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Non-destructive biological and chemical traits to track down long term effects of environmentally relevant low concentrations of pharmaceutical mixtures using two freshwater species

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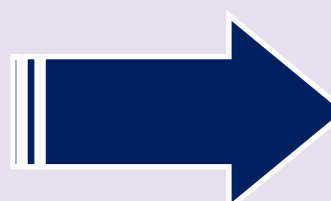
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Context & Objectives

Currently, there are a lot of normalised **acute bioassays** that evaluate ecotoxicity:

- Of single pollutants
- Under short time exposures (72h or 96h)
- At high concentrations (mg.L⁻¹)



There is a **need for new models** to evaluate :

- Pollutant mixtures
- Chronic toxicity
- Low environmental concentrations

Pollutants & Freshwater species

Study of 2 pharmaceuticals

not removed in wastewater treatment plants

Carbamazepine (CBZ), OxCarbazepine (OxCBZ)

and one of their metabolite **Acridine-9-carboxylic acid (9-CAA)**



Lemna minor L.

- ❖ Cosmopolitan species and fast growth rate
- ❖ Normalised bioassay for acute & chronic toxicity

Growth conditions:

Light : 9h/day, Moisture : 60%, Temperature : 15°C, Hoagland media (10%)

- ❖ Optical measurement of early changes in primary and secondary metabolites (anthocyanin, chlorophyll, flavonol, phenol and nitrogen balance indexes)

Published ecotoxicity values

Only For CBZ^{1,2}

Lemna minor L., EC₅₀ : 25.5 mg.L⁻¹, growth inhibition, 7d, (chronic toxicity)

Hydra attenuata Pall., EC₅₀ : 15.52 mg.L⁻¹, 96h (chronic toxicity)

LC₅₀ : 29.4 mg.L⁻¹, 96h (acute toxicity)

Hydra attenuata Pall.



- ❖ Minimized genetic variability (polyps propagate asexually by budding)
- ❖ Diploblastic organisation

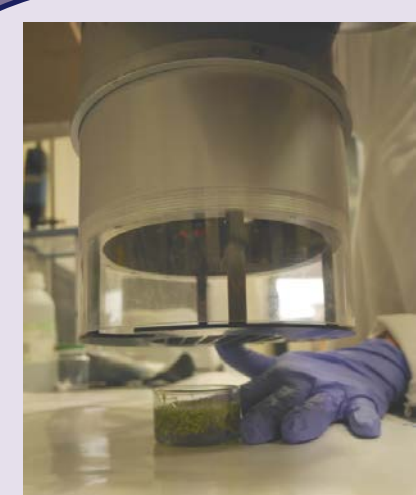
Growth conditions:

Single strain, Light : 12h/day, Temperature : 19.5°C, TES* buffer, EDTA, CaCl₂

- ❖ Monitoring of morphological alterations, reproductive rates, feeding behaviour

* 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid

Results



Compounds	Concentration range		
	600 ng.L ⁻¹	600 µg.L ⁻¹	60 mg.L ⁻¹
CBZ	27 ng.L ⁻¹	270 µg.L ⁻¹	24 mg.L ⁻¹
OxCBZ	900 ng.L ⁻¹	900 µg.L ⁻¹	15 mg.L ⁻¹
Mixture	Σ _{CBZ+OxCBZ+9CAA}		



Compounds	Effluent realistic concentrations
CBZ	605 ng.L ⁻¹
OxCBZ	27 ng.L ⁻¹
9-CAA	900 ng.L ⁻¹
Mixture	Σ _{CBZ+OxCBZ+9CAA}



Phenolic index of compounds and control during a 17 day-long exposure

➤ Phenolic index:

The highest concentration of 9-CAA induced an early metabolic **stress** in *Lemna minor* L. as shown by the increase of the phenolic index probably due to a **defence mechanism**.

For OxCBZ, a slight metabolic stress was observed latter whereas neither CBZ nor the mixture at low concentrations impacted this index.

➤ Nitrogen balance index:

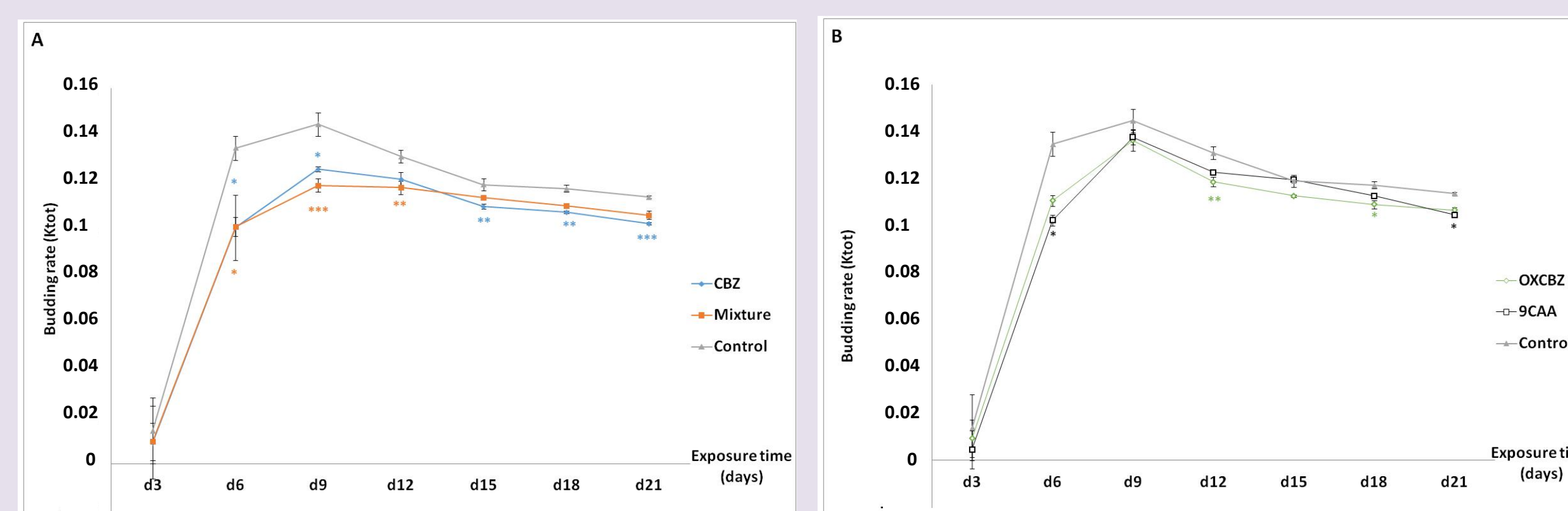
OxCBZ and CBZ at the highest concentrations increased the nitrogen balance index after 11 days. It shows a modification of **allocation of nitrogen and carbon** that may **impact photosynthesis** in of duckweed.

➤ Chlorophyll index:

This primary metabolite was **stable** for all the compounds and their mixture.

➤ Anthocyanin and flavonol indexes were below detection limits.

➤ Morphological changes, reflecting **progressive intoxication**, were observed after only one day of exposure : 21% of polyps exposed to CBZ, OxCBZ or 9-CAA showed first signs of intoxication and 16,6% of polyps exposed to the mixture showed more pronounced signs of intoxication. Interestingly, after 6 days of exposure morphological signs of intoxication tended to disappear and polyps exhibited normal morphological stage until the end of exposure (21 days). These observations suggest that *H. attenuata* possesses **defence mechanisms** to face intoxication.



A-B : Budding rate (Ktot) of exposed polyps and controls followed during 21 days.

K_{tot} values were calculated³ with the following formula $K_{tot} = (\ln(n_{tot}) - \ln(n_0)) / d$
n: number of polyps at the beginning of the experiment (d0); n_{tot}: the sum of attached buds plus the number of polyps (i.e. detached buds and initial polyps) after x days of exposure (d3 to d21).
*:P< 0,05, **:P< 0,01, ***:P< 0,001, significantly different from control (n= 24 in triplicate; Kruskal-Wallis followed by Dunn test).

➤ In exposed polyps, **slowing down and decrease of budding rate were observed** (Figs A-B) that could be related to morphological changes. Indeed, as the decrease of budding rate occurred from the d6 after exposure, i.e. the time required to the *Hydra* polyps to restore their normal morphology, trade-offs between the survival of polyps and the reproductive success of the population could be hypothesized. **As polyps exposed to CBZ and mixture never reached the maximal budding rate observed in control**, these compounds have negative effect on the reproductive success.

➤ Whatever the exposure conditions, all *Hydra* polyps were still able to catch and ingest prey : no alteration of feeding behaviour was observed.

Conclusion

❑ Both models enable to study **non-destructive biological pertinent endpoints**.

- ❑ The macrophyta, *Lemna minor*, showed **deleterious effects only at high concentrations**. This specie does not seem highly sensitive to these compounds under the tested conditions.
- ❑ Bioassays using *Hydra attenuata* showed that the selected **compounds at environmental concentrations induced deleterious effects** at early (morphological changes) and later stages (budding rates). Such negative effects raise the question of the impact on natural populations that are chronically exposed to pollutants.
- ❑ These preliminary results could help to develop **new tools** for water quality monitoring and **for chronic toxicity assessment at realistic environmental concentrations**.

References

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³ De Jong L., Pech N., de Aragão Umbuzeiro G., Moreau X. 2016. Multi-scale biomarker evaluation of the toxicity of a commercial azo dye (Disperse Red 1) in an animal model, the freshwater cnidarian *Hydra attenuata*. *Water Research* 96 : 62-73.

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