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Effects of environmental concentrations of chlordecone (insecticide), glyphosate-based herbicide and imazalil (fungicide) in mixture on reproductive rate, morphology and DNA damage in the freshwater *Hydra vulgaris*

Laetitia De Jong, Virginie Tassistro, Maëlys Cadat, Thierry Orsière, Hervé Macarie, Blandine Courbiere, Xavier Moreau

Aix Marseille Université, Avignon Université, CNRS, IRD, IMBE, Marseille, France. (xavier.moreau@imbe.fr)

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INTRODUCTION

Between 1972 and 1993, 300 tons of the organochlorine insecticide chlordecone ($C_{10}Cl_{10}O$) were dumped in the banana plantations of the French West Indies (FWI) to eradicate the black banana weevil (*Cosmopolites sordidus*). In 1999, the massive water pollution in the FWI was revealed, along with the contamination of most of the population. Today, almost all of the water and soil is still contaminated and will remain so until remedial action is taken, if at all. The results of a monitoring conducted by the French Water Board showed that 130 phytosanitary products were detected in FWI rivers: 40% herbicides, 30% insecticides and 23% fungicides (Defontaines & Mottes, 2017). Among them, the herbicide glyphosate and the fungicide imazalil were the still frequently detected (Wintz & Pak, 2021). The aim of this work was to evaluate, in the cnidarian *Hydra vulgaris*, Pallas, 1766, the effects of a chronic exposure of mixtures containing chlordecone (CLD), imazalil (IMAZ) and a glyphosate-based herbicide (GBH) at realistic environmental concentrations as found in FWI rivers on reproduction rate, morphology alteration and DNA strand breaks.

MATERIALS & METHODS

Chronic toxicity assay (14 days)

Healthy non-budding *Hydra vulgaris* polyps were placed in well of a 6 well microplates either in 6 mL of TES buffer (0.1 mM, pH 7: negative control) or of pesticides in mixture or alone at the same respective concentrations (Table 1). Herbicide concentrations refer to nominal concentrations (µg/L) of the active ingredient (a.i.) glyphosate-isopropylammonium. Polyps were exposed during 14 days at 22 ± 0.1°C under a 12-12h light-dark cycle. During the bioassay, polyps were fed every 3-4 days with living *Artemia* sp. larvae with a renewal of solutions. At the end of exposure period, the morphology and the asexual reproduction rate were determined. Then, polyps were immediately treated for comet assay to evaluate primary DNA lesions.

Table 1

Name of Mixtures	CLD µg/L	IMAZ µg/L	GBH µg a.i./L
M1	0.5	1.5	1
M2	0.5	3	2
M3	10	1.5	1
M4	10	3	2
M5	20	1.5	1
M6	20	3	2

Asexual Reproductive Hydra Rate (RHR) (Fig. 1)

The RHR was calculated according the following formula: $RHR = ((N_{14} - N_0) / N_0) \times 100$

with Nx number of Hydra polyps at day 14, and N0 number of Hydra polyps at day 0 (Colpaert et al. 2020)

Morphological Alteration Index (I_{MA}) (Fig.2)

As toxicity increases, morphological signs can be observed. Six morphological stages ranging from *a* (normal morphology) to *f* (decay) have been described. Stages *b* to *d* are reversible and are considered sublethal, while irreversible stages *e* and *f* are considered as lethal (Colpaert et al. 2020). The I_{MA}, proposed here for the first time, considers the percentage of each morphological stage after 14-day exposure and the severity of morphological damage reflecting toxicity: $I_{MA} = \sum (S_{MA} * w_i)$ *with* S_{MA} *= a to f morphological stage (%);* w_i *= severity factor of each stage:* $w_a = 1$; $w_b = 1.25$; $w_c = 1.5$; $w_d = 1.75$; $w_e = 2$; $w_f = 2$

DNA strand breaks: comet assay (Fig. 3)

The hydra were dilacerated with trypsin-EDTA allowing the cell individualization. Cells were embedded in an agarose gel and placed on a microscope slide. They were lysed with an alkaline agent (pH 10) to release the nuclei. The nuclei were then incubated in a basic electrophoresis buffer (pH 13) to facilitate denaturation, untwisting of the helix and exposure of alkali sensitive sites. The released DNA is subjected to electrophoresis and then revealed by a fluorescent intercalant (propidium iodide). Intact DNA appears as a sphere (Fig 3A). Damaged nuclei appear as comets, the distal part of which is proportional to the number of strand breaks (Fig 3B). DNA damage assessed by quantification of %Tail DNA (Komet 7 Andor technologies).

RESULTS & INTERPRETATIONS

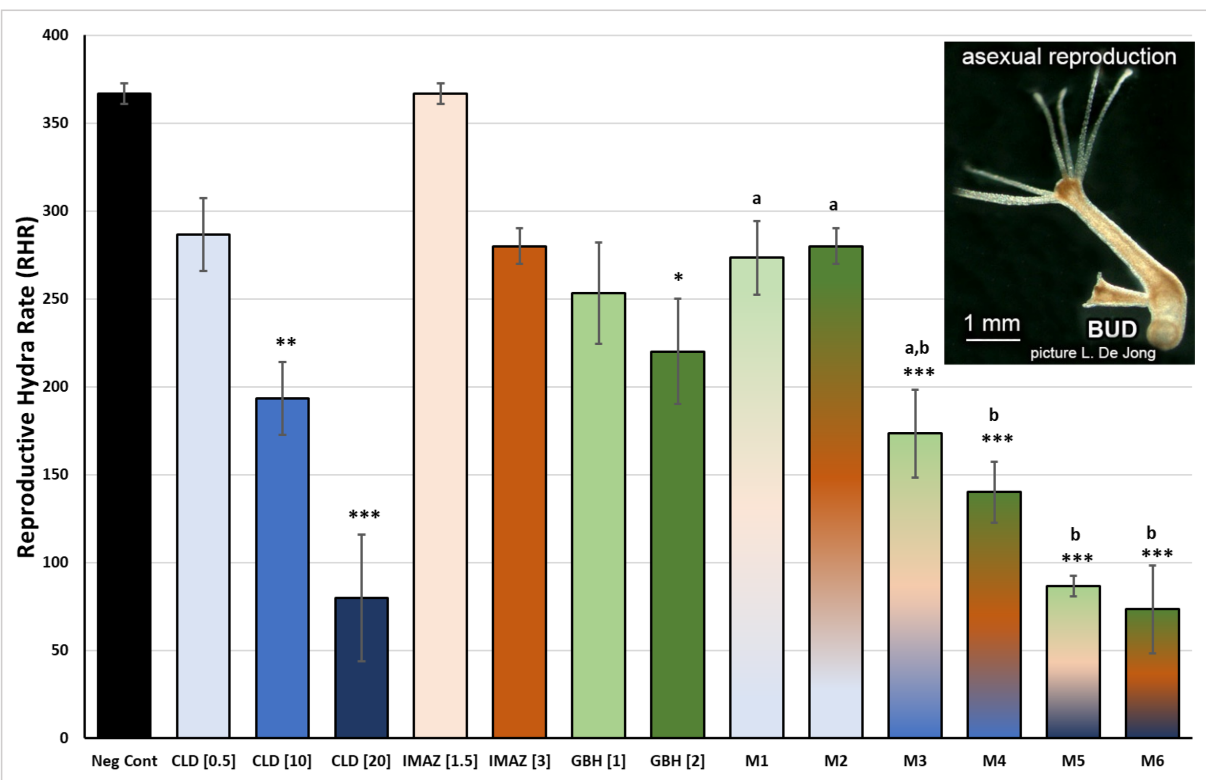

Fig 1. Reproductive Hydra Rate (RHR).
A similar and significant decrease of RHR is observed after exposure to CLD[10], CLD[20], M3, M4, M5 and M6. A significant but lower decrease is observed after exposure to GBH[2].

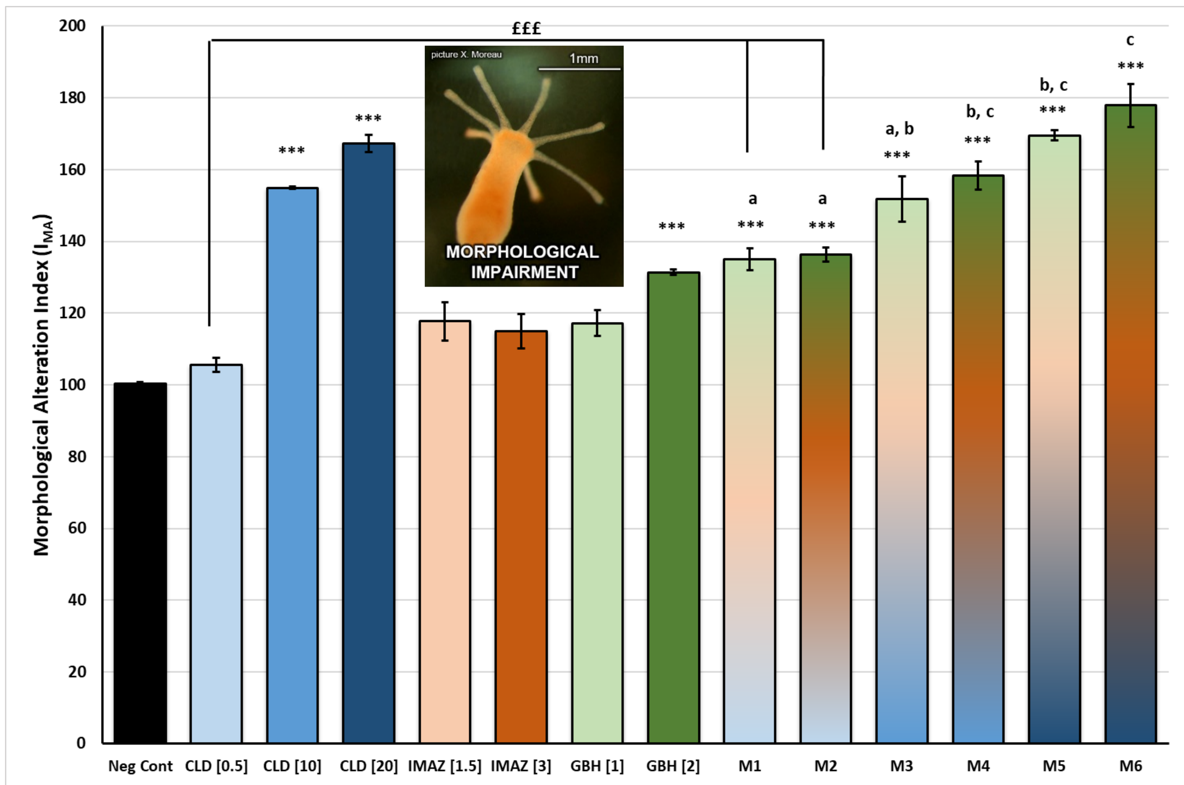

Fig 2. Morphological Alteration Index (I_{MA}).
A significant increase of I_{MA} was observed after exposure to CLD[0.5], CLD[10], GBH[2] and all tested mixtures. Among the mixtures, M6, which contains 20 µg/L of CLD, was the most toxic but I_{MA} was not different from CLD[20] alone. Morphological alterations after exposure to M2, containing 2µg/L of GBH, were not different from hydra exposed to GBH[2] alone. Interestingly, M1 altered the hydra morphology while the 3 pesticides tested alone at the same concentration did not induce a morphological alteration.

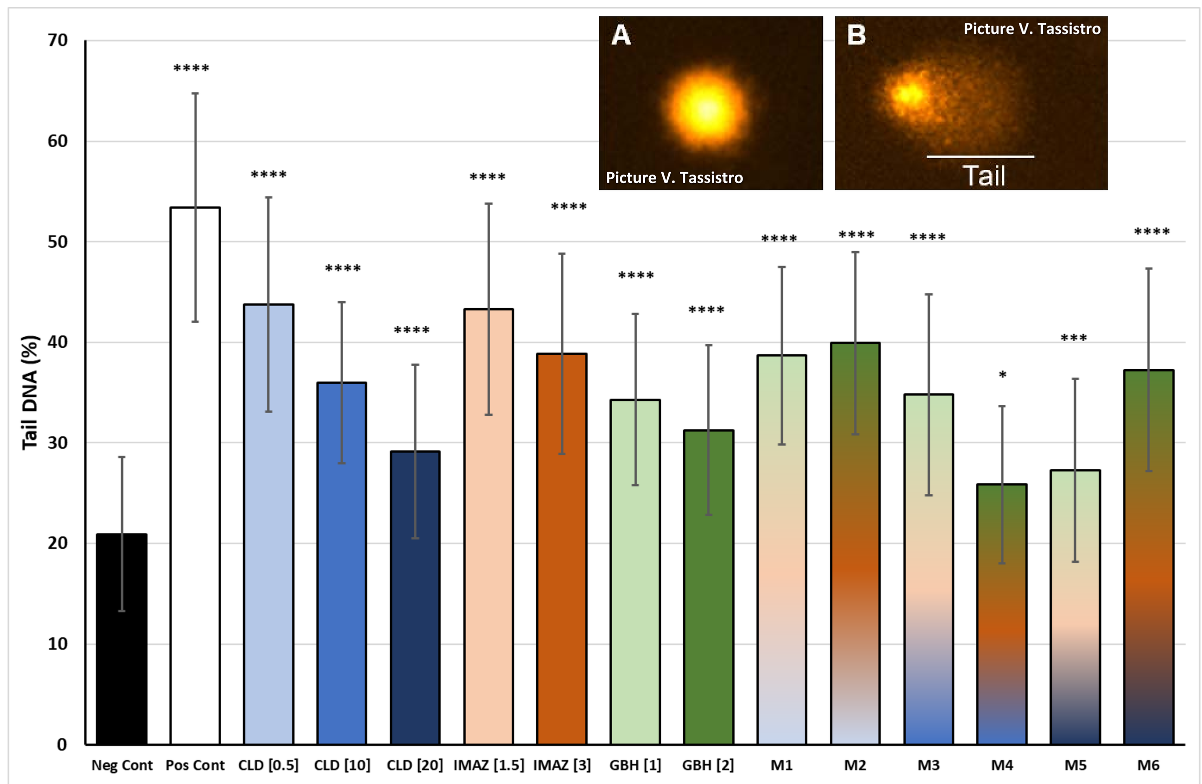

Fig 3. DNA damage assessed by the comet assay measuring tail DNA (%). All exposure conditions resulted in significantly higher DNA damage than in the negative controls.

Legends for figures 1 to 3:
CLD[0.5], CLD[10], CLD[20] correspond to 0.5, 10 and 20 µg/L of CLD
GBH[1] and GBH[2] correspond to 1 and 2 µg a.i. /L GBH
IMAZ[1.5] and IMAZ[3] correspond to 1.5 and 3 µg/L of IMAZ
For composition of mixtures (M1 to M6) see Table 1

All values were compared by one-way ANOVA followed by post-hoc Tukey HSD test for paired comparisons.
Statistical significance between exposed groups and negative controls are indicated by asterisks (p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001); Different letters indicate a significant difference between groups exposed to mixtures. £££ indicates significant difference (p< 0.001) between groups exposed to mixtures and those exposed to their respective CLD concentration alone.*

- ✓Regardless tested CLD concentration, **mixtures do not impact reproduction more than CLD alone**.
- ✓Morphology results suggest **a coalition effect of all three pesticides in mixtures at the lowest concentrations** tested (M1). They show the importance of studying mixtures at low environmental concentrations especially when the compounds tested separately have no effect on the evaluated biological endpoint. The other mixtures have no greater morphological impact than CLD alone, except for M2 which contains 2µg a.i. /L GBH. However, morphological alterations in M2 are not different from those observed in hydra exposed to 2 µg a.i. /L GBH (GBH[2]).
- ✓The results of the comet assay are difficult to interpret and the fact that all conditions induce primary DNA damage does not necessarily mean that the compounds alone or in mixtures are genotoxic. The lesions observed after 14 days may be due to indirect factors, *e.g.,* oxidative stress and the poor cellular state of the hydra, whose morphology is very altered.

CONCLUSION & PERSPECTIVES

Multiscale studies evaluating multiple biological responses, from the molecular to the population level, should be favored in environmental toxicology, as they help define the mode of action explaining the observed physiological perturbations. Since the induction of DNA damage appears to be influenced by cytotoxicity, more research is needed before a conclusion can be drawn on the issue of genotoxicity. The next steps will be (i) short exposure (2h, 24h) to analyze DNA damage before cytotoxicity appears; (ii) similar chronic exposures to very low environmental concentrations of CLD-containing pesticide mixtures to explore coalition effects. Taken together, these preliminary results, obtained in *Hydra vulgaris*, suggest that CLD in these mixtures is the agent inducing most of the observed toxicity and that remediation programs to remove CLD *in situ* would be valuable for environmental quality in the FWI.

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