respectively. In co-exposure, the concentration of PHB-NPs was positively correlated with the toxic effect of the mixture.

Keywords: nanoparticles; pharmaceutically active compound; ecotoxicity, cytotoxicity, *Xenopus laevis*

Introduction

Plastic pollution

- Pollution is a relevant driver of both Human and Environmental Health (Hardesty et al., 2017);

- Plastic contaminants are a target of extensive hazard assessment research, because of their persistence and fragmentation to nano sizes in the environment (Beiras et al., 2021), potentiating its uptake by biota, including humans (Tussellino et al., 2015; Ragusa et al., 2021; Leslie et al., 2022);

- A decrease in particles size constitute a serious threat to all biota due to an increase in bioavailability, and hindrance of their removal (Almeida et al., 2019);

- As a mitigation measure, efforts have been allocated to the development of biodegradable polymers as replacement candidates for synthetic plastics.

Introduction

Polyhydroxybutyrate Nanoparticles (PHB-NPs)

- Polyhydroxybutyrate (PHB) is a bio-based and biodegradable polyester;
- Strong mechanical proprieties, and the potential to be synthesised exclusively by renewable feedstocks (Tokiwa et al., 2009), potentiates its industrial applications in the eco-friendly circular economy (Mourão et al., 2021);
- Although praised in the medical field due to its biocompatibility and non-toxicity to humans, little is known regarding its toxicity when used as nanoparticles (Fernández et al., 2022);
- In addition to the threat posed by its individual exposure, NPs may serve as vectors for chemicals, promoting its incorporation by Humans and other biota (Magara et al., 2019).

Introduction

Caffeine

- Caffeine is considered the world's most widely consumed psychoactive drug and the best pharmaceutically active compound pollutant representative (Li et al., 2019);

- Its presence on daily products of high consumption, together with the limited efficiency of wastewater treatment plants, makes this compound an ubiquitous contaminant in freshwater systems, as well as in tap drinking water (Metcalfe et al., 2003; Li et al., 2019; 2020)

- It can readily cross the placenta barrier causing embryotoxic effects (e.g., increase in congenital malformations). Although verified in some mammals, these effects have not been fully confirmed in humans (Burdan, 2000).

Introduction

Amphibians ecological relevance

- Amphibians are a globally threatened group (41% of species; IUCN, 2022);
- Amphibians are especially susceptible to contaminants, due to the permeable, and highly irrigated nature of the skin, gills, and eggs, allowing for diffusion off gases and electrolytes;
- Due to this high sensitivity, amphibians may serve as sentinel species and good biological systems to assess toxicity of emergent pollutants;
- There is a need to establish *in vitro* methodologies as an alternative to animal experimentation for first tiers of amphibian aquatic stages risk assessment.

Introduction

Objectives and how to achieve them

i) Provide data on the PHB-NPs and caffeine potential risks to aquatic life stages of amphibians;

ii) Allow validation of cell lines as tools to allow extrapolation to *in vivo* data.

In vivo

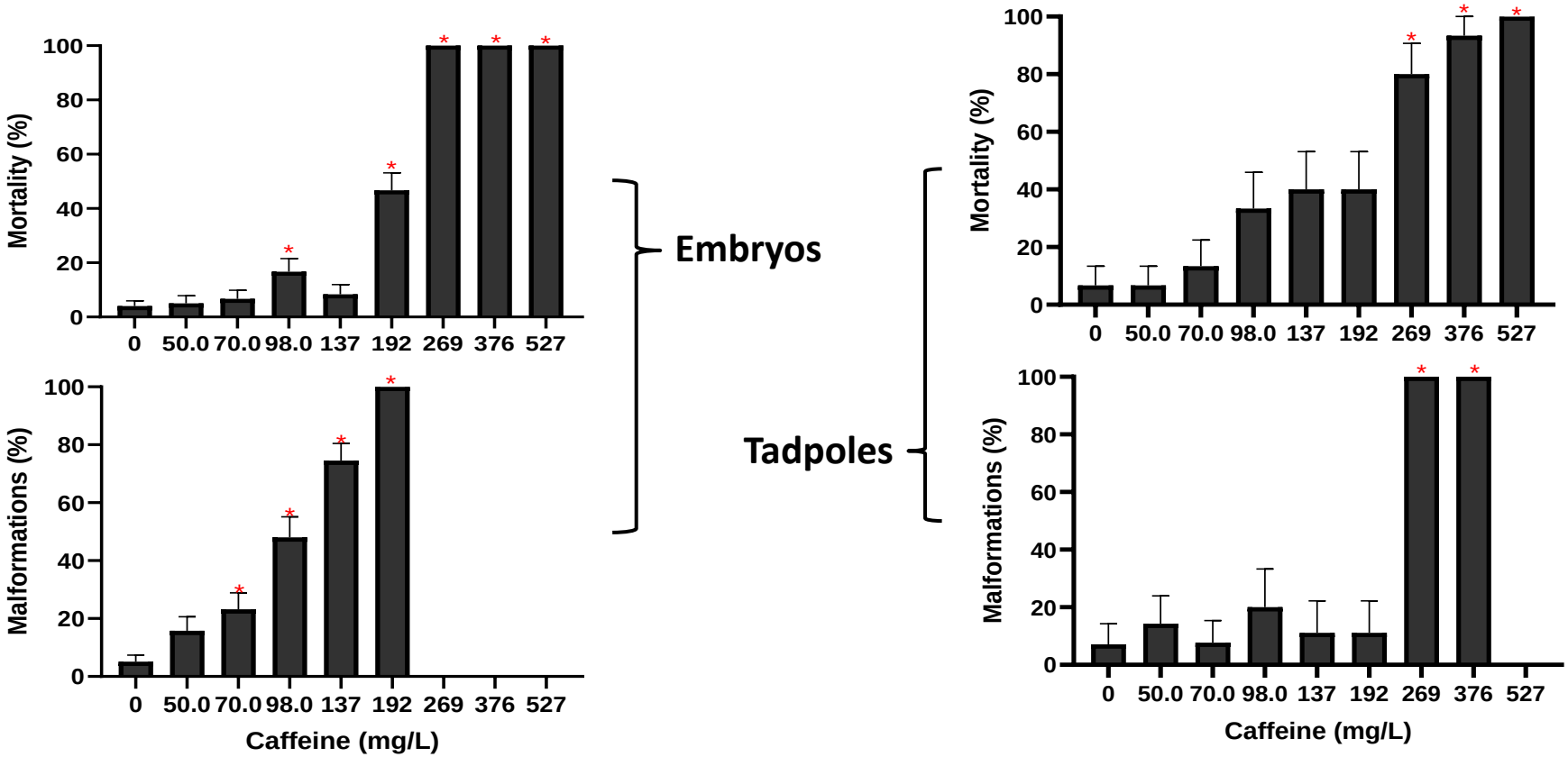
Assay: Teratogenic Ecotoxicity assay
Biological model: Embryos and Tadpoles of *X. laevis*
Contaminants: PHB-NPs and caffeine (in single and co-exposures)
Duration: 96-h, renewal at 48-h
Endpoints: Mortality, malformations, Heartbeat, Weight, Body Lengths

In vitro

Assay: Cytotoxicity, Resazurin and MTT viability assay
Biological model: A6 and XTC-2 cell lines of *X. laevis*
Contaminants: PHB-NPs and caffeine (in single and co-exposures)
Duration: 72-h
Endpoints: cell viability

Results and discussion

In vivo - Caffeine

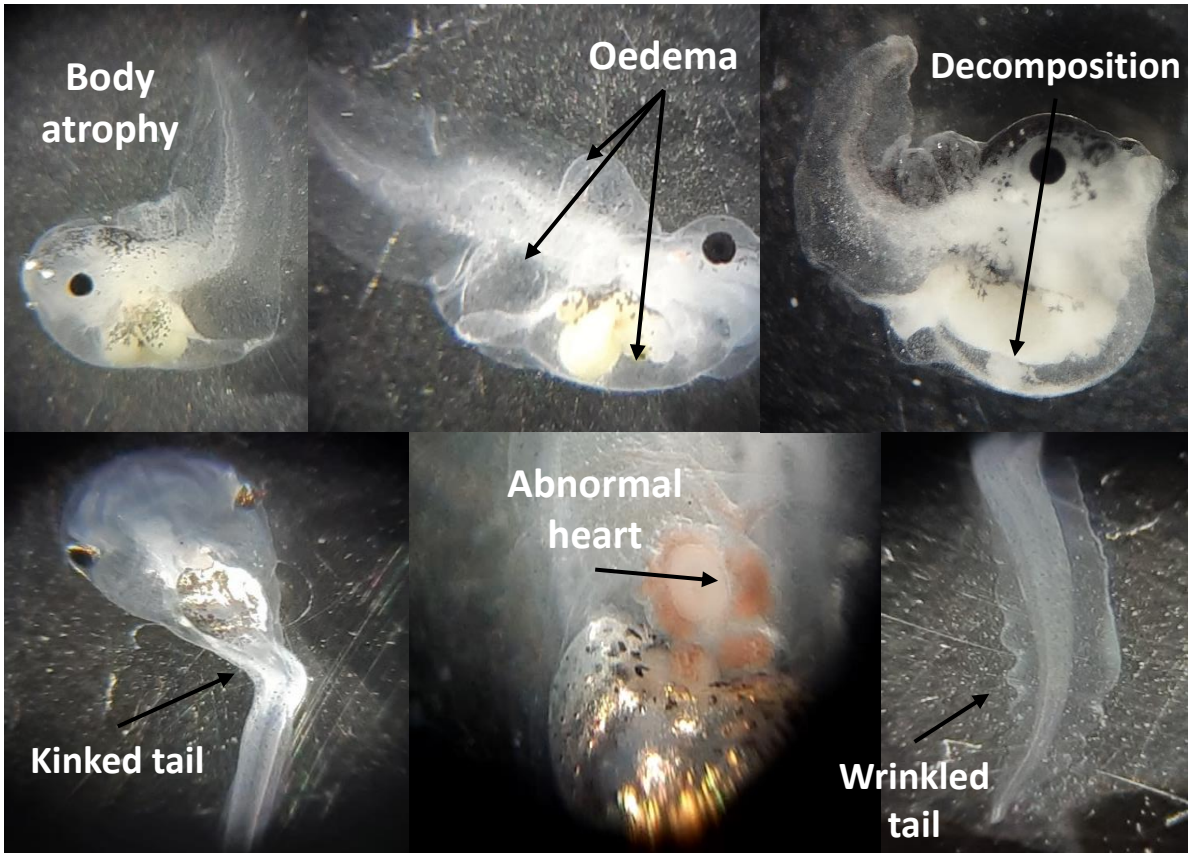


* indicates significant differences relatively to the control ($p < 0.05$).

Caffeine had a significant impact in both mortality and malformations. Embryos were more susceptible (LOEC= 98.0 and 70.0 mg/L) than tadpoles (LOEC= 269 mg/L).

Results and discussion

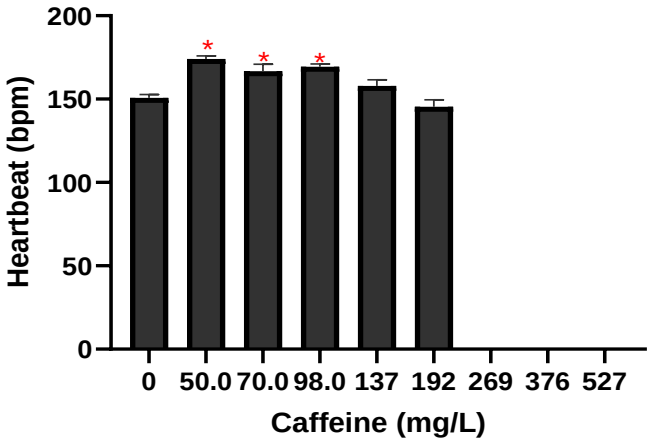
In vivo - Caffeine



Malformations in embryos (top) and tadpoles (bottom) of Xenopus laevis exposed to caffeine.

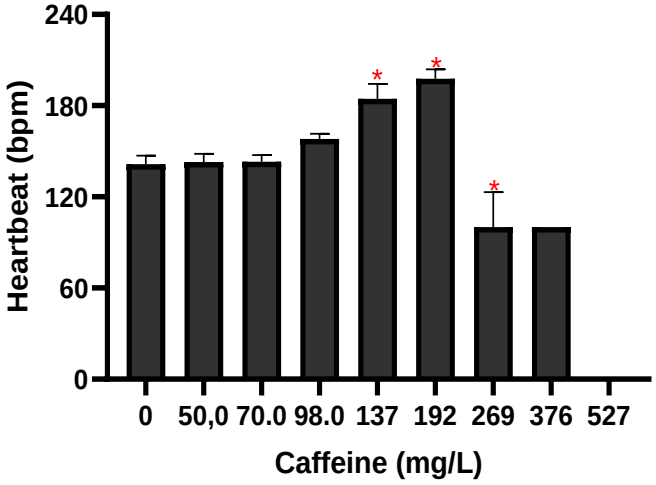
Results and discussion

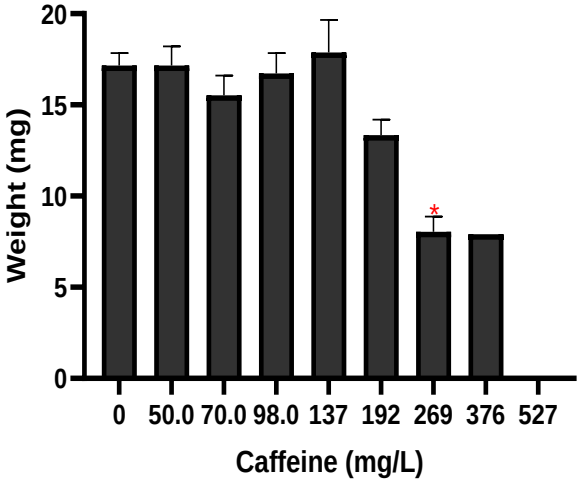
In vivo - Caffeine



Embryos

Tadpoles

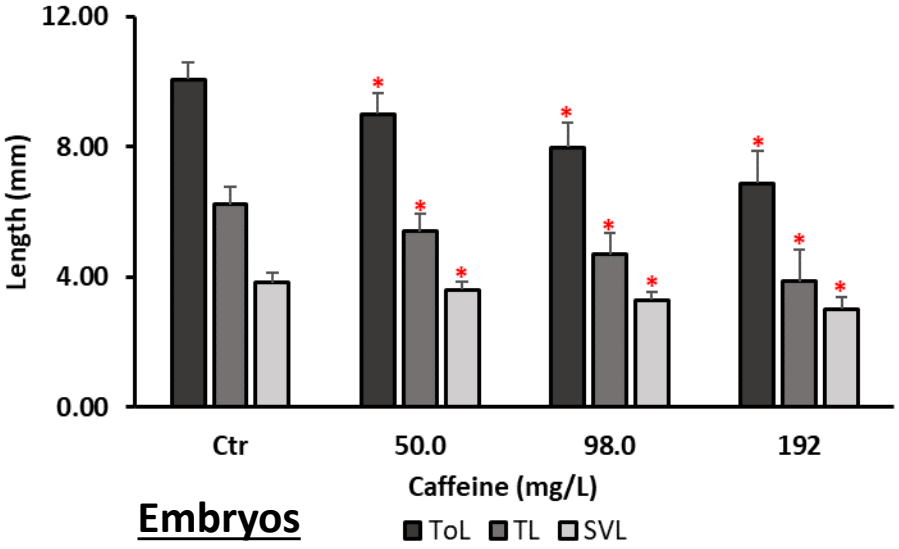




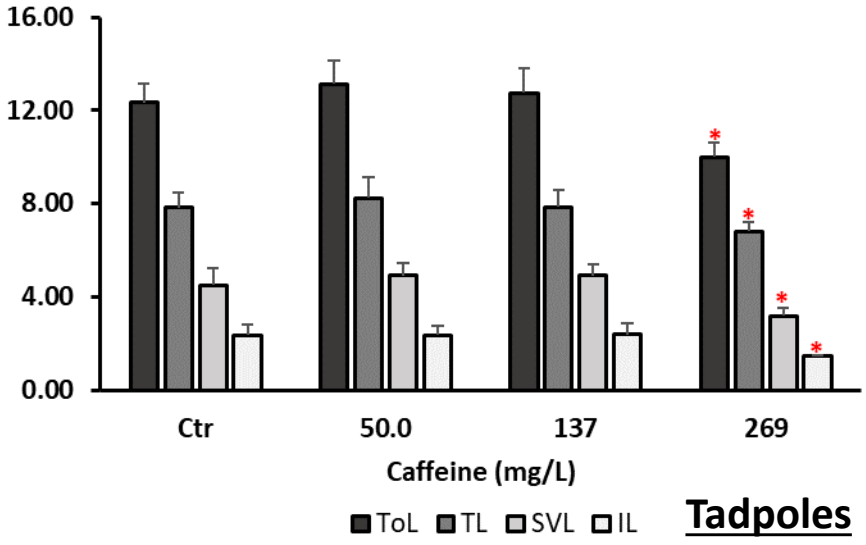
* indicates significant differences relatively to the control ($p < 0.05$). Caffeine had a significant impact in tadpole's weight (LOEC= 269 mg/L). Regarding heartbeat, caffeine had a dichotomic (i.e., stimulant and inhibitor) effect on both life stages. Embryos were more susceptible (LOEC= 50.0) than tadpoles (LOEC= 137 mg/L).

Results and discussion

In vivo - Caffeine



Embryos



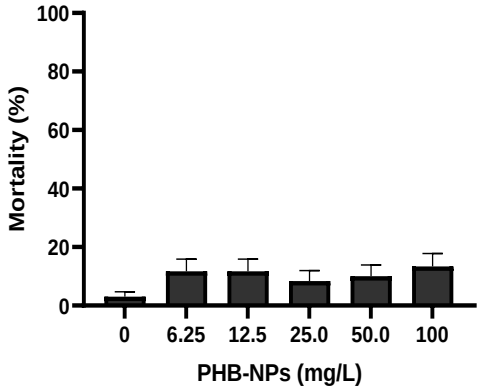
Tadpoles

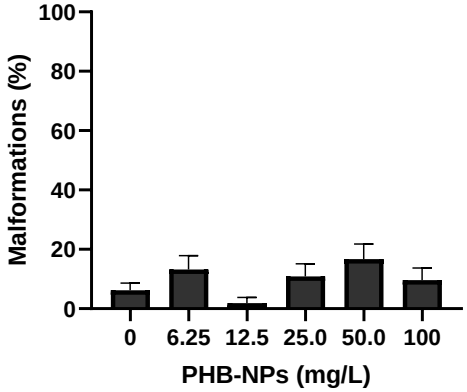
* indicates significant differences relatively to the control ($p < 0.05$). ToL – Total length; SVL – snout to vent length; TL – tail length; IL – interocular length.

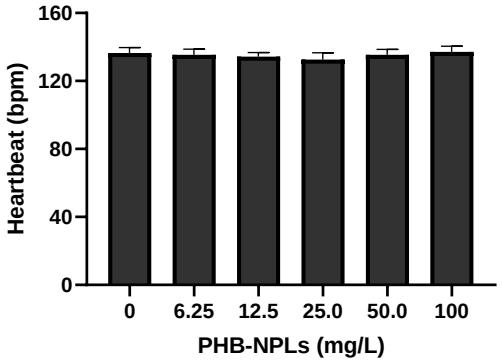
Caffeine had a significant impact in all body lengths assessed. Embryos were more susceptible (LOEC= 50.0 mg/L) than tadpoles (LOEC= 269 mg/L).

Results and discussion

In vivo – PHB-NPs

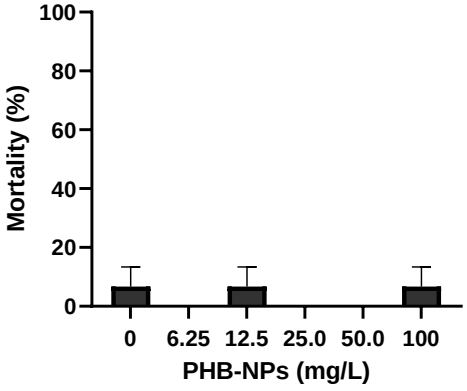


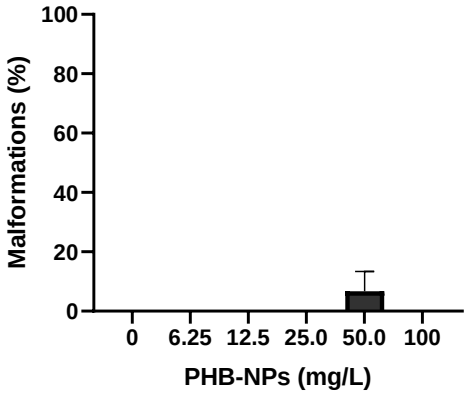


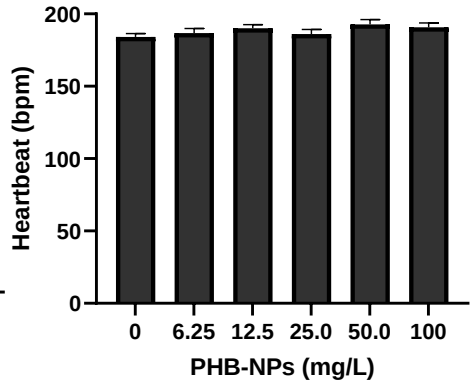


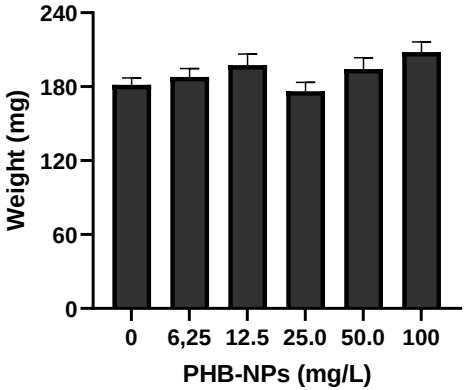
← Embryos

Tadpoles ↓







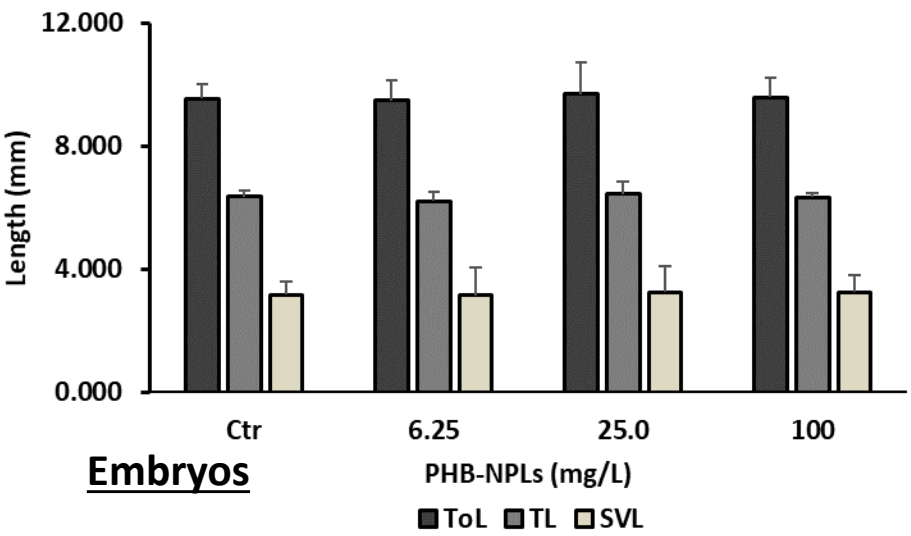


* indicates significant differences relatively to the control ($p < 0.05$).

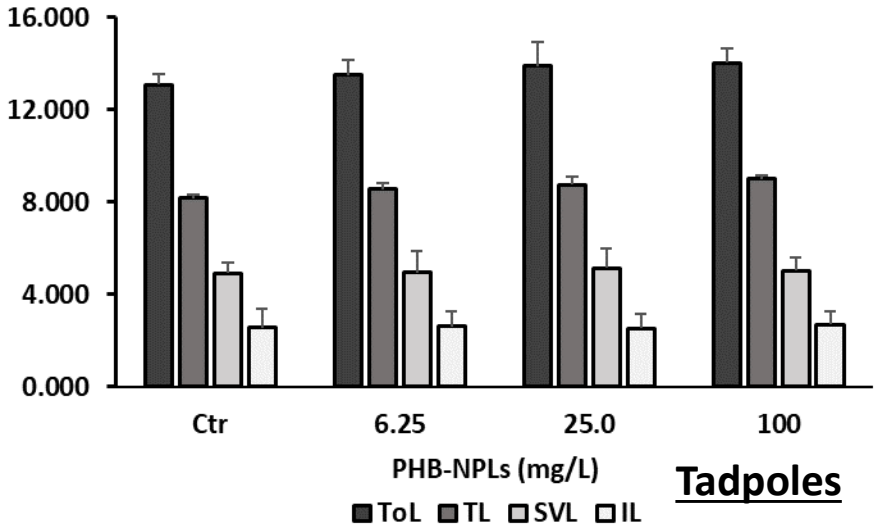
PHB-NPs had no significant impact on mortality, malformations, heartbeat and weight.

Results and discussion

In vivo – PHB-NPs



Embryos

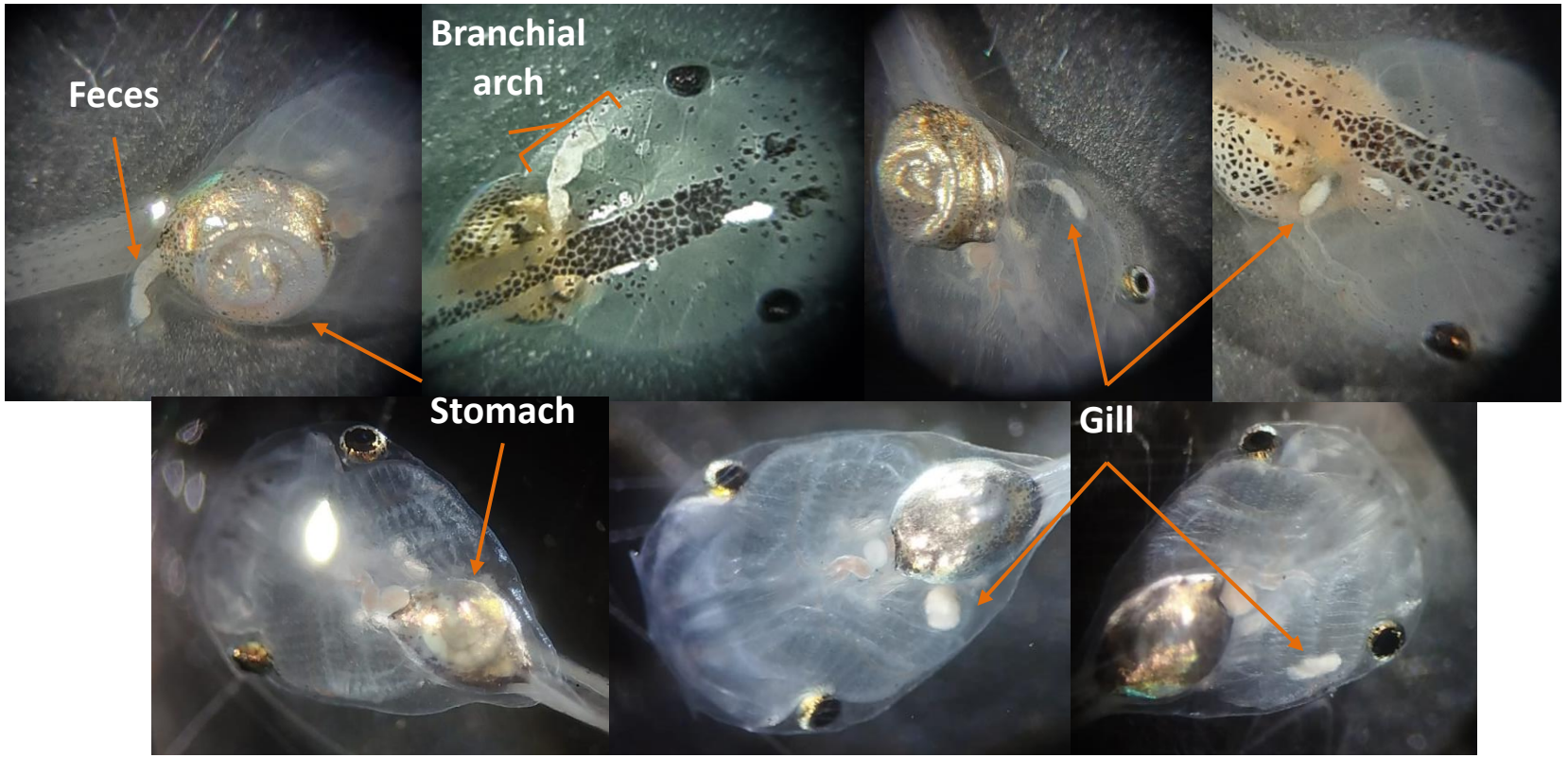


Tadpoles

* indicates significant differences relatively to the control ($p < 0.05$). HTL – head to tail length; SVL – snout to vent length; TL – tail length; IL – interocular length. PHB-NPs had no significant impact on any of the tested body lengths.

Results and discussion

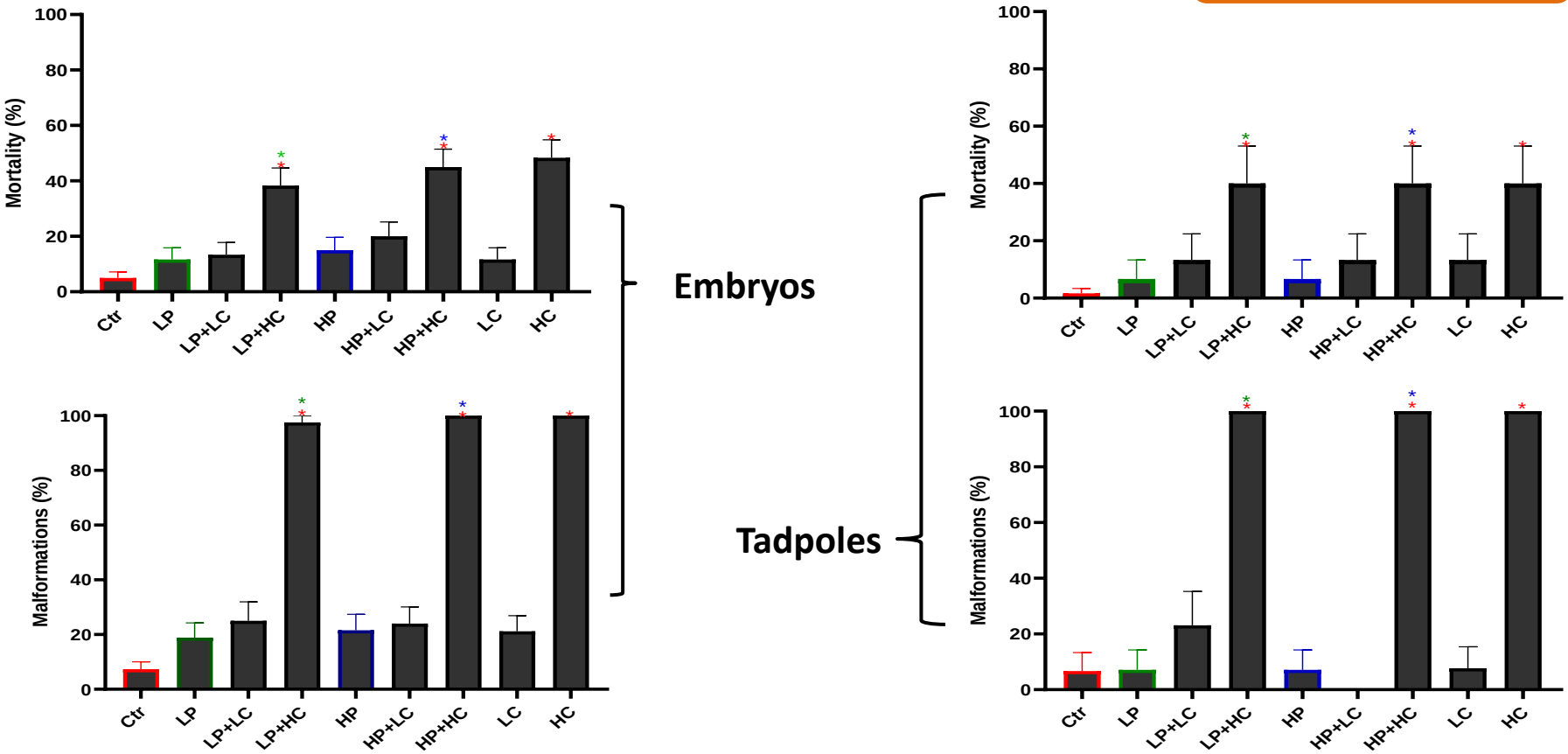
In vivo – PHB-NPs



Accumulation and elimination of PHB-nanoparticles by Xenopus laevis tadpoles exposed to PHB-NPs alone (top) and in combination with caffeine (bottom).

Results and discussion

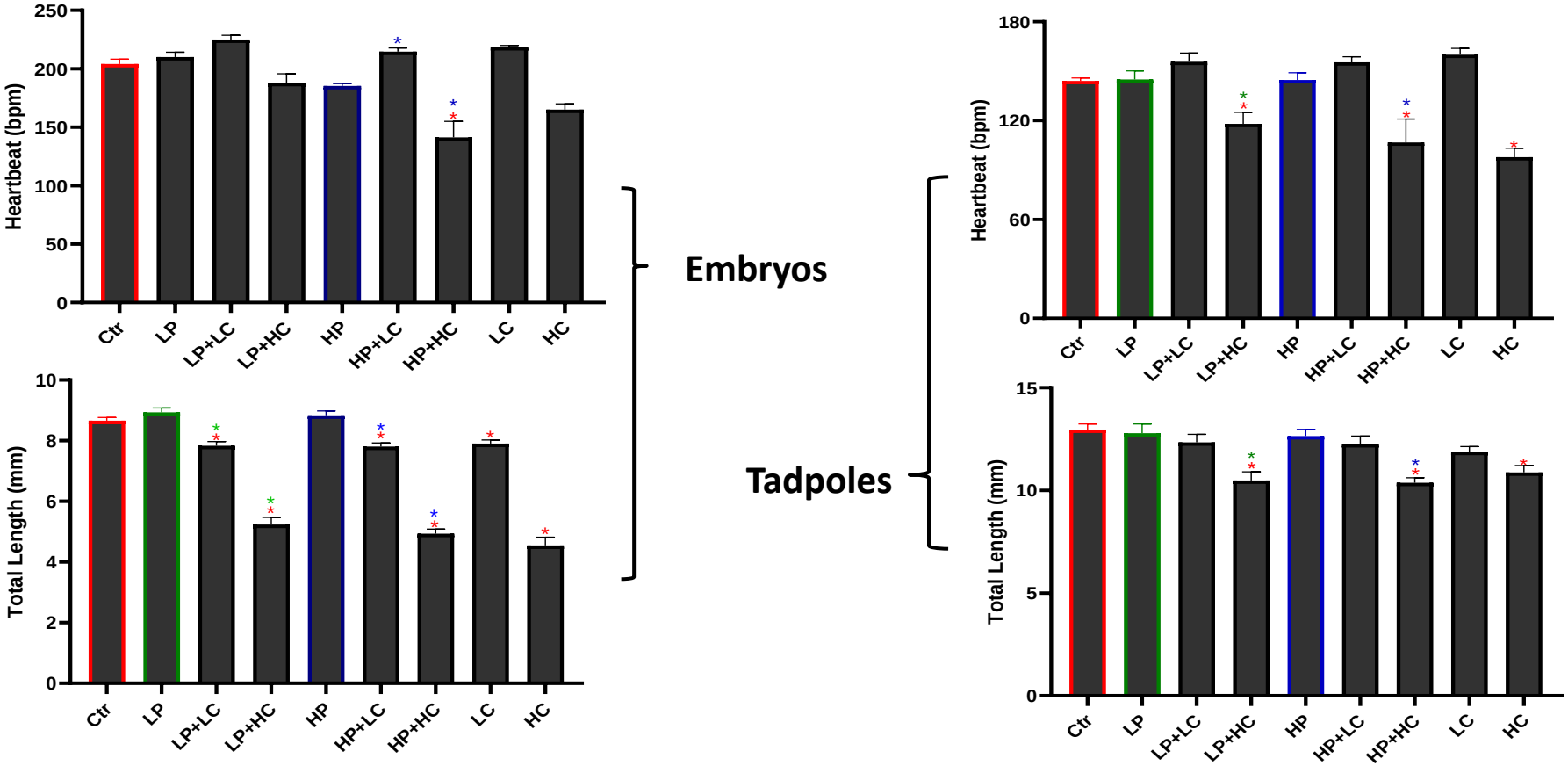
In vivo – Mixture



* indicates significant differences relatively to the control, LP, HP, LP and HP ($p < 0.05$). LP – Low PHB-NPs; HP – High PHB-NPs; LC –Low caffeine; HC – High caffeine. High caffeine had a significant impact alone an in mixture. PHB-NPs had no impact on the mortality and malformation.

Results and discussion

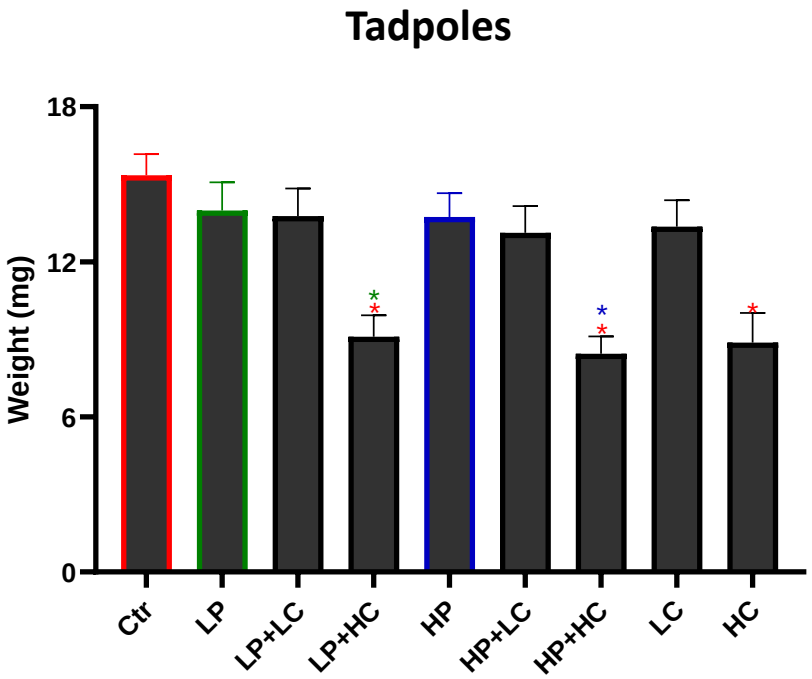
In vivo - Mixture



* indicates significant differences relatively to the control, LP, HP, LP and HP ($p < 0.05$). LP – Low PHB-NPs; HP – High PHB-NPs; LC –Low caffeine; HC – High caffeine. Caffeine had a significant impact alone an in mixture. PHB-NPs had no impact on the heartbeat and head-tail length.

Results and discussion

In vivo - Mixture



* indicates significant differences relatively to the control, LP, HP, LP and HP ($p < 0.05$). LP – Low PHB-NPs; HP – High PHB-NPs; LC –Low caffeine; HC – High caffeine. High caffeine had a significant impact alone an in mixture. PHB-NPs had no impact on weight of tadpoles.

Results and discussion

In vivo

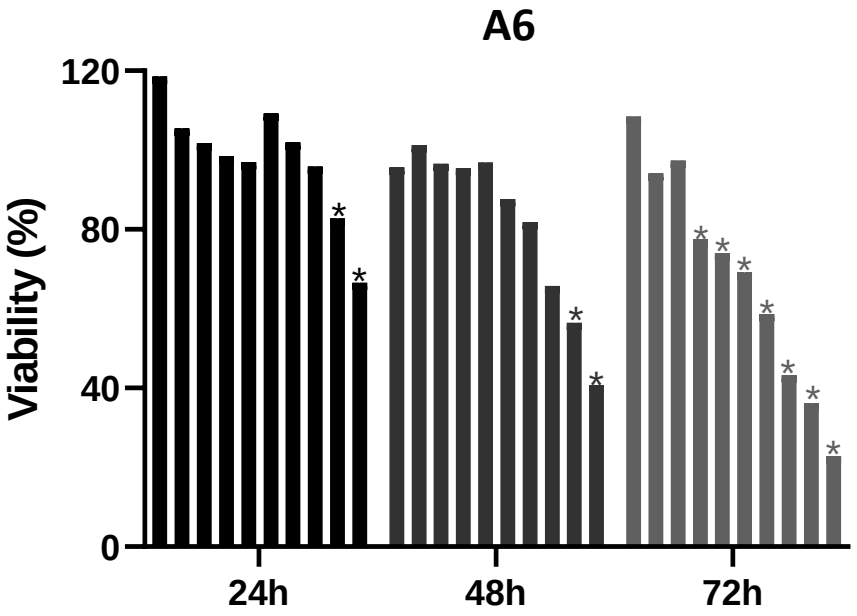
Lethal and sublethal concentrations. LC_{50}, EC_x and MCIG are represented in mg/L. Underline values represent EC_{25}, while double underline represents EC_{15}, and no underline represents EC_{50}.

		Lc50		Ec50						TI[j]
		Mt [a]	Mf [b]	Ht [c]	Wg [d]	ToL [e]	SvL [f]	TL [g]	IL [h]	
Caffeine	Embryos	196	124	<u>143</u>	NC [k]	<u>170</u>	<u>138</u>	<u>118</u>	NC [k]	1.58
	Tadpoles	226	241	<u>96.2</u>	194	<u>199</u>	<u>196</u>	<u>232</u>	238	0.94
PHB-NPs	Embryos	N/A	N/A	N/A	NC [k]	N/A	N/A	N/A	NC [k]	N/A
	Tadpoles	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

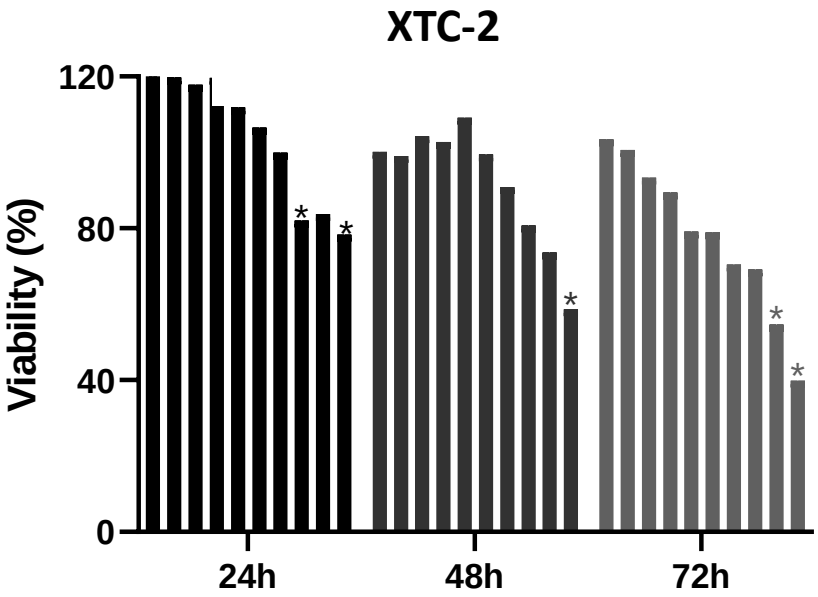
[a] Mortality; [b] Malformations; [c] Heartbeat; [d] Weight; [e] Total length; [f] Snout-vent length; [g] Tail length; [h] Interocular length; [i] Minimum concentration to inhibit growth = LOEC of Total length; [j] Teratogenicity index based on the LC_{50}/EC_{50}; [k] Not calculated.

Results and discussion

In vitro - Caffeine



72-h LOEC = 262 mg/L

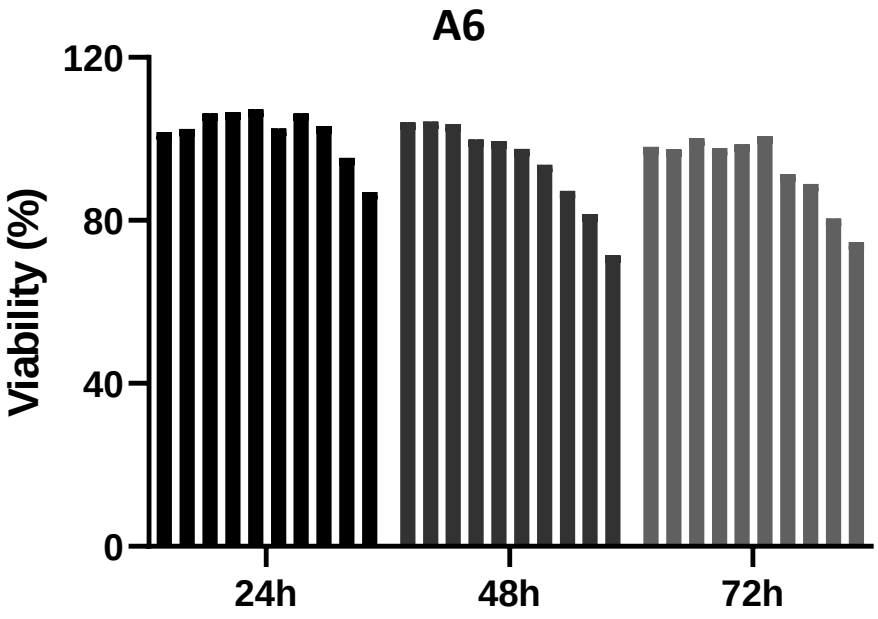


72-h LOEC = 800 mg/L

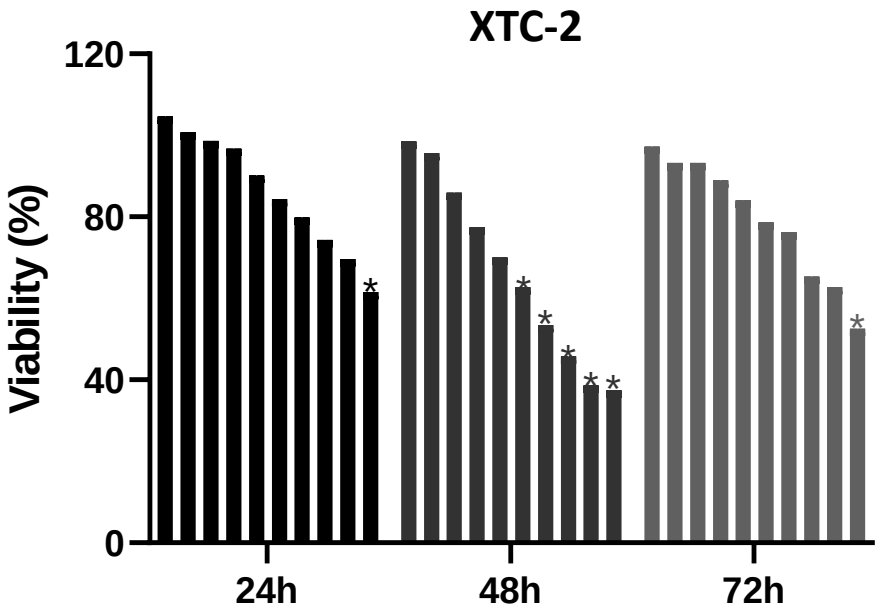
* indicates significant differences relatively to the control ($p < 0.05$). Caffeine reduced viability of both cell lines in all tested timepoints. A6 cells were more susceptible to caffeine than XTC-2

Results and discussion

In vitro – PHB-NPs



72-h LOEC = N/A

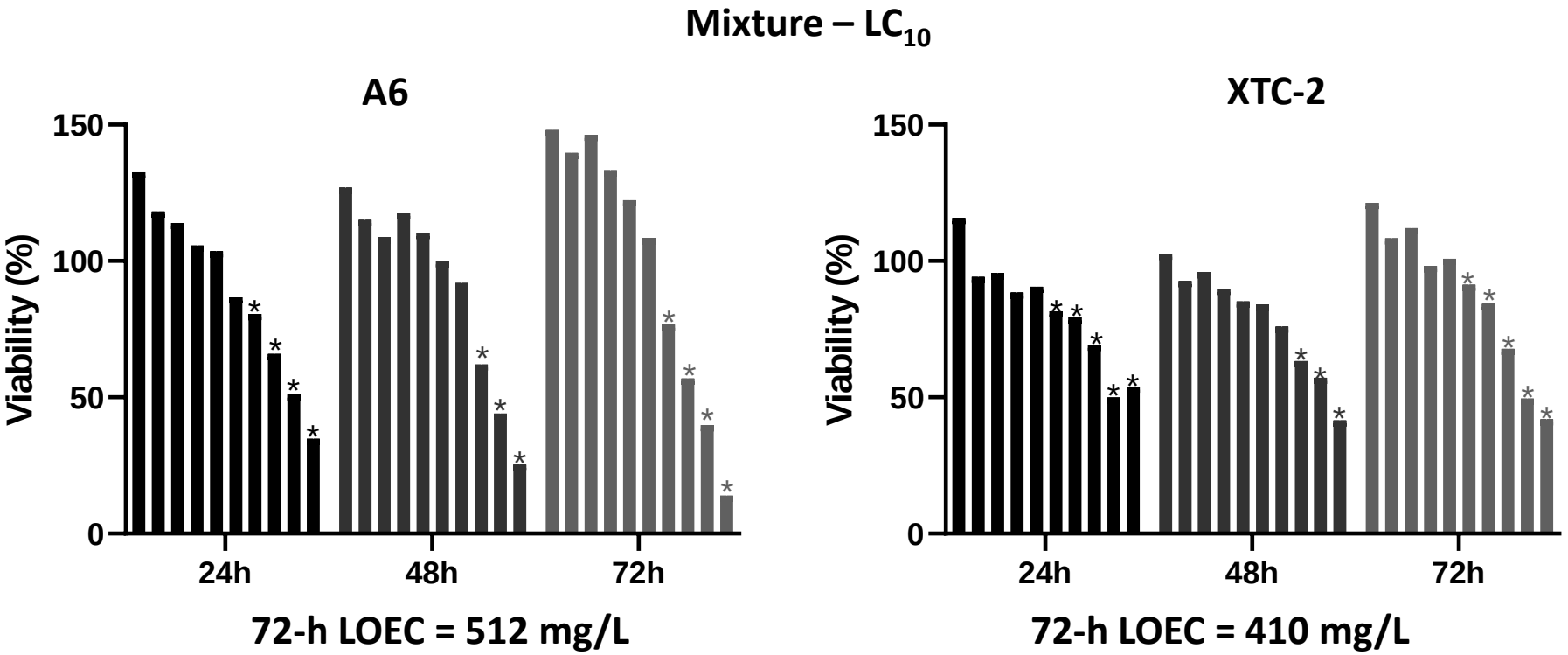


72-h LOEC = 100 mg/L

* indicates significant differences relatively to the control ($p < 0.05$). PHB-NPs reduced viability of XTC-2 cell lines in all tested timepoints. However, it had no significant effect on A6 cell line

Results and discussion

In vitro - Mixture

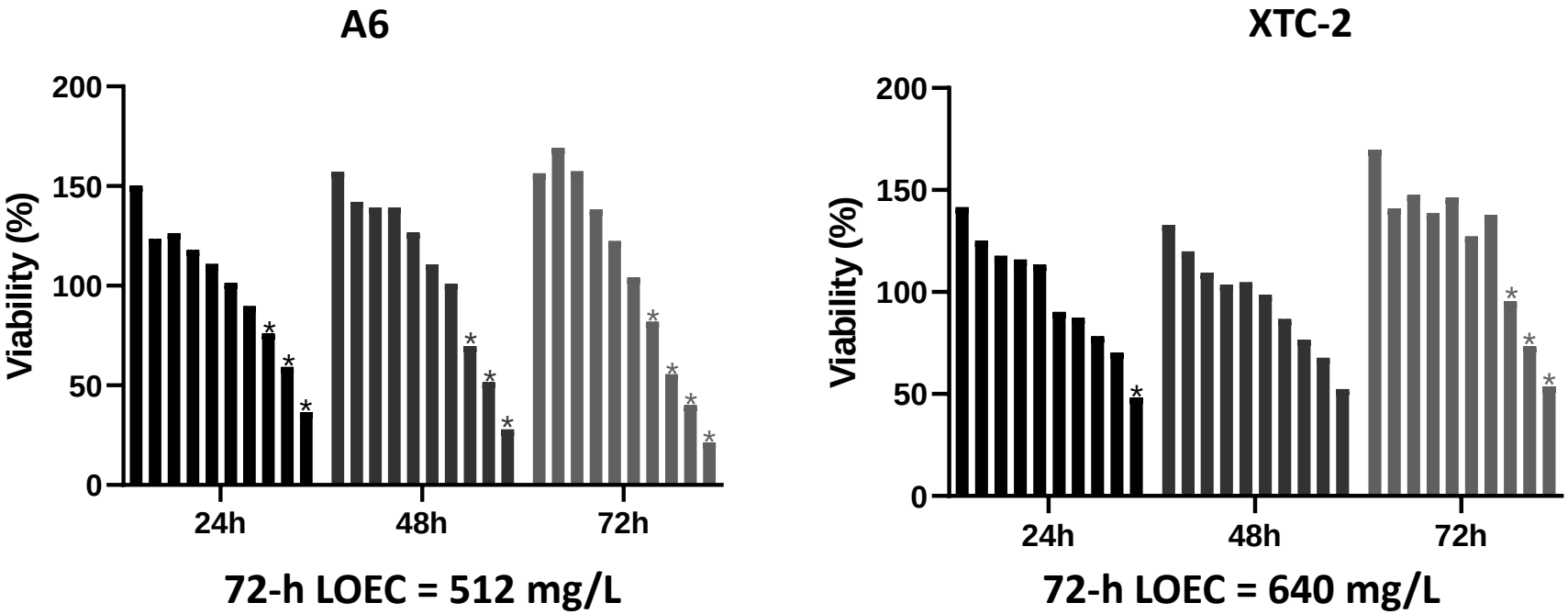


* indicates significant differences relatively to the control ($p < 0.05$). Caffeine and PHB-NPs (LC_{10}) mixture reduced viability of both cell lines in all tested timepoints. XTC-2 cells showed an overall higher sensibility to the mixture.

Results and discussion

In vitro - Mixture

Mixture – LC_{25}



72-h LOEC = 512 mg/L

72-h LOEC = 640 mg/L

* indicates significant differences relatively to the control ($p < 0.05$). Caffeine and PHB-NPs (LC_{25}) mixture reduced viability of both cell lines in all tested timepoints with the exception for XTC-2 cell at 78-h. A6 cells showed an overall higher sensibility to the mixture.

Results and discussion

In Vitro

Lethal Concentrations (LC_x) of caffeine and PHB-NPs, after 72 hours of single and mixture exposure, in cell lines of *Xenopus laevis* – XTC-2 and A6. Values represented in mg/L.

Assay		Cell line	MTT			Resazurin		
			LC_{10}	LC_{25}	LC_{50}	LC_{10}	LC_{25}	LC_{50}
Single	Caffeine	XTC-2	262	459	864	420	701	-
		A6	219	328	587	424	499	621
	PHB-NPs	XTC-2	1.18	11.4	131	-	-	-
		A6	20.9	96.7	-	-	-	-
Mixture	LC10	XTC-2	-	7.63	241	413	569	857
		A6	-	-	371	477	546	682
	LC25	XTC-2	-	-	102	734	839	1006
		A6	-	-	-	469	534	680

Results and discussion

In vitro - Mixture

Alteration in cell viability of cell lines exposed to caffeine alone and in combination with PHB-NPS (LC10 and LC25 respectively). One-way ANOVA results. Significant differences ($p < 0.05$) expressed as "yes".

Cell line	Time Point	Caff only x Caff + PHB-NPs (LC_{10})	Caff only x Caff + PHB-NPs (L_{C25})	Caff + PHB-NPs (LC_{10}) x Caff + PHB-NPs (LC_{25})
A6	24-h	no	no	yes
	48-h	no	yes	Yes
	72-h	yes	yes	No
XTC-2	24-h	yes	no	yes
	48-h	yes	no	yes
	72-h	yes	yes	yes

Conclusions

- *In vivo*
  - Caffeine displayed lethal and sublethal toxicity (e.g., teratogenesis, growth inhibitor, heartbeat disrupter) effects on both amphibian early life stages;
  - PHB-NPs had no impact on the tested endpoints, alone an in mixtures;
- *In vitro*
  - Both xenobiotics reduced viability in the two amphibian cell lines;
  - The presence of PHB-NPs reduced the overall toxicity of caffeine;
- The *in vitro* methodology demonstrated a lower sensitivity to caffeine exposure than both amphibian early life stages. For PHB-NPs the opposite was observed.

Acknowledgments

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