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# Hydra bioassay for the evaluation of chlordecone toxicity at environmental concentrations, alone or in complex mixtures with dechlorinated byproducts: experimental observations and modeling by experimental design

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## Abstract

In chlordecone (CLD)-contaminated soils of the French West Indies, if microbial remediation or a physicochemical remediation process, e.g., *in situ* chemical reduction, is implemented, concentrations of degradation byproducts, such as hydrochlordecones, are expected to increase in the ecosystems. To study their impact in mixtures with CLD, bioassays were carried out. They consisted in evaluating the regenerative capacity of hydra polyps, from a clone whose phylogenetic analysis confirmed that it belonged to the species *Hydra vulgaris* Pallas, 1766. Hydra gastric sections were exposed to CLD alone or CLD plus dechlorinated byproducts (CLD-BP) for 96 h to assess regeneration. Based on chromatographic analysis, the CLD-BP mix was composed of the 5-monohydrochlordecone isomer (CAS nomenclature), four dihydrochlordecone isomers, and one trihydrochlordecone isomer representing 50%, 47%, and 3% of the total chromatographic area, respectively. A total of 18 mixtures of CLD and CLD-BP were tested. Six environmental concentrations of CLD ( $2.10^{-4}$   $\mu\text{M}$  to  $4.10^{-2}$   $\mu\text{M}$ ) and a similar range of CLD-BP were used. Results from exposures to CLD alone showed the following: (i) a significant decrease in the regenerative capacity of hydra, except at the lowest concentration ( $2.10^{-4}$   $\mu\text{M}$ ); (ii) a concentration-independent deleterious effect. The regeneration scores obtained after the exposure to the addition of CLD-BP were not significantly different from those obtained after exposure to CLD alone. Using an experimental design, a modeling of the regeneration scores of hydra exposed to mixtures is proposed. Interpreted carefully, since they are limited to only one type of bioassay, the present results suggest that the situation in the aquatic environments should not become worse in terms of toxicity, if soil remediation programs resulting in the formation of hydrochlordecones are put in place.

**Keywords** Hydra regeneration · Ecotoxicity · Freshwaters · Insecticide · Organochlorine · Modeling · Experimental design

## Introduction

Chlordecone ( $\text{C}_{10}\text{Cl}_{10}\text{O}$ , CLD) is a persistent organochlorine insecticide which was formerly manufactured in the USA under the trade name of Kepone®. One of the main uses of CLD was to control black banana weevil populations. For this specific use, the CLD was formulated as a very fine powder diluted to 5% by weight in a mineral matrix, which was applied to the soil surface around the banana pseudostem (Clostre et al. 2014a; Epstein 1978). Due to the mismanagement of the production process, most of the workers making CLD in the USA were poisoned, which led to the complete ban on the production, marketing, and use of CLD in that country in 1977 (Cannon et al. 1978; Dawson et al.

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Jean-Pascal Andraud, Hervé Macarie, Daniel E. Martínez, Maxime Robin, and Michelle Sergent have contributed equally to the work and are listed in alphabetical order.

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1979). In 1982, to cope with the shortage of CLD, a French consortium of banana producers managed to restart the production of this molecule under a new brand, Curlone®, with the aim of using it mainly in the French West Indies (FWI). It is estimated that approximately 17% (i.e., 300 t) of the world's CLD production was used in FWI from 1972 to 1993, either in the form of Kepone® or Curlone® (Le Déaut and Procaccia 2009; Devault et al. 2016). Due to its physicochemical properties, in particular a strong affinity for organic matter, CLD is very persistent in soils. In FWI, if no remediation action is taken and considering that natural *in situ* degradation of CLD is negligible, it has been estimated that, depending on soil composition, 60 to 700 years would be required for its near complete disappearance (Cabidoche et al. 2009). These duration estimates have recently been lowered (Comte et al. 2022) to account for natural degradation of CLD for which evidence has accumulated over the past years (Chevallier et al. 2019; Lomheim et al. 2020; Macarie et al. 2016). And although these new estimates predicted that all the soil types would be decontaminated by the 2070s (Comte et al. 2022), the fact remains that the FWI population will continue to be exposed to this persistent organochlorine insecticide over the next three to five decades. Due to rainfall resulting in leaching, runoff, and erosion, CLD is extracted from the soil, and although it has low water solubility (Dawson et al. 1979), it reaches surface and groundwater and even reaches coastal waters (Crabit et al. 2016; Mottes et al. 2020). Through its presence in the environment, it contaminates the entire food chain, i.e., crops, farm animals as well as terrestrial, freshwater, and marine fauna, and also humans (Cabidoche and Lesueur-Jannoyer 2012; Coat et al. 2011; Dromard et al. 2019; Dyc et al. 2015; Jondreville et al. 2014; Lavison-Bompard et al. 2021; Méndez-Fernandez et al. 2018).

The latest study, carried out in 2013–2014, to assess the level of impregnation of the FWI population showed that CLD was present in the blood of more than 92% of the inhabitants of Guadeloupe and Martinique (Dereumeaux et al. 2020). These populations fear for their health since this compound is recognized as “likely to be carcinogenic to humans” (US EPA 2009) and is known as a neurotoxicant and endocrine disruptor (ATSDR 2020). To avoid human exposure to CLD through trophic transfer, the authorities have taken measures such as installing activated carbon filters in water purification plants, restricting the type of crops according to the level of soil contamination, banning fishing in rivers and coastal areas, closing most aquaculture farms, and strengthening carcass control in slaughterhouses. The economic impact of these measures has created a social crisis within the FWI. Because soil is the primary source of contamination in the FWI, several remediation processes have been tested to address the problem: (i) addition of CLD adsorbents to soil (activated carbon, biochar, compost) to decrease or eliminate CLD transfers to

water, crops, and breeding animals (Clostre et al. 2014b; Ranguin et al. 2020); (ii) extraction of CLD from soil using plants (Liber et al. 2018), and (iii) destruction of CLD by microbial or physicochemical processes (Chevallier et al. 2019; Lomheim et al. 2020; Macarie et al. 2016; Mouvet et al. 2017). To date, of all these approaches, beside the addition of compost, the physicochemical *in situ* chemical reduction (ISCR) process, which consists of adding Fe<sup>0</sup> to the soil, is the only one that has been tested with promising results under real environmental conditions in FWI, up to the plot scale (Mouvet et al. 2020). Indeed, nearly 70% of CLD removal was achieved on a nitisol and an alluvial soil allowing the cultivation of radishes, cucumbers, and sweet potatoes and often in compliance with the residue limits considered as safe for human health according to the French regulations (Mouvet et al. 2016, 2020). The ultimate fate of CLD during this process remains to be established, but it is known to generate a series of dechlorinated byproducts, called hydrochlorocones. These byproducts, which have kept the bishomocubane structure of the parent molecule, could lose up to 7 chlorine atoms that are replaced by hydrogen atoms. In the absence of complete mineralization, there is always a risk that the byproducts may retain a similar toxicity or acquire a higher toxicity than the parent molecule (Benoit et al. 2017; Dolfin et al. 2012). To answer this question, the genotoxic, mutagenic, and proangiogenic<sup>1</sup> properties of 3 major CLD derivatives, formed during the ISCR process (i.e., isomers having lost 1, 3, and 4 chlorine atoms), were compared to those of CLD (Alibrahim et al. 2020; Legeay et al. 2017). These studies showed that, like CLD, these derivatives were non-genotoxic and non-mutagenic, while their proangiogenic properties tested both *in vitro* and *in vivo* were strongly reduced. These studies are fundamental to assess the respective toxicity of each byproduct, but because the compounds are tested individually, no information can be given about their possible effects when they are found together in the environment. Indeed, during the ISCR process, their simultaneous presence in soil, in proportions that may fluctuate over time, has been reported (e.g., Mouvet et al. 2020). In addition, these byproducts will be transported to other environmental compartments as mixtures in water (Ollivier et al. 2020a, b) and chemical interactions could occur. For example, Yang et al. (2017) using complex insecticide mixtures (i.e., neonicotinoid-organophosphorus-organochlorine-carbamate) reported synergistic effects. Thus, it can be hypothesized that due to potential chemical interactions, mixtures containing CLD and its byproducts may have a greater toxic effect than CLD alone. To evaluate this possibility, ecotoxicity tests were performed on hydra. Prior to our ecotoxicological investigations, we performed a phylogenetic analysis of our clone to confirm its belonging to the species *Hydra vulgaris* Pallas,

<sup>1</sup> Ability to promote vascularization and tumor growth.

1766. Using 96-well polycarbonate microplates, the ecotoxicity bioassays consisted of evaluating the regenerative capacity of gastric sections of hydra exposed to CLD alone or to 18 complex mixtures of CLD and dechlorinated byproducts (CLD-BP). Six environmental concentrations of CLD (ranging from  $2.10^{-4}$   $\mu\text{M}$  to  $4.10^{-2}$   $\mu\text{M}$ ) were selected. These CLD concentrations were in the range of those measured in FWI surface freshwaters (Lesueur-Jannoyer et al. 2016; Mottes et al. 2020). The CLD-BP mix used here contained the 5-monohydrochlordecone (CAS nomenclature) isomer, four dihydrochlordecone isomers and one trihydrochlordecone isomer. In the 18 mixture combinations, the total molar concentrations of hydrochlordecones were in a similar range to that of CLD. The CLD-BP used in our study could be considered as a good proxy of the cocktail derivatives observed during CLD treatment by ISCR (Belghit et al. 2015; Mouvet et al. 2020). Hydra was chosen as the freshwater animal model for the study because it has already been used in recent work to assess the effect of long-term (14 days) CLD exposure on several biological markers such as stress gene expression, oxidative stress parameters, and reproductive rate (Colpaert et al. 2020). The relevance of this choice was recently reinforced by the finding that *Hydra* species are ubiquitous in FWI surface waters (Macarie and Martinez 2019).

This study was conducted to answer the following fundamental questions: (i) Are environmental concentrations of CLD, which could be found in FWI freshwaters, capable of causing poor regenerative capacity of hydra? (ii) What is the effect of mixtures of CLD and CLD-BP on the regenerative capacity of the hydra? (iii) Is the regenerative capacity of hydra more affected by mixtures of CLD and CLD-BP than by CLD alone? (iv) How can hydra regeneration scores be modeled to investigate the most likely effects of increased CLD-BP mixed with environmental concentrations of CLD, which have been found in FWI rivers? To answer this last question, a modeling of hydra regeneration scores was performed using an experimental design, which allows (i) to limit the number of experimental conditions and thus considerably reduce the amount of waste generated during the experimental procedure and (ii) to gain the most information from a limited, well-chosen number of mixtures with varied concentrations of components. This method contrasts with many classical methods applied in ecotoxicology and environmental toxicology, where one concentration is changed at a time while keeping the others constant.

## Materials and methods

### Hydra clone culture conditions

The hydra clone (strain IMBE1) was not collected in FWI. As far as we know, this clone has been used in several

laboratories in North America and Europe since at least the last four decades (first mention in the literature by Johnson in 1980) and has not been exposed to CLD previously. Therefore, it could not have developed tolerance to CLD. The hydra clone (strain IMBE1) was reared in TES buffer (0.1 mM; pH 7) (Sigma-Aldrich, Saint Quentin-Fallavier, France) at  $20 \pm 0.1$  °C and under a 12/12 h light-dark cycle according to the procedure of De Jong et al. (2016) which was adapted from Trottier et al. (1997). Polyps were fed *ad libitum* every three to four days with nauplii of *Artemia* sp. hatched within 24 h. All specimens used in this experiment were derived from asexual reproduction and belong to the same clone.

### DNA extraction, sequencing, and phylogenetic analysis

DNA from the IMBE1 hydra clone was extracted using the QIAGEN DNAeasy Blood & Tissue Kit following manufacturer's protocol (final elution was 150  $\mu\text{L}$ ). Ribosomal DNA (the ITS region comprising 18S partial; ITS1, 5.4S complete, ITS2 and 28S partial) was amplified by PCR (94 °C for 3 min; and 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 60 s, extension at 72 °C for 90 s). The amplified fragment was verified by 1% agarose electrophoresis and cloned into a PROMEGA pGEM-T Easy vector (ligation was performed overnight at 4 °C). Sequencing was carried out by Eurofins Genomics.

The IMBE1 ITS sequence was first aligned with CLUSTAL Omega to hydra sequences from all 4 major clades of hydra: Viridissima, Braueri, Oligactis, and Vulgaris (Martínez et al. 2010). Once the specific hydra group was determined, a second alignment was performed to investigate the geographic origin of the IMBE1 strain. This second alignment included a total of 76 hydra ITS sequences, 7 of which were used as outgroups in the phylogenetic analyses. A maximum likelihood analysis of the phylogenetic relationships was implemented using Garli 2.0 (Zwickl 2006). For this analysis, the invariant, and hence uninformative, 5.4S portion of the sequence was not included in the alignment. The best likelihood model was selected based on the Akaike information criteria (AIC; Akaike 1987) using JModelTest (Guindon et al. 2010; Posada 2008). We used FigTree (version 1.3.1; Rambaut 2009) to plot the best phylogram produced by Garli. Maximum likelihood bootstrap values were calculated based on 1000 pseudo replicates using IQtree (Hoang et al. 2018; Nguyen et al. 2015) and Garli. Neighbor Joining bootstrap values were calculated based on 1000 pseudo replicates using PAUP (Swofford 2003).

## Chemicals and reagents

CLD was provided by Azur Isotope (Marseille, France) in powder form with a purity greater than 97%. The mix of CLD-BP was synthesized by Alpha Chimica (Châtenay-Malabry, France) and was also provided in powder form. The product was received labeled as a sample of a trihydrochlordecone isomer with a chromatographic purity (GC-EI-MS)<sup>2</sup> of 90%. To verify the purity of this powder, we performed a LC-MS analysis<sup>3</sup>. The analysis was done with a UHPLC instrument (Dionex Ultimate 3000 equipped with RS pump, autosampler, thermostatically controlled column compartment and UV diode array, Thermo Scientific, USA) coupled to a precision mass spectrometer (QqToF) equipped with an ESI source (Impact II, Bruker Daltonics, Germany). UHPLC separation was performed on an Acclaim C18 column (100 mm × 2.1 mm, 2.2 μm, Thermo Scientific, USA). The injection was set at 1 μL and the elution rate at 0.8 mL/min at a constant temperature of 40 °C. The chromatographic solvents were composed of A: water (LCMS grade, Carlo Erba) and B: acetonitrile (LCMS grade, Carlo Erba). The chromatographic gradient was defined as follows: 10% B for 2 min, then increasing the proportion of solvent B to 100% at 15 min. Each analysis was followed by a return to initial conditions phase for 1 min, and column equilibration for 4 min for a total run time of 20 min. The mass spectrometer parameters were set as follows: nebulizing gas, N<sub>2</sub> at 1.2 bar; dry gas, N<sub>2</sub> at 12 L/min, capillary temperature at 200 °C and voltage at 4500 V. The mass spectrometer was calibrated with a cluster-forming formate/acetate solution over the mass range of interest before analysis. The mass spectra were recorded in negative mode in full scan mode from 50 to 1200 amu at 2 Hz. Surprisingly, LC-MS analysis of the CLD-BP product synthesized by Alpha Chimica revealed the presence of 6 compounds with monoisotopic *m/z* characteristics of hydrochlordecone gem-diols in different proportions, estimated as percentage of total peak area: 3% of a trihydrochlordecone isomer (CLD-3Cl, peak 1, *m/z* 400.8036), 47% of four dihydrochlordecone isomers (CLD-2Cl, peaks 2, 3, 4, 5, *m/z* 434.7650) and 50% of a monohydrochlordecone isomer (CLD-1Cl, peak 6, *m/z* 468.7260) (Fig. 1). The retention times of the CLD-1Cl and CLD-3Cl isomers obtained by LC-MS were identical to those of the pure standards provided by Alpha Chimica (data not shown) which are known to be identical to the CLD-1Cl and CLD-3Cl isomers generated in the soil by the ISCR process (Sébastien Bristeau, BRGM, France, personal communication). However, these pure standards were not available in sufficient quantity to

be used in the hydra exposure experiments. Further analysis of the Alpha Chimica CLD-BP product by GC-EI-MS (data not shown) confirmed that the mass spectrum of the CLD-1Cl isomer was typical of that of 5-monohydrochlordecone (CAS nomenclature) generated by the ISCR process or microbial degradation (major fragments at *m/z* 202 and 238 and presence of the molecular ion of CLD-1Cl that has lost one Cl at *m/z* 420.7; Belghit 2014; Chevallier et al. 2019; Mouvet and Bristeau 2012). Overall, the composition of the CLD-BP product provided by Alpha Chimica could be considered as a good proxy of the cocktail of derivatives that can be formed during CLD treatment by ISCR (Belghit et al. 2015; Mouvet et al. 2017, 2020).

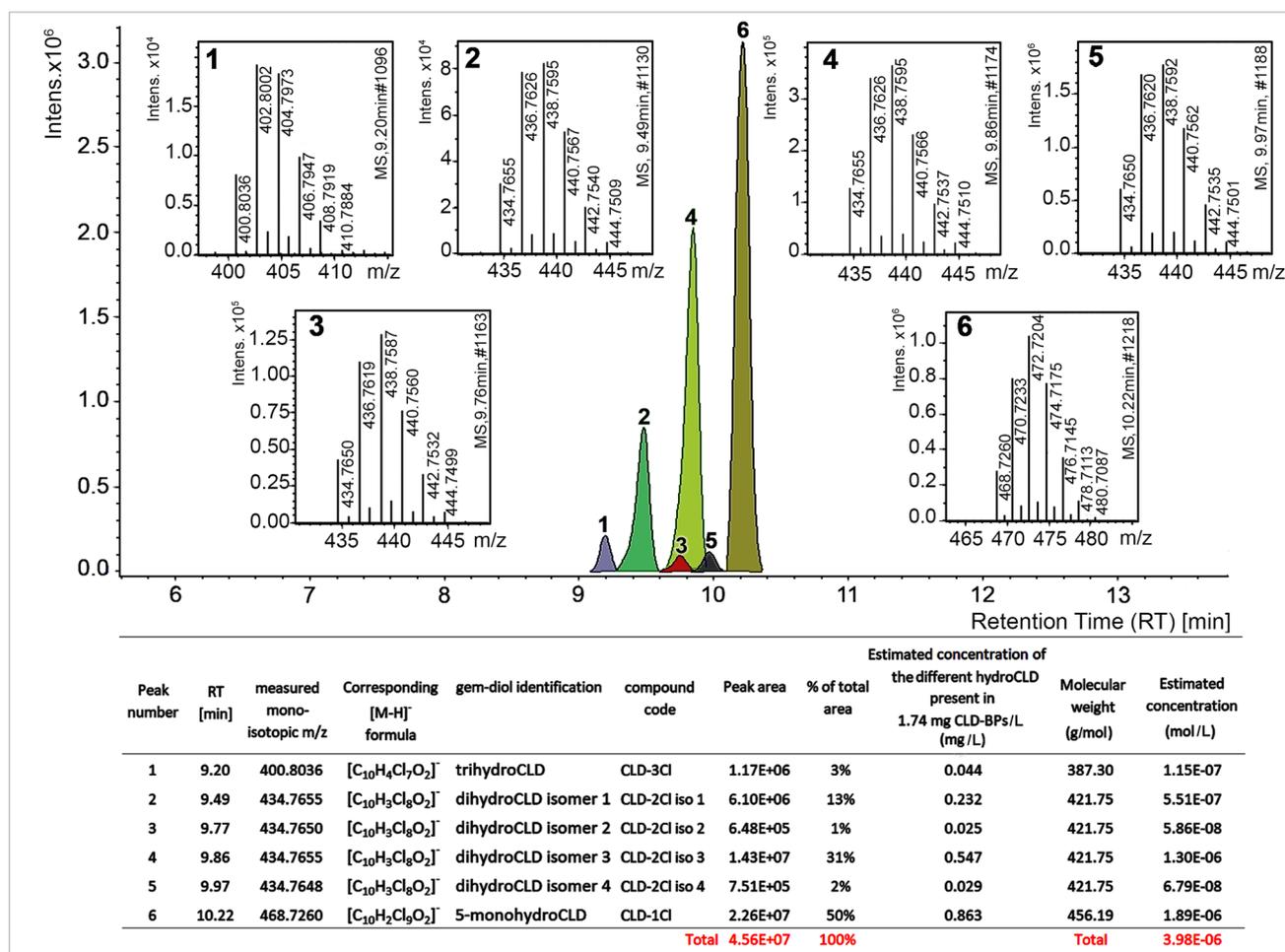
Stock solutions of CLD and CLD-BP were prepared at 1 mg/L (2 μM) of CLD or 1.74 mg/L (about 4 μM) of CLD-BP in pure water. For CLD-BPs, the molar concentration was estimated according to the following formula: Total molar concentration =  $\sum$  (total mass-weighted CLD-BP) × (% chromatographic area of a specific hydrochlordecone isomer)/(molecular weight of the corresponding hydrochlordecone isomer) (Fig. 1). In exposure experiments, the stock solutions of CLD and CLD-BP were freshly diluted in the hydra breeding media, i.e., 0.1 mM TES buffer (Sigma-Aldrich, Saint Quentin-Fallavier, France), to reach the target concentrations. The concentrations of CLD and CLD-BP used in bioassays are shown in “Toxicity evaluation” and “Experimental design” sections.

## Toxicity evaluation

The regenerative capacity of hydra was assessed using a score scale ranging from 0 (dead polyp) to 10 (healthy and completely regenerated polyp). Our score scale was constructed according to score scales proposed in previous studies (Pachura-Bouchet 2005; Park and Yeo 2012; Quinn et al. 2008; Vasseur and Pachura 2006; Wilby and Tesh 1990) and considers delayed regeneration, abnormalities, and morphological alterations due to toxicity of exposure conditions (Table 1). One day prior to the start of the experiment, the population was fed with 24-h-old hatched *Artemia* sp. nauplii. Budding polyps of similar size were randomly collected from a dense, healthy population. Dissection was performed under a stereomicroscope. Only the central section of the polyp, known as the gastric region, was retained to assess the regenerative capacity of the hydra (Fig. 2). Each gastric region was carefully placed in a well of a 96-well microplate in 250 μL of TES buffer (0.1 mM; pH 7) for controls or in 250 μL of solutions containing CLD diluted in TES buffer or in 250 μL of solutions containing CLD in mixture with CLD-BP diluted in TES buffer (see “Chemicals and reagents”). Finally, the 96-well microplates were placed in a temperature-controlled incubator at 20 ± 0.1 °C under a 12/12 h light-dark cycle for 96 h.

<sup>2</sup> GC-EI-MS: Gas Chromatography – Electronic Impact – Mass Spectrometry.

<sup>3</sup> LC-MS: Liquid Chromatography – Mass Spectrometry.



**Fig. 1** Deconvoluted chromatogram of the 6 hydrochlordecone isomers detected in a water/acetonitrile (50/50 v/v) solution of Alpha Chimica product (CLD-BP) analyzed by LC-MS. The insets in the graph are the isotopic distribution of the [M-H]<sup>-</sup> mass spectra of the 6 peaks on which the raw formula of the corresponding compounds

and their identification were based. The peak area and estimated concentration of each of the hydrochlordecones (hydroCLD) present in the stock solution of CLD-BP used for the hydra exposure experiments are presented in the table below the chromatogram

The choice of the working range of CLD and CLD-BP concentrations is explained in the “Experimental design” section. The following molar concentrations of CLD were used:  $2.10^{-4}$ ,  $2.88.10^{-3}$ ,  $1.02.10^{-2}$ ,  $2.04.10^{-2}$ ,  $3.06.10^{-2}$ ,  $4.10^{-2}$   $\mu\text{M}$ . CLD was used either alone or in 18 mixture combinations containing various concentrations of CLD-BP (Table 2). Considering the relative proportions of the different dechlorinated compounds in the CLD-BP stock solution, the final molar concentrations in the wells were in a similar range to that of CLD. Due to the very low concentrations of toxicants, the small volume of mixtures used, and the complexity of the mixtures, it was not possible to verify the concentrations of compounds in each well by chemical analysis. Number of replicates were the following: eight replicates in control condition, five replicates in CLD exposures, and 3 to 6 replicates in mixture exposures (see Table 2).

## Experimental design

A screening of the results of the different sampling campaigns shows that 56% of the rivers in Martinique contain CLD concentrations ranging from values just above the analytical limit of quantification, e.g., 0.003 up to 20  $\mu\text{g/L}$  and that the annual average of CLD concentrations in all these rivers varies between 0.6 and 0.86  $\mu\text{g/L}$  depending on the year (Arqué and Bocaly 2020; ARS 2018; Cattani et al. 2019; Mottes et al. 2017). Because the idea was to obtain hydra regeneration score values over a range of environmental concentrations of CLD observed in FWI rivers, we chose 0.1  $\mu\text{g/L}$  (i.e.,  $2.10^{-4}$   $\mu\text{M}$ ; limit of water potability) and 20  $\mu\text{g/L}$  (i.e.,  $4.10^{-2}$   $\mu\text{M}$ ) for the lowest and highest CLD concentrations, respectively. A similar range of molar concentrations was used with CLD-BP (Table 2). Because CLD and its dechlorinated byproducts have different molar

**Table 1** Scale of regeneration scores of hydra gastric sections (IMBE1 clone). Under normal conditions, complete regeneration of the polyps is observed in 96 h and the scores (PSR<sub>96h</sub>) reach the maximum value of 10 points. During a 96-h exposure to toxic substances, the regeneration capacities can be negatively impacted leading to a delay and/or a blockage of regeneration resulting in a decrease of the regeneration score. If tentacle malformations are present, points will

be deducted from the observed score, as they reflect signs of toxicity. The absence of a mouth will not result in a score higher than 5. Similarly, impairment of the basic physiological function of osmoregulation of the hydra will not result in a score greater than 5. Finally, an absence of healing will result in a score of 1 and disintegration of the gastric section will result in a score of 0

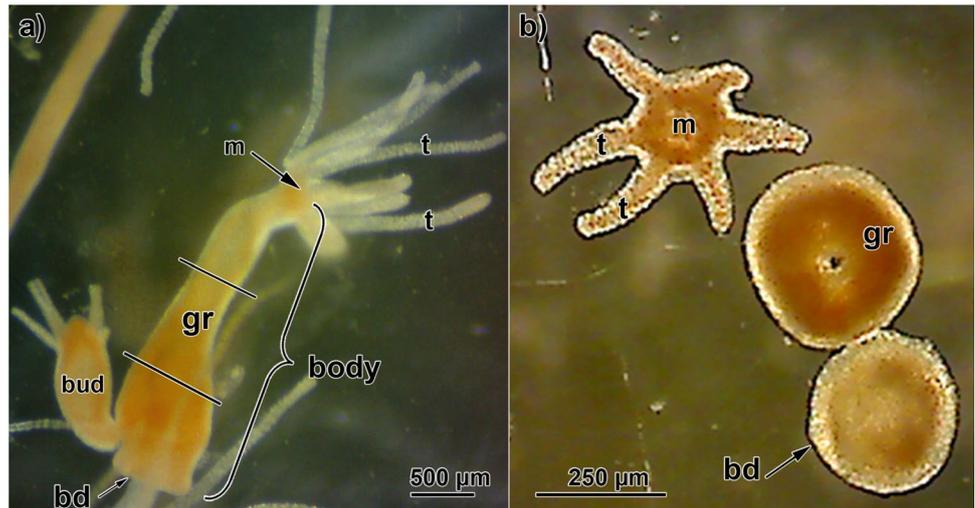
	Shape of polyp at the end of the 96 h exposure time	Regeneration score	
Progressive Steps of Regeneration: PSR <sub>96h</sub>	Mouth, four to height long tentacles (length > ½ of body length), body length > 1.1 mm	10	
	Mouth, four to height long tentacles (length > ½ of body length), body length < 1.1 mm	9	
	Mouth, two to three long tentacles (length > ½ of body length), body length > 1.1 mm	8.5	
	Mouth, two to three long tentacles (length > ½ of body length), body length < 1.1 mm	8	
	Mouth, one long tentacle (length > ½ of body length), body length > 1.1 mm	7.5	
	Mouth, one long tentacle (length > ½ of body length), body length < 1.1 mm	7	
	Mouth, short tentacles, hydra body length > 1.1 mm	6.5	
	Mouth, short tentacles	6	
	Basal disk and short tentacles	5	
	Basal disk and beginning of tentacle	4	
	Basal disk only or beginning of tentacle only	3	
	Normal wound healing	2	
	Signs of toxicity: abnormalities, morphological alterations and loss of physiological functions	no basal regeneration or normal tentacles but at wrong location	PSR <sub>96h</sub> - 1
		Number of long normal tentacles > number of abnormal tentacles (bulbed or split into two...)	PSR <sub>96h</sub> - 1
Number of long normal tentacles = number of abnormal tentacles (bulbed or split into two...)		PSR <sub>96h</sub> - 2	
Number of long normal tentacles < number of abnormal tentacles (bulbed or split into two...)		PSR <sub>96h</sub> - 3	
No mouth, basal disk, long tentacles (length > ½ of body length) and/or body > 1.1 mm		5	
Loss of osmoregulation (bleaching, abnormal conic shape...), mouth		5	
Loss of osmoregulation (bleaching, abnormal conic shape...), no mouth		4	
No wound healing	1		
Disintegrated	0		

These regeneration scores can be interpreted as follows: 0 to 1 = death; 2 to 5.9 = extremely toxic; 6 to 6.9 = very toxic; 7 to 7.9 = toxic; 8 to 8.9 = slightly toxic, viable polyp; 9 to 10 = no toxicity, healthy polyp. In the modeling steps of this study, the experimental value of each regeneration score is called Y<sub>exp</sub>

weights, it was necessary to express the concentrations in μM instead of μg/L to work with comparable concentrations. To allow the possibility of modeling the regeneration scores of hydra exposed to mixtures of CLD and CLD-BP, we used an experimental design. In the experimental design, CLD and CLD-BP concentrations are two predictor variables. Each predictor variable is associated with coded variables (X<sub>1</sub>, X<sub>2</sub>). The lowest level of each coded variable corresponds to - 1, while the highest level corresponds to + 1 and the intermediate coded variables (0.87, - 0.5, 0, + 0.5, + 0.87) are presented in Table 2. Within this experimental range, i.e., domain of interest, the different concentrations

of mixtures between CLD and CLD-BP represent an infinite number of experimental conditions. Therefore, in order to predict the most likely effects of these mixtures in the domain of interest, we chose an empirical model that allows us to gather the maximum amount of information using a minimum number of experimental conditions. Knowing that in hydra CLD exposure can lead to non-monotonic dose-response curves, i.e., a nonlinear concentration-response relationship (Colpaert et al. 2020), we cannot postulate that the result of each experimental condition is a linear combination of the effects of the coded variables X1 and X2. Therefore, instead of using a first-order polynomial model,

**Fig. 2** Stereomicroscopy images of *Hydra vulgaris*. **a** Healthy, budding hydra polyp showing the level of the two transverse sections (black lines) made to obtain the fragment that will allow monitoring of regeneration. **b** The three parts of the polyp obtained after the cross sections. Only the central section of the hydra body, also called “gastric region” (*gr*), is kept for the regeneration experiments. *bd*, basal disk; *m*, mouth; *t*, tentacle



**Table 2** The eighteen mixture combinations (M1 to M18) containing chlordecone (CLD) and dechlorinated byproducts (CLD-BP) at different ratios of concentrations. M1 to M13 (in bold) were used for modeling steps and M1 to M18 for comparisons with the control group and with groups exposed to CLD alone. The byproduct solution (CLD-BP) contains a cocktail of dechlorinated molecules (5-monohydrochlordecone (CLD-1Cl), four dihydrochlordecone isomers (CLD-2Cl), one trihydrochlordecone (CLD-3Cl) at the following ratios, 50%, 47%, 3% respectively, based on chromatographic area)

Name of binary mixture	Coded variables		Experimental conditions		Number of replicates
	X <sub>1</sub>	X <sub>2</sub>	Predictor variables: concentrations in µM		
			[CLD]	[CLD-BP]	
<b>M1</b>	-1	-1	2.10 <sup>-4</sup>	2.10 <sup>-4</sup>	3
<b>M2</b>	+1	-1	4.10 <sup>-2</sup>	2.10 <sup>-4</sup>	6
<b>M3</b>	-1	+1	2.10 <sup>-4</sup>	4.10 <sup>-2</sup>	6
<b>M4</b>	+1	+1	4.10 <sup>-2</sup>	4.10 <sup>-2</sup>	3
<b>M5</b>	-1	0	2.10 <sup>-4</sup>	2.04.10 <sup>-2</sup>	3
<b>M6</b>	+1	0	4.10 <sup>-2</sup>	2.04.10 <sup>-2</sup>	3
<b>M7</b>	0	-1	2.04.10 <sup>-2</sup>	2.10 <sup>-4</sup>	3
<b>M8</b>	0	+1	2.04.10 <sup>-2</sup>	4.10 <sup>-2</sup>	3
<b>M9</b>	0	0	2.04.10 <sup>-2</sup>	2.04.10 <sup>-2</sup>	3
<b>M10</b>	-0.5	-0.5	1.02.10 <sup>-2</sup>	1.02.10 <sup>-2</sup>	3
<b>M11</b>	-0.5	0.5	1.02.10 <sup>-2</sup>	3.06.10 <sup>-2</sup>	3
<b>M12</b>	0.5	-0.5	3.06.10 <sup>-2</sup>	1.02.10 <sup>-2</sup>	3
<b>M13</b>	0.5	0.5	3.06.10 <sup>-2</sup>	3.06.10 <sup>-2</sup>	6
M14	-1	-0.87	2.10 <sup>-4</sup>	2.88.10 <sup>-3</sup>	3
M15	+1	-0.87	4.10 <sup>-2</sup>	2.88.10 <sup>-3</sup>	3
M16	-0.87	-1	2.88.10 <sup>-3</sup>	2.10 <sup>-4</sup>	6
M17	-0.87	+1	2.88.10 <sup>-3</sup>	4.10 <sup>-2</sup>	6
M18	-0.87	-0.87	2.88.10 <sup>-3</sup>	2.88.10 <sup>-3</sup>	6

we postulated a quadratic polynomial model that allows for possible curvature effects (Carboué et al. 2018; Hamrouni et al. 2020). This mathematical model based on two predictor variables, CLD and CLD-BP concentrations, associated with their coded variables X<sub>1</sub> and X<sub>2</sub> was written as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

in which Y expresses the calculated value of the experimental response, i.e., the calculated value of hydra regeneration score.

To estimate the coefficients (b<sub>n</sub>) of the mathematical model, the experimental conditions were carefully chosen by the experimental design. Indeed, the quality of the coefficient estimation and the quality of the prediction depend only on the choice of the experimental points and are independent of the experimental results (Box 1954; Box and Hunter 1957; Droesbeke et al. 1997; Sarabia and Ortiz 2009). From the set of optimal designs for a quadratic model, we chose a centered composite design with nine points (M1 to M9, Table 2). To validate the model, we added four “validation points” within the domain (M10 to M13, Table 2). Only these thirteen mixture combinations (M1 to M13) are needed to predict the most likely effects, i.e., the regeneration scores, after 96 h of exposure to the mixtures in the experimental range. Three steps were required for the modeling. The first step has used the nine composite design points (M1 to M9, Table 2) to calculate the model coefficients (b<sub>n</sub>) by multilinear regression on the coded variables (X<sub>1</sub> and X<sub>2</sub>). In the second step, the experimental values obtained with the “validation points” have been compared to the values calculated using a Student test. At this stage, if a non-significant difference is observed between the experimental and calculated values, then the four “validation points” are included for the calculation of the coefficients (b<sub>n</sub>). The third step has used the Azurad® software

to produce a graphical representation showing the isoscore lines, i.e., the most likely combinations of mixtures leading to similar regeneration scores.

To take this a step further, knowing that the concentrations around  $2.88 \cdot 10^{-3} \mu\text{M}$  of CLD could lead to a “stochastic effect” for several biological parameters after a 14-day exposure of whole hydra polyps (Colpaert et al. 2020), five other mixture combinations were studied (M14 to M18, Table 2). Because these 5 points were not expected in a centered composite design, they were not required in the modeling steps to express the model equation.

Finally, the effects of the 18 mixture combinations were studied as follows: (i) M1 to M13 for the modeling steps to obtain response surfaces, i.e., the most likely isoscore lines of hydra regeneration within the experimental range; (ii) M1 to M18 for comparisons with the control group and with the groups exposed only to CLD.

## Statistical analyses

Shapiro–Wilk tests were performed to check whether the data distributions of the different groups to be compared were normal or not. Because Shapiro–Wilk tests were significant (with at least  $P < 0.05$ ), indicating that the distribution of values was not normal, all values were compared with the Kruskal–Wallis test accompanied by Dunn’s test for pairwise comparisons. Regeneration score values between two groups were compared with the Mann–Whitney  $U$  test. All values are expressed as mean  $\pm$  SEM. For modeling steps, significance of the regression has been tested with a Fisher test and significance of residual values between experimental scores and calculated ones has been tested using Student tests.

## Results

### Phylogenetic affinities and geographical origin of the IMBE1 strain

The observation of the IMBE1 clone nematocysts under the microscope suggested that the strain could belong to the *Vulgaris* clade. However, the morphological characterization of hydra is not always reliable and does not permit to establish the geographic origin of a particular strain. An initial alignment of the IMBE1 ITS region to sequences of hydra from each of the four known clades (Viridissima, Braurei, Oligactis, and Vulgaris, Martínez et al. 2010) unequivocally placed the IMBE1 strain within the Vulgaris group. Fortunately, hydra within this clade show distinct ITS sequence patterns depending on their geographical distribution. We generated a second alignment which included 67 *H. vulgaris* sequences from all continents inhabited by the species. Sequences of 7 strains of the Oligactis clade (sister clade to Vulgaris)

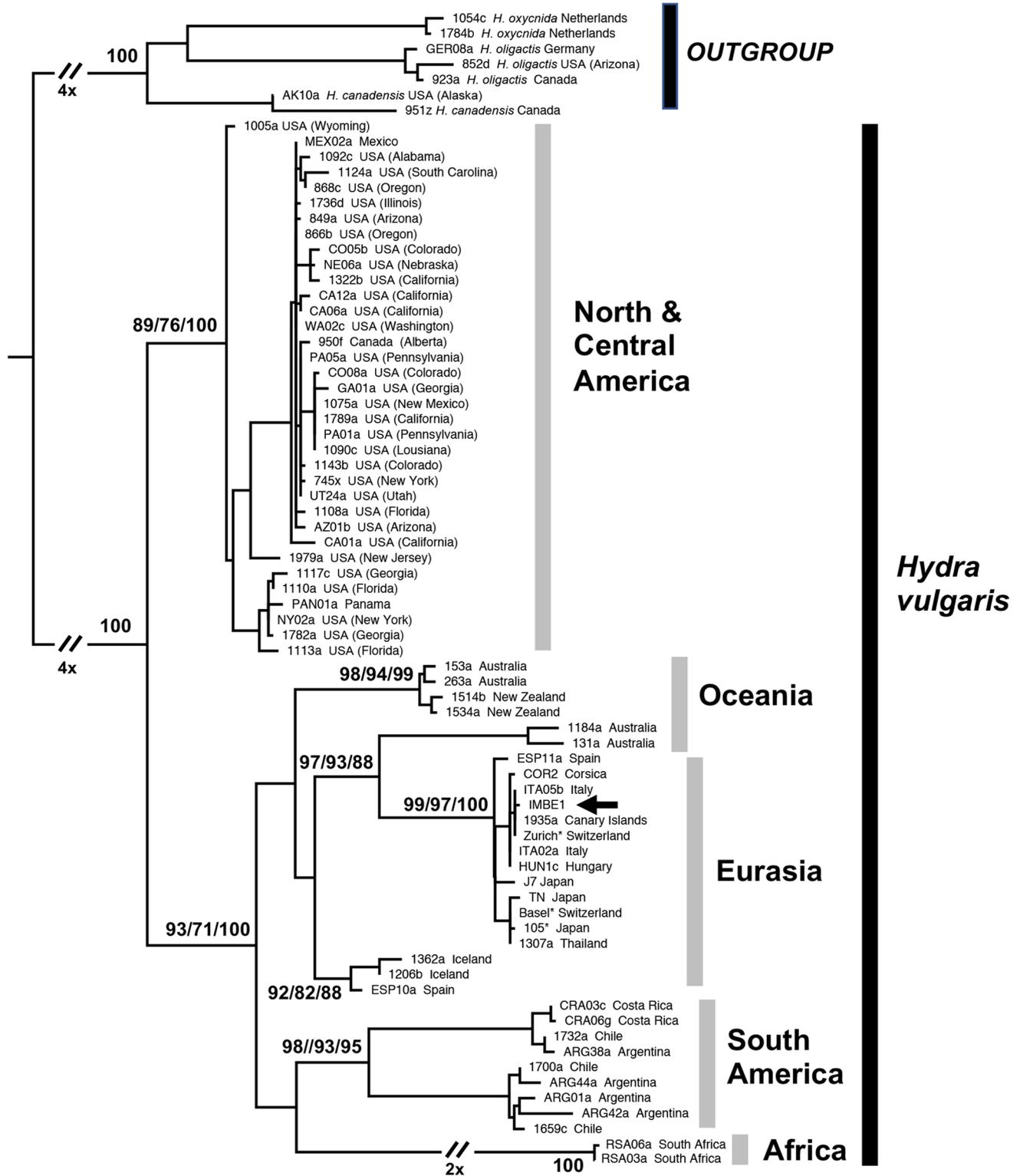
belonging to the three known species in that group were also included as outgroups. Using the Akaike information criteria (Akaike 1987) implemented by JModelTest (Posada 2008; Guindon et al. 2010), we determined that the best substitution model for this data set was HKY+G (Hasegawa et al. 1985). The variation in substitution rates between different sites was Gamma distributed. The maximum likelihood phylogram generated implemented using Garli 2.0 (Zwickl 2006) clearly showed that strain IMBE1 is a Eurasian hydra most likely from Europe (Fig. 3). The bootstrap values calculated by three different methods indicate the topology of the tree is quite robust which adds a high degree of certainty to our conclusion.

### Effects of CLD on the regenerative capacity of hydra

Figure 4 shows the hydra regeneration scores after 96 h of exposure to environmental concentrations of CLD compared with controls. In controls, the mean value of regeneration scores observed after 96 h was  $9.8 \pm 0.5$  ( $n = 8$ ) showing complete regeneration and indicating that the polyp population was healthy at the beginning of the exposures. The mean values of regeneration scores observed after 96 hours were  $8.4 \pm 1.2$ ,  $7.1 \pm 0.7$ ,  $6.6 \pm 2.2$ ,  $7.3 \pm 0.8$ ,  $7.4 \pm 1.0$ , and  $7.1 \pm 1.3$  for groups exposed to  $2 \cdot 10^{-4}$ ,  $2.88 \cdot 10^{-3}$ ,  $1.02 \cdot 10^{-2}$ ,  $2.04 \cdot 10^{-2}$ ,  $3.06 \cdot 10^{-2}$ , and  $4 \cdot 10^{-2} \mu\text{M}$  CLD, respectively (Fig. 4). At the lowest concentration of CLD ( $2 \cdot 10^{-4} \mu\text{M}$ ), no significant difference was observed either from the control group or from all other groups exposed to CLD. For the next five increasing concentrations of CLD ( $2.88 \cdot 10^{-3}$ ,  $1.02 \cdot 10^{-2}$ ,  $2.04 \cdot 10^{-2}$ ,  $3.06 \cdot 10^{-2}$ ,  $4 \cdot 10^{-2} \mu\text{M}$ ), a significant decrease in regeneration scores was observed compared to controls and complete regeneration of hydra polyps was never observed. Therefore, these experimental conditions could be considered as “harmful”. No significant difference was observed between these five groups, indicating a toxic effect independent of CLD concentrations. For the five concentrations of CLD exposure resulting in significantly decreased regeneration scores, of the 25 gastric sections used, 20%, 44%, 24%, and 12% had scores considered extremely toxic, very toxic, toxic, and slightly toxic, respectively. On the contrary, at the CLD concentration of  $2 \cdot 10^{-4} \mu\text{M}$ , only 20% of the scores were in the very toxic range while 80% were in the slightly toxic/healthy range.

### Effects of mixtures (CLD and CLD-BP) on the regenerative capacity of hydra

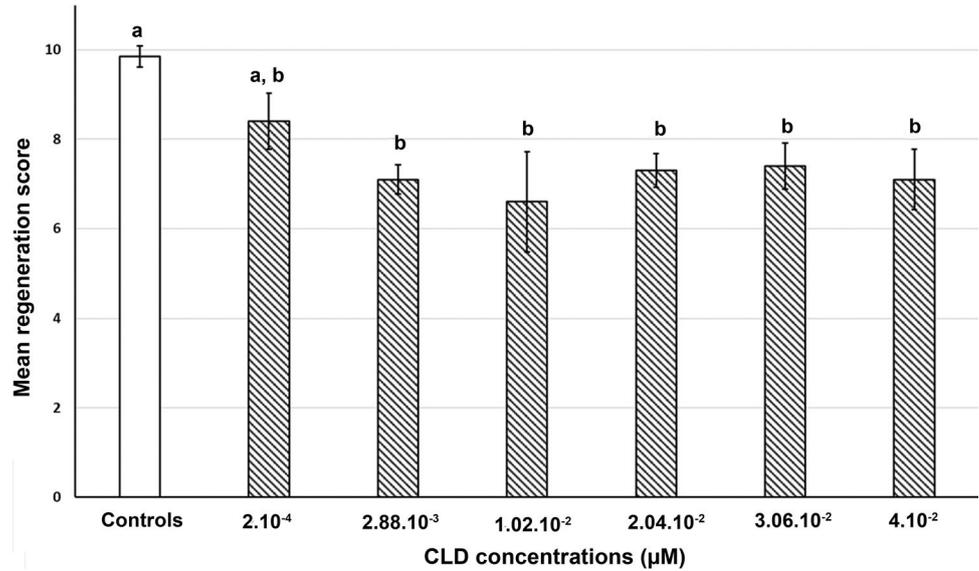
Table 3 shows comparisons of hydra regeneration score values between controls and groups exposed for 96 h to the 18 mixture combinations. No significant differences were observed between the controls and the following nine mixture combinations: M3, M5, M6, M7, M8, M10, M11,



**Fig. 3** Rooted maximum likelihood phylogram generated using ITS1 and ITS2 regions of ribosomal DNA. The IMBE1 strain used in this study (arrow) is nested within a European *Hydra vulgaris*. The numbers on the internal nodes represent bootstrap values calculated over 1000 pseudo replicates with three different methods: Maximum Likelihood implemented using IQtree / maximum likelihood implemented

using Garli / neighbor junction implemented using PAUP. Previously unpublished sequences have the following GenBank accession numbers: *IMBE1* OM470517; *TN* OM470518; *ITA02a* OM470519; *ESP11a* OM470520; *ITA05b* OM470521; *ESP10a* OM470522; *1362a* OM470523

**Fig. 4** Regeneration scores of *Hydra vulgaris* after 96 h of exposure to increasing environmental molar concentrations of chlordecone (CLD). Different letters above the bars indicate significant differences between groups ( $P < 0.001$ ), values are expressed as mean  $\pm$  SEM ( $n = 8$  for control group,  $n = 5$  for exposed groups)



**Table 3** Regeneration scores of *Hydra vulgaris* (mean  $\pm$  SEM), after 96 h of exposure to 18 mixture combinations (M1 to M18) containing chlordecone (CLD) and dechlorinated byproducts (CLD-BP). A significant decrease in regeneration score values was observed in nine

mixture combinations (in bold). Under control conditions, the mean  $\pm$  SEM regeneration score values recorded after 96 h were  $9.8 \pm 0.5$  ( $n = 8$ )

		CLD-BP concentrations ( $\mu\text{M}$ )					
		$2.10^{-4}$	$2.88.10^{-3}$	$1.02.10^{-2}$	$2.04.10^{-2}$	$3.06.10^{-2}$	$4.10^{-2}$
CLD concentrations ( $\mu\text{M}$ )	$2.10^{-4}$	<b>M1*</b> $7.5 \pm 1.0$	M14 <sup>NS</sup> $9.2 \pm 0.8$		M5 <sup>NS</sup> $8.3 \pm 0.8$		M3 <sup>NS</sup> $8.3 \pm 2.2$
	$2.88.10^{-3}$	<b>M16***</b> $4.9 \pm 1.9$	<b>M18***</b> $4.8 \pm 0.7$				<b>M17***</b> $5.7 \pm 1.7$
	$1.02.10^{-2}$			M10 <sup>NS</sup> $8.3 \pm 0.8$		M11 <sup>NS</sup> $7.3 \pm 3.8$	
	$2.04.10^{-2}$	M7 <sup>NS</sup> $8.7 \pm 0.3$			<b>M9**</b> $6.5 \pm 1.5$		M8 <sup>NS</sup> $8.5 \pm 0.5$
	$3.06.10^{-2}$			<b>M12*</b> $7.7 \pm 1.0$		<b>M13***</b> $5.5 \pm 2.0$	
	$4.10^{-2}$	<b>M2**</b> $6.8 \pm 1.5$	M15 <sup>NS</sup> $8.7 \pm 0.3$		M6 <sup>NS</sup> $7.8 \pm 1.6$		<b>M4***</b> $5.0 \pm 0.9$

NS = not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared with controls; Kruskal–Wallis test followed by Dunn’s test

M14, M15. Therefore, these mixtures could be considered as “non-harmful” experimental conditions. Compared to the controls, a significant decrease in the regeneration score values was observed for the following nine mixture combinations: M1, M2, M4, M9, M12, M13, M16, M17, M18. These latter mixtures could therefore be considered as “harmful” experimental conditions. It is interesting to recall that no significant difference could be observed between the groups exposed to these combinations of “harmful” mixtures and the groups exposed to the same molar concentration of CLD alone. The deleterious effects on regeneration caused by the nine “harmful” mixtures (M1, M2, M4, M9, M12, M13, M16, M17, M18) could not be explained by a simple increase in the molar concentration of CLD or CLD-BP in the mixtures. For example, exposure to M4, which was composed of the two highest molar concentrations ( $4.10^{-2} \mu\text{M}$  CLD,  $4.10^{-2} \mu\text{M}$  CLD-BP), resulted in a significant decrease in regeneration

scores compared to the control group, and the same was true after exposure to M1, yet composed of the two lowest molar concentrations ( $2.10^{-4} \mu\text{M}$  CLD,  $2.10^{-4} \mu\text{M}$  CLD-BP). To go further, comparisons were performed between groups exposed to “harmful” combinations and to “non-harmful” combinations of mixtures. They can be summarized as follows:

- The regeneration scores after exposure to the M18 mixture (“harmful” combination) were significantly lower than those observed after exposure to the following nine mixtures: M3, M5, M6, M7, M8, M10, M11, M14, M15 (“non-harmful” conditions).
- The regeneration scores after exposure to the M16 mixture (“harmful combination”) were significantly lower than those observed after exposure to the following eight mixtures: M3, M5, M6, M7, M8, M10, M14, M15 (“non-harmful” conditions).

**Table 4** Comparison between experimental and modeled values of Y for each replicate of the four experimental conditions that were not used for the initial formulation of the quadratic equation of the model. The calculated values of Y, called  $Y_{Fcalc}$ , were determined with a first expression of the mathematical model that was obtained with the nine points of the composite design (M1 to M9). Then, the experimental values ( $Y_{exp}$ ) were compared to the calculated values ( $Y_{Fcalc}$ ) (Student test)

Experimental condition	Experimental value of Y ( $Y_{exp}$ )	Modeled value of Y ( $Y_{Fcalc}$ )	$(Y_{exp}) - (Y_{Fcalc})$	P value %
M10_replicate 1	8.500	8.042	0.458	69.77
M10_replicate 2	9.000	8.042	0.958	41.89
M10_replicate 3	7.500	8.042	- 0.542	64.53
M11_replicate 1	10.000	8.464	1.536	19.85
M11_replicate 2	9.000	8.464	0.536	64.85
M12_replicate 1	8.000	7.454	0.546	64.21
M12_replicate 2	6.500	7.454	- 0.954	41.90
M12_replicate 3	8.500	7.454	1.046	37.63
M13_replicate 1	7.000	7.100	- 0.100	93.19
M13_replicate 2	7.000	7.100	- 0.100	93.19
M13_replicate 3	6.000	7.100	- 1.100	35.41
M13_replicate 4	7.000	7.100	- 0.100	93.19

- The regeneration scores after exposure to the M13 or M4 mixtures (“harmful” combinations) were significantly lower than those observed after exposure to the following five mixtures: M3, M7, M8, M14, M15 (“non-harmful” conditions).
- Regeneration scores after exposure to the M17 mixture (“harmful” combination) were only significantly lower than those observed after exposure to the M14 mixture (“non-harmful” condition).
- Finally, no significant difference could be observed between the regeneration scores after exposure to the M1, M9, or M12 mixtures (“harmful” combinations) and those observed after exposure to the M3, M5, M6, M7, M8, M10, M11, M14, M15 mixtures (“non-harmful” conditions). Despite these comparisons between groups, we could not conclude that among the combinations of “harmful” mixtures, some led to a greater decrease in the values of the regeneration score because no significant difference between the groups exposed to the “harmful” experimental conditions (M1, M2, M4, M9, M12, M13, M16, M17, M18) was revealed. Similarly, we could not conclude that among the “non-harmful” mixture combinations, some led to a better regeneration score because no significant difference between the groups exposed to the “non-harmful” experimental conditions (M3, M5, M6, M7, M8, M10, M11, M14, M15) was revealed.

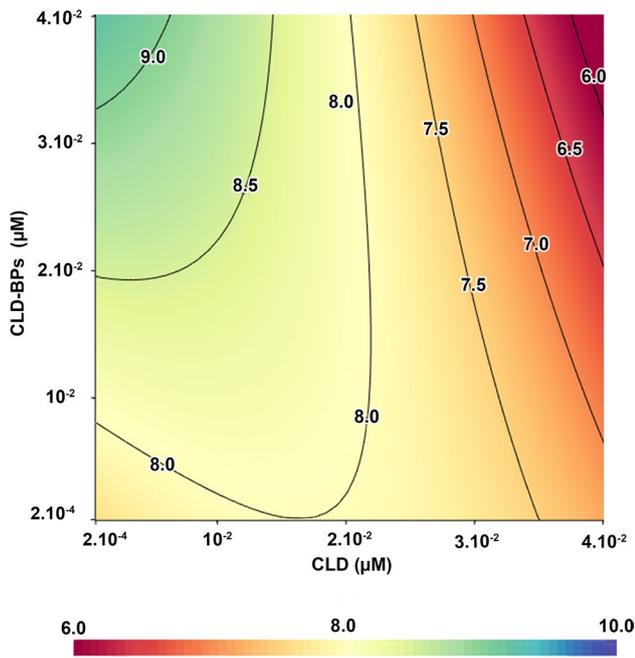
In summary, statistical comparisons between groups exposed to “harmful” mixtures and those exposed to “non-harmful” mixtures (i) reveal differences between groups but fail to identify the most “harmful” mixtures; (ii) show no significant relationship between increasing concentrations of compounds in mixtures and decreasing values of regeneration scores, suggesting a nonlinear concentration-response. Furthermore, even for mixtures classified as “not harmful” on the

basis of no significant difference from controls (M3, M5, M6, M7, M8, M10, M11, M14, M15), individually examined score values indicate evidence of damage (data not shown). Thus, it is interesting to note that of the 30 hydra sections used for these nine exposure conditions, only 47% had scores identified as nontoxic, while 37%, 6%, and 10% had scores reflecting slightly toxic, toxic, and extremely toxic conditions, respectively. Therefore, it appears that the characterization of these mixtures as “non-harmful” may not be fully justified and that it may be more appropriate and less risky to consider them as “slightly harmful.”

Another method to distinguish the most probable “harmful” combinations of mixtures containing CLD and CLD-BP is offered by score regeneration modeling. For the modeling steps, we considered the nine points of the composite design (M1 to M9) to calculate the model coefficients using multilinear regression on the coded variables ( $X_1$  and  $X_2$ ). This initial expression of the mathematical model was then validated using the four “validation points” (M10 to M13) (Table 2). In examining the experimental results, three replicates that appeared to be outliers were not included in the validation steps. Thus, only three and two replicates were considered for the M13 and M11 experimental conditions, respectively. The experimental values that were obtained for the validation points were compared to the calculated values (Table 4). The data showed no significant difference (Student test) with significance values well above 5% indicating that the calculated and experimental values were close. This result made it possible to include the four “validation points” in the calculation of the coefficients of the final mathematical model.

The final expression of the model based on all experimental conditions can be written as follows:

$$Y_{calc} = 8.040 - 1.059 X_1 + 0.004 X_2 + 0.593 X_1^2 - 0.093 X_2^2 - 0.830 X_1 X_2$$



**Fig. 5** Modeling of regeneration scores of *Hydra vulgaris* exposed 96 h to mixtures of CLD and CLD-BP showing isoscore lines, i.e., similar regeneration scores for mixtures at various concentrations (Azurad® software). The limits of the experimental range are  $2.10^{-4}$   $\mu\text{M}$  and  $4.10^{-2}$   $\mu\text{M}$ . The regeneration scores can be interpreted as follows: 0 to 1 = death; 2 to 5.9 = extremely toxic; 6 to 6.9 = very toxic; 7 to 7.9 = toxic; 8 to 8.9 = slightly toxic, viable polyp; 9 to 10 = no toxicity, healthy polyp

Because the quantitative relationship between the variation in score and the variation in CLD and CLD-BP concentrations is significant with a  $P$  value  $< 0.001$  (Fisher's test), this mathematical model can be used to predict, regardless of the proportions of CLD and CLD-BP in the mixtures, the most likely calculated regeneration scores ( $Y_{\text{calc}}$ ) within the experimental range, the limits of which are  $2.10^{-4}$   $\mu\text{M}$  and  $4.10^{-2}$   $\mu\text{M}$  for each component (CLD and CLD-BP).

The surface responses, given in Fig. 5, show a graphical representation of isoscore lines, i.e., the most likely combinations of mixtures leading to similar regeneration scores. The model predicts the following: (i) very toxic (scores between 6 and 6.9) to extremely toxic (scores below 6) conditions with mixtures containing concentrations of each component (CLD and CLD-BP) at the upper limits of the experimental range; (ii) healthy conditions (scores above 9) with mixtures containing concentrations of CLD at the lower limits of the experimental range and concentrations of CLD-BP at the upper limits of the experimental range; (iii) toxic conditions (scores between 7 and 7.9) with mixtures containing concentrations of both CLD and CLD-BPs at the lower limits of the experimental range; and finally (iv), conditions of toxicity (scores between 7 and 7.9) or slightly toxic (scores between 8 and 8.5) with most mixtures.

The direction of the isoscore lines gives indications on the influence of the components in the mixtures: preponderant influence of CLD or CLD-BP on the regeneration score? or influence of both? Here, for example, the vertical direction of the isoscore line 8.0 indicates that for these concentrations of components in the mixtures, the presence of CLD-BP has no particular influence on the regeneration scores, i.e. on toxicity. Therefore, in the area on either side of this isoscore line, the slightly toxic or toxic conditions could be primarily attributed to the presence of CLD in the mixtures. The diagonal direction of the isoscore lines 7.5, 7.0, 6.5, and 6.0 indicates the influence of both CLD and CLD-BP when the concentrations are at the upper limits of the experimental range.

Finally, although the observed “stochastic effects” of some mixtures (M14, M15, M16, M17, M18) correspond to 5 points that were not expected in a centered composite design and were therefore not included in the modeling steps, we can note that the interpretation of the modeled values for these five mixtures is risky for a single mixture, namely M17. Indeed, the model predicts: (i) toxic conditions for M14 and M15, which were experimentally considered “non-harmful” and “slightly harmful” conditions; (ii) toxic conditions for M16 and M18, which were experimentally considered “harmful” conditions; (iii) a non-toxic condition for M17, which was experimentally considered a “harmful” condition.

## Discussion

In the present study, the classification of our hydra clone IMBE1 was re-evaluated to remove doubts about its species name. At the time of writing, this clone has been in breeding for at least the last four decades. In the early 1980s, it was used at the University of Philadelphia (USA) under the name *Hydra attenuata* in a pre-screening test for substances with teratogenic potential in mammals (e.g., Johnson et al. 1982). The same clone was subsequently used by Environment Canada and, in France, by the Universities of Lorraine and Aix Marseille in standardized tests to assess the toxicity of polluted fresh and waste waters as well as pure chemical compounds (Blaise and Kusui 1997; Blaise et al. 2018; Colpaert et al. 2020; De Jong et al. 2016; Pachura et al. 2005; Pachura-Bouchet et al. 2006; Quinn et al. 2008). Due to the confusion created by Schulze in 1917 around the name “*attenuata*” which was used to describe specimens belonging to both the species *Hydra vulgaris* and *Hydra circumcincta* (Campbell 1989), the clones used in the previously cited work were named either *Hydra attenuata* or *Hydra circumcincta*. The phylogenetic tree generated in our study clearly showed that the closest relatives of our IMBE1 clone, which had traveled between North America and Europe, belonged to the species *Hydra vulgaris* and were of European origin, suggesting that it itself had the same geographic origin.

To our knowledge, this is the first time that a phylogenetic analysis has been performed in an ecotoxicological study using hydra, whereas many studies have proposed hydra as a model invertebrate in ecotoxicity bioassays (e.g., Blaise and Kusui 1997; Blaise et al. 2018; De Jong et al. 2016; Desbilles et al. 2020; Pachura et al. 2005; Pachura-Bouchet et al. 2006; Quinn et al. 2008). This phylogenetic determination is of great interest, as data supporting a different sensitivity to toxicants depending on strain exist for other species. For example, the study by Cuhra et al. (2013) using *Daphnia magna* clones shows small differences in clonal sensitivity to glyphosate. Therefore, it would be interesting to evaluate the impact of CLD and its derivatives using native FWI hydra that may have been in contact with chlordecone and acquired some resistance to these compounds.

The exceptional regenerative ability of species in the genus *Hydra* has been successfully used in ecotoxicology by several authors to detect the teratogenic potential of many chemicals. To our knowledge, the pioneer in this field is Johnson (1980) who used pellets of dissociated hydra cells as “artificial embryos.” Interestingly, using eight pharmaceuticals already evaluated in mammals (dexamethasone, aspirin, retinol acetate, methotrexate, actinomycin D, vinblastine, and unacetylated isoniazid), he demonstrated that inexpensive hydra tests, performed on both whole adult polyps and “artificial embryos” could be useful in identifying non-coeffective and coeffective teratogens<sup>4</sup> as adequately as expensive mammalian tests performed on rodents. In later studies (Johnson et al. 1982; Johnson and Gabel 1983), Johnson and colleagues extended their initial findings to a larger family of compounds including formaldehyde, benzene, lithium carbonate, lithium chloride, DMSO, flavor enhancers, food additives (e.g., monosodium glutamate), phenylenediamine (hair dye product), saccharine, colchicine, as well as several phthalates (dimethyl phthalate, diethyl phthalate, dibutyl phthalate) that are known endocrine disruptors (EDs) (e.g., Mathieu-Denoncourt et al. 2015, for review). Johnson and collaborators showed that hydra tests, using adult polyps and “artificial embryos,” were sensitive enough to identify compounds capable of posing developmental hazards and concluded that these tests could be used as predictive of the hazard potential of a putative teratogen before standard mammal tests were performed. Later, Wilby et al. (1986) used “gastric sections” as “artificial embryos” as a pre-screen for teratogenic potential of several compounds and confirmed the interest of hydra bioassays. For example, in the case of thalidomide, which is teratogenic to humans and was used as a remedy for the discomfort of pregnant women, the hydra regeneration test proved to be more sensitive than

the results of a test on mice, which did not show teratogenicity. Based on the test on mice, the use of the drug unfortunately caused developmental alterations in children (Cera et al. 2020). Because the hydra bioassay based on “gastric sections” was easier to perform than using of dissociated cell pellets, it has been reused by several authors (e.g., Pachura-Bouchet 2005; Park and Yeo 2012; Quinn et al. 2008; Vasseur and Pachura 2006). Although, unlike most metazoans, cnidarian cells are not generally organized into organs or systems, the hydra regeneration bioassay can, with care, be a useful indicator of the potential risk to a developing vertebrate embryo (Bowden et al. 1995). But of course, as Tarrant (2005) pointed out “care must be taken not to assume processes will be identical in all organisms.” In other words, the teratogenic effects on “artificial hydra embryos” exposed to toxic substances could mirror the teratogenic effect of mammal embryos, but no conclusions could be drawn regarding the physiological mechanisms involved. In our study, we used “gastric sections” as “artificial embryos,” and therefore a decrease in regeneration scores could be interpreted as a potential teratogenic effect.

Within the range of CLD concentrations studied, our results do not show a concentration–response relationship, as increasing concentrations of the toxicant do not result in a greater decrease in hydra regeneration capacity.

Thus, here, the teratogenic effect is not dependent on an increase in CLD concentration. Such a phenomenon has also been described recently for several other biological parameters studied in entire hydra polyps exposed to CLD: the reproductive rates, morphological changes, and expression of target genes involved in oxidative stress, detoxification, and neurobiological processes (Colpaert et al. 2020). Our results on “artificial hydra embryos” thus confirm the previous observations of Colpaert et al. (2020) and lead to the same conclusion: the biological effects observed in hydra after exposure to CLD are not dependent on increasing concentrations. Such an observation has been reported with xenobiotics that affect the endocrine system of animals, the endocrine disruptors (EDs). Although *Hydra* species do not possess a complex endocrine system, these organisms have neurosecretory and glandular cells capable of secreting peptides that stimulate budding, regeneration, and growth (Galliot 2013). Although physiological regulation and potential disruptions are poorly understood in cnidarians, hormones common to vertebrates (e.g., steroids, iodinated organic compounds, neuropeptides, and indolamines) have been identified in their tissues, and chemical stressors could also impact the physiology of these invertebrates (Tarrant 2005). Thus, keeping in mind that CLD is proved to be an endocrine disruptor with an effect on human reproduction (Multigner et al. 2016), the non-concentration-dependent relationship of the teratogenic response obtained here in hydra is not so surprising. Other studies have also reported that EDs may

<sup>4</sup> A coeffective teratogen is a substance that is not primarily targeted at development but can disrupt it.

have greater effects at low doses rather than at higher doses (e.g., Klimenko 2021; Varret et al. 2018). The sensitivity of the “artificial hydra embryo” bioassay demonstrated in this study was also demonstrated by Pachura-Bouchet et al. (2006) in the case of exposure to nonylphenol, a compound belonging to a family of EDs found in wastewater (e.g., Filali-Meknassi et al. 2004). Pachura-Bouchet et al. (2006) showed that hydra regeneration was disrupted at concentrations three times lower than those causing adult lethality. The present study confirms the interest of this bioassay, and thus reaffirms the value of such investigations for the assessment of risks related to environmental concentrations of xenobiotics. Therefore, our study fits a need to investigate the toxic effects of CLD and its dechlorinated byproducts at environmental concentrations. Similarly, using the giant freshwater prawn *Macrobrachium rosenbergii*, a crustacean living in the FWI, Gaume et al. (2015) also highlighted the need to study the biological effects of environmental concentrations of CLD. Indeed, after exposure to concentrations of CLD similar to those tested here, these authors demonstrated an induction of genes involved in biotransformation processes and in defense mechanisms against oxidative stress in *M. rosenbergii*. Interestingly, the European Union establishes three categories to define freshwater quality: (1) A river is considered to be in “good chemical status” if any pesticide concentration does not exceed 0.1 µg/L; (2) a river is considered to be in “poor chemical status” when the sum of pesticide concentrations exceeds 0.5 µg/L; and (3) a river is considered untreatable for drinking water if any pesticide concentration exceeds 2 µg/L or the sum of pesticide concentrations exceeds 5 µg/L. Thus, according to the European Union Water Quality Directive and considering only CLD concentrations, all quality categories are encountered in FWI freshwaters. In this context, our results indicate that in mixtures with CLD-BP, even the lowest environmental concentrations of CLD that we used ( $2 \cdot 10^{-4}$  µM, i.e., 0.1 µg/L) could impair the regenerative capacity of *H. vulgaris*. This observation supports the need to encourage further investigations for a better understanding of the effects of CLD at environmental concentrations on aquatic fauna.

According to Martin et al. (2021), a review of studies conducted from 2007 to 2017 in ecotoxicology and in toxicology (mammals including humans), conventional designs with two-component mixtures generally consist of a simple combination of concentrations. For example, (1) two chemicals are tested alone at low, medium, and high concentrations, and then as a binary mixture, in identical proportions (e.g., low concentrations together, medium concentrations together, and high concentrations together); (2) the concentration of one compound is fixed while the concentrations of the other compound vary, but under a limited number of conditions. Although methods using various ratios are preferable to conventional design mixtures,

they account for only 5% of experimental designs (Martin et al. 2021). Similarly, the advance offered by studies using response surfaces of biological effects of mixtures is still underrepresented in the literature (Martin et al. 2021). Thus, in our study, the experimental design, where the concentrations of each component are varied, coupled with a mathematical model to construct response surfaces (i.e., isoscore lines), corresponds to one of the challenges in the field of ecotoxicology (Eggen et al. 2004). Here, modeling of hydra regeneration scores predicts the most likely “harmful” combinations of mixtures containing CLD and CLD-BP, regardless of their proportions in the mixture within the experimental range from  $2 \cdot 10^{-4}$  to  $4 \cdot 10^{-2}$  µM. The model predicts very toxic (scores between 6 and 6.9) to extremely toxic (scores below 6) conditions for mixtures containing concentrations of both CLD and CLD-BP at the upper limits of the experimental range. According to the surface response profile analysis, for the highest concentrations studied, the presence of CLD-BP in the mixtures has a deleterious influence on the regeneration scores, whereas this is not the case for the other mixture combinations. However, such conditions are unlikely to occur in the environment as a result of a soil remediation process such as the ISCR. This is because dechlorination of CLD to generate CLD-BP will result in a decrease in the concentration of CLD in the soil while that of the dechlorinated byproducts will increase, so that a concentration of CLD and CLD-BP at the upper limits of the experimental range cannot be present at the same time. In any case, since it will only be possible to apply the remediation processes to a few tens of centimeters of the upper part of the soil, the stock of CLD in the lower parts will remain intact. Under these conditions, the work of Ollivier et al. (2020a, b) with columns of surface-treated soils by ISCR has clearly shown that concentrations of dechlorinated byproducts will always be lower than the CLD in soil leachates that contaminate surface and groundwater even though these products are more soluble and therefore more mobile than the CLD. For combinations of mixtures more likely to occur in environmental conditions, modeling most often predicts regeneration scores between 7 and 8.5 reflecting slightly toxic or toxic conditions. However, it should be noted that the very toxic to toxic conditions predicted with some mixtures containing a concentration of CLD at the upper limits of the experimental range warrant a special warning against agricultural practices that could remobilize CLD and lead to increased concentrations of CLD in freshwater. Since the ban on use of CLD, concentrations of CLD in freshwaters could increase only if CLD stored in the soil matrix is released. In a recent study in the FWI, Sabatier et al. (2021) demonstrate a resurgence of CLD due to the widespread use of glyphosate-containing herbicides since the late 1990s. This agricultural practice, still in use today, is

considered responsible for an unprecedented increase in soil erosion and for a significant release of CLD, which was previously stabilized in polluted soils (Sabatier et al. 2021). The mathematical model predicts likely toxic conditions (score less than 8) for mixtures containing concentrations of CLD and CLD-BP at the lower limits of the experimental range and healthy conditions (scores greater than 9) for mixtures containing concentrations of CLD at the lower limits of the experimental range and concentrations of CLD-BP at the upper limits of the experimental range. However, for these latter mixtures, it is risky to assert a score improvement related to an antagonistic effect of CLD-PBs, because the experimental values obtained with the M17 mixture ( $2.88 \cdot 10^{-3} \mu\text{M}$  CLD/ $4 \cdot 10^{-2} \mu\text{M}$  CLD-BP) did not confirm the model prediction and indicate “harmful conditions.” Thus, although predictions must be taken with caution, and despite the difficulty of modeling the effects of mixtures containing compounds belonging to EDs, our mathematical model associated with response surfaces is an interesting tool for predicting the likely effects of most combinations of mixtures in a domain of interest and gives indications of the influence of components in mixtures. Here, depending on the concentrations, the deleterious influence could be attributed either to CLD alone or to CLD and CLD-BP together.

In addition, our study helps to answer the question: are harmful mixtures containing CLD and CLD-BP more toxic than CLD alone with respect to the regenerative capacity of hydra? The answer is no, as the nine mixtures (i.e., M1, M2, M4, M9, M12, M13, M16, M17, M18) considered “harmful” did not show greater toxicity than CLD alone. Regardless of the level of toxicity of these mixtures, containing different concentrations of CLD and CLD-BP, our overall results suggest that the presence of CLD-BP in the mixtures, at the concentrations expected after the application of a remediation process such as ISCR, has no additional deleterious effect on the regenerative abilities of hydra than CLD alone.

## Conclusion

Our work confirms that the hydra regeneration bioassay is a screening tool inexpensive and appropriate for the evaluation of the toxicity of mixtures. Moreover, the use of a mathematical model, even empirical as developed here, offers perspectives in environmental toxicology, as it is another way to study complex mixtures (with more than two products) and to have the possibility of obtaining surface responses.

This type of experimental design could also be used to study the variation of abiotic factors such as temperature or exposure duration. For example, using a similar experimental design, De Jong et al. (1994) studied the toxicity

of binary mixtures containing calcium and methylmercury or mercury(II) chloride to a brown alga as a function of exposure duration.

The data presented here on hydra suggest that mixtures of CLD with hydrochlorocones, which could be generated by remediation processes such as ISCR, do not appear to be more toxic than CLD itself, and more importantly that their presence, at least for the concentrations that would be expected to be found in the environment, would not increase the deleterious effect on hydra. However, further studies on different organisms are still needed to confirm this observation.

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**Author contribution** Laetitia De Jong and Xavier Moreau contributed to the overall conception and design of the study and carried out most of the exposure experiments with the help of Jean-Pascal Andraud. Magalie Claeys-Bruno and Michelle Sergent contributed to the design of the experimental matrix and the development of the mathematical model. Daniel E Martínez performed the phylogenetic analysis of the hydra clone IMBE1. Hervé Macarie contributed to the selection of (1) the dechlorinated byproducts (CLD-BP) to be used in the study and (2) the range of CLD and CLD-BP concentrations that are representative of the French West Indies environments. He also contributed to the literature search, reconstruction of the laboratory history of the IMBE1 hydra clone, and analysis of mass spectra of CLD-BPs to identify possible isomers. Maxime Robin synthesized the CLD standard used in the study. All authors contributed to the analysis of the results. The first version of the manuscript was written by Laetitia de Jong and Xavier Moreau, and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials** Not applicable.

## Declarations

**Ethical approval** The manuscript is an original study and has not been submitted elsewhere in any form or language, but some of the results were presented, in French, at the congress of the “Groupement Français de Recherche sur les Pesticides,” GFP (May 20, 2021; <http://www.gfppesticides.org/congres/579/593-20-mai-2021.html>) in the form of a commented slideshow.

**Consent to participate** All authors approved the content and gave their explicit consent to submit the manuscript. They obtained consent from their research laboratory and the university where the work was performed.

**Conflict of interest** The authors declare no competing interests.

**Consent to publish** All the authors consent to publish the present research as Traditional publishing model—published articles are made available to institutions and individuals who subscribe to *Environmental Science and Pollution Research* or who pay to read specific articles.

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