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Abstract

Chlordecone (CLD) is an organochlorine pesticide widely used by the past to control pest insects in banana plantations in the French West Indies. Due to its persistence in the environment, CLD has contaminated the soils where it has been spread, as well as the waters, and is still present in them. The objective of our study was to evaluate the effects of chronic exposure to environmentally relevant CLD concentrations in an animal model, the freshwater hydra (*Hydra circumcincta*). In a multi-marker approach, we have studied the expression of some target stress genes, the morphology, and the asexual reproduction rates. Our data showed that exposure to low concentrations of chlordecone leads to *i*) a modulation of the expression of target genes involved in oxidative stress, detoxification, and neurobiological processes, and *ii*) morphological damages and asexual reproduction impairment. We have observed non-monotonic dose-response curves, which agree with endocrine disrupting chemical effects. Thus, "U-shaped" dose-response curves were observed for SOD, GRed, Hym355, and potentially GST gene expression; inverted "U-shaped" curves for GPx and CYP1A gene expression, and reproductive rates; as well as a biphasic dose-response curve for morphological damages. Therefore, in the range of environmental concentrations tested, very low concentrations of CLD can produce equally or more important deleterious effects than higher ones. Finally, to our knowledge, this study is the first one to fill the lack of knowledge concerning the effects of CLD in *Hydra circumcincta*, and confirms that this diploblastic organism is a pertinent freshwater model in the risk assessment.

Keywords : *Hydra circumcincta*, Chlordecone, endocrine disrupting chemicals, non-monotonic dose-response curves, freshwater risk assessment, pesticides, environmental toxicology, persistent organic pollutant

Introduction

Chlordecone (CLD) belongs to the well-known organochlorine pesticides, which are potent Persistent Organic Pollutants (POPs). POPs are chemicals of global concern, due to (1) one or several toxic impacts on human health, (2) the long-range transport, (3) the persistence in the environment and (4) the ability to bio-magnify in food webs, leading to high bioaccumulation in apex predators, even though environmental water concentrations are low and ranged from ng/L to µg/L (Yu et al. 2010). In the French West Indies, soils, freshwaters and coastal areas have been polluted by extensive use over a twenty-year period (1972-1993) of this pesticide, especially in banana plantations to limit black weevil populations (*Cosmopolites sordidus*). In soils, CLD can binds strongly with organic matter and resists to biodegradation and degradation under environmental conditions (Jablonski et al. 1996; Cabidoche et al. 2009). Thus, soil contaminations lead to subsequent contamination of water, plants and animals (Dubuisson et al. 2007; Coat et al. 2011). Major impregnation of human populations occurs through daily diet of contaminated crops and animals such as fish and other aquatic food (Guldner et al. 2010). In the French West Indies, contamination of foodstuffs by CLD was associated to an increase of the prevalence of prostate cancer (Multigner et al. 2010), and to an impaired cognitive and motor development of children exposed during pregnancy and/or through breast feeding (Dallaire et al. 2012; Boucher et al. 2013). Therefore, monitoring the CLD and its effects in contaminated human environments is of a great concern. In Martinican waterways, the highest concentrations of CLD measured are around 6 µg/L and could arise 20 µg/L after major cyclonic events such as typhoons (Gourcy et al. 2009; Arnaud et al. 2013). In a recent study in the Martinique island (Mottes et al. 2017), the CLD detection frequency at the catchment outlet was 100%, and concentration varied from 0.05 to 0.77 µg/L. Furthermore, numerous organochlorine pesticides are referred as Endocrine Disrupting Chemicals (EDCs) (Gore et al. 2015). EDCs are well known compounds able to alter physiological functions of the endocrine system. Consequently, EDC exposures lead to severe adverse health effects in adults, but also on their progeny and therefore impact reproduction and fitness of wild populations. It has been reported that EDCs could exert deleterious effects in both human and fauna at low environmental concentrations, and that the effects do not follow classical dose-response curves (Gore et al., 2015). In this context, it would be of interest to use water sentinel organisms able to respond to environmental low concentrations of pollutants. Among candidates, the freshwater *Hydra sp*. is a suitable animal model for ecotoxicological investigations (Galliot, 2012; Quinn et al., 2012; de Jong et al. 2016), as it allows to study multi-scale biological endpoints from genes to population, which makes this cnidarian a sensitive environmental indicator. Numerous advantages of this model include the asexual reproduction, leading to clone populations with a negligible genetic interindividual variations, and the simple body

plan organization of the diploblastic polyp, leading to direct contact between all body cells and the exposure media. Finally, the evaluation by qRT-PCR analyses of the expression of some stress genes can be easily performed and is inexpensive (Woo et al. 2012).

The objective of our study was to evaluate in the freshwater hydra (*Hydra circumcincta*), the effects of chronic exposure to low concentrations of CLD, compatible with environmental exposure, on the expression of some target stress genes, the morphology, and the asexual reproduction rates. According to Eggen et al. (2004), low concentrations of pollutants and long exposure time (i.e. chronic effects) as well as multiple effects by single pollutants are two great challenges in ecotoxicological studies.

Materials and Methods

Chemicals and reagents

Pure CLD was provided as a powder from Azur Isotopes (Marseille, France), with a purity greater than 97 %, and was diluted in pure water at a concentration of 1 mg/L. All the solutions were stored in the dark at 4°C. The stock solutions of CLD for expositions were freshly prepared in TES buffer (Sigma-Aldrich, Saint Quentin-Fallavier, France), the hydra breeding media. Isopropanol was purchased from Sigma (L'Isle d'Abeau, France). Chloroform (99%), and ethanol (75%) were from Carlo Erba® Reagents (Val-de-Reuil, France). Moloney Murine Leukemia Virus Reverse Transcriptase was from In Vitrogen/Thermo-Fischer (Cergy-Pontoise, France). PCR Water Nuclease Free, 5x HOT Pol Evagreen® qPCR Mix Plus, and Tri-Reagent™ were from Euromedex (Souffelweyersheim, France).

Biological material and culture conditions

Before expositions, the population of *Hydra circumcincta* polyps was raised at a constant temperature of 20°C ± 0.1°C, with a 12/12h light-dark cycle in TES buffer (0.1 mM; pH 7), as previously described by de Jong et al. (2016). Culture maintenance procedure was adapted from that developed by Trottier et al. (1997). The polyps were fed every three days with 24h hatched *Artemia sp*. nauplii. All specimens used in this experiment arise from a single polyp and thus belong to the same clone.

Chlordecone chronic toxicity assay

Exposure protocol is based on that of de Jong et al. (2016). All solutions were prepared in TES buffer, and the CLD concentrations ranged from 0.1 µg/L to 20 µg/L. Experiments were carried in sterile 6-well polystyrene

microplates. Using a stereo-microscope, non-budding *H. circumcincta* polyps of similar size were randomly collected among a dense healthy population (Trottier et al. 1997). The multi-well microplates were placed into a thermo-regulated incubator at 20 ± 0.1°C under a 12/12h light-dark cycle for 14 days. The chronic toxicity assay was done in six replicates of each concentration. Although the standardized chronical exposure test on freshwater invertebrate is done with 21 days exposures on Daphnids (OPPTS 850.1300 Daphnid chronic toxicity test; US EPA), covering the first instar stage to the sexual maturity of the animal, this could not be done with the hydras since the sexually mature and immature polyps could not be differentiated. In this case, a 14 days exposure was chosen, corresponding to two-time the duration needed for the replacement of all the epithelial cells in the animal, since this duration is of about a week in the hydra species (Martinez & Bridge. 2012). This allowed to ensure that all the cells of all the animals at the end of the experiment were fully grown on a contaminated media. About 10 living 24h hatched *Artemia sp*. nauplii were used to feed polyps on the day before the beginning of the experiment and then each three days (day 3, 6, 9 and 12). All solutions were renewed after the 4-5 hour feeding period. Daily, morphology was recorded, and the number of buds and new polyps was counted to determine the asexual reproduction. At the end of the 14-days exposure period, the media was carefully removed. About 50 living polyps of each condition were harvested in 1 mL Tri-Reagent™ (Euromedex®; Souffelweyersheim; France), and stored at -80°C until RNA extraction.

Expression of some stress gene evaluated by means of qRT-PCR analyses

Total RNA extraction was done in accordance with the Tri-Reagent manufacturer guideline. The RNA concentrations were quantified by spectrophotometric analyses on a Nanovue Plus Spectrophotometer (GE Healthcare Life Science®; Marlborough, US-MA; USA).

The conversion of RNA to cDNA was carried out in the presence of nucleotides (dNTPs), random primers, M-MLV Reverse Transcriptase, and incubation buffer. The obtained cDNA were used as a template for PCR amplification.

The cDNA fragments of 10 genes, obtained from the conversion of the *Hydra circumcincta* RNAs, were amplified by qRT-PCR, with dedicated sets of primer for each gene, as described by Woo et al. (2012). The studied genes were the genes coding for : Tubulin α-1, as housekeeping gene (TUBA1, GenBank accession no. CV660826), Catalase (CAT, CN631284), Glucose-6-phosphate dehydrogenase (G6PD, DT614664), Glutathione peroxidase (GPx, DQ286040), Glutathione reductase (GRed, XM_002159979), Glutathione-S-transferase (GST, XM_002153968), Superoxide dismutase (SOD, XM_002157471), Cytochrome P450 1A (CYP1A, AB049403), Acetylcholinesterase (AChe, AB513182), Hym-355 (mRNA coding for the precursor protein of Hym-355 peptide,

AB025945) and Ubiquitin (UBI, CB888414). The sequences of the primer used for qRT-PCR, as well as their expected melting temperature (Tm) are shown in Table 1. Primers defined by Woo and coworkers were designed for *Hydra magnipapillata* (or *Hydra vulgaris*). Unfortunately, the genome of *Hydra circumcincta* (or *Hydra attenuata*) have not been sequenced yet. In order to assume the specificity of the primers, during preliminary experiments we have verified that the size of the amplification products was consistent with the expected size. Moreover, during qPCR experiments, we have analyzed the melting curves to assume that there was only one amplification product.

PCR experiments were performed using a Biometra T-Personal Thermal Cycler (Eurobio Ingen®; Les Ullis; France) in 96 well-plates. The PCR was performed with 0.4 µM of each primer, and 5x HOT Pol Evagreen® qPCR Mix Plus (Euromedex). Cycling conditions included an initial PCR activation (1 cycle) at 95°C for 15 min, followed by 40 cycles of amplification (15 s denaturation at 95°C, 20 s primer annealing at 44°C, and 20 s fragment elongation at 72°C). A melt program was performed at the end of the PCR to check the specificity. The raw fluorescence data were analyzed using T-Personal Thermal Cycler LightCycler (Biometra, Göttingen, Deutschland). Target gene mRNA expression was normalized to Tubulin α-1, and data were quantified using the 2-ΔΔCt method (Schmittgen and Livak, 2008).

Asexual reproduction rates of hydra

The budding rates (Ktot) were calculated as follow:

$$Ktot = (\ln(n_{tot})-\ln(n))/d$$

in which n is the number of polyps at the beginning of the experiment (d_0), and n_{tot} the sum of the number of attached buds and the number of polyps (detached buds and initial polyps) after d days of exposure (de Jong et al. 2016).

The reproductive *Hydra* rate (RHR) during the two-weeks exposure to CLD were calculated according the following formula:

$$RHR = ((Nx - N_0) / N_0) \times 100$$

with Nx number of *Hydra* polyps at day « x », and N_0 number of *Hydra* polyps at day 0 (according to Arkhipchuk et al. (2006)).

Morphological stages after CLD exposures

In *Hydra sp.,* an increase in toxicity lead to progressive morphological changes that have been codified in previous studies (de Jong et al. 2016; Pachura-Bouchet et al. 2006). Briefly, six morphological stages are observed (Figure 1): *a* stage, normal polyps characterized by an extended body and tentacles; *b* stage, appearance of bulbs at tentacle

tips; *c* stage, slightly shortened body and tentacles; *d* stage, very shortened body and tentacles; *e* stage, body and tentacles drastically shortened; *f* stage, disintegrated polyp.

Statistical analyses

All values of each group on *H. circumcincta* were expressed as mean ± SD, and were compared by one-way ANOVA, followed by Tukey HSD post-hoc test for paired comparisons. When the data did not follow a Normal distribution, they were compared with the Kruskal-Wallis test accompanied by the bilateral Dunn test for paired comparisons. Analyses were done on Graphpad Prism Software Version 7, or XLSTAT-Ecology (ver 19.02.44449).

Results

Chlordecone effects on the expression of genes involved in oxidative stress response

The effects of environmental doses of CLD on the expression of some genes involved in oxidative stress response are summarized in Figure 2. Interestingly, we have observed different kinds of responses, depending on the studied gene. CAT expression was significantly reduced by 2-fold at 1 µg/L (p=0.0160), and approximately by 5-fold at 20 µg/L (p=0.0051). Similarly, G6PD expression was significantly repressed by 2-fold at 10 µg/L (p=0.0241). Inversely, SOD expression was significantly induced by 2-fold at 1 µg/L (p=0.0241), by 2.5-fold at 20 µg/L (p=0.0111), and by 3-fold at 0.5 µg/L (p=0.0241) and 10 µg/L (p=0.045). GPx and GRed displayed variable responses, depending on the studied concentrations. GPx was significantly enhanced by 1.9-fold at 1 µg/L (p=0.0113), and repressed by 2-fold decrease at 10 $\mu g.L^{-1}$ (p=0.0004). In the same way, GRed expression was induced by 2-fold at 0.1 µg/L (p=0.003), while it was reduced by 3-fold at 5 µg/L (p=0.001). No significant difference from the control was observed for GST.

Chlordecone effects on the expression of genes involved in detoxification

The effects of environmental doses of CLD on the expression of some genes involved in detoxification are summarized in Figure 3. The results show an induction of CYP1A expression by 1.5-fold at 0.1 µg/L (p=0.07) and at 5 µg/L (p=0.0076), and by 2-fold at 0.5 µg/L (p=0.0065), while its expression is reduced by 2-fold at 20 µg/L (p=0.0264) and by 3-fold at 10 µg/L (p=0.002). As CYP1A, UBI was induced at low doses, and repressed at higher doses, with an expression enhanced by 1.75-fold at 0.5 µg/L (p=0.0048), while it was decreased by 2-fold at 10 µg/L (p=0.05) and by 3-fold at 1 µg/L (p=0.0027).

CLD effects on the expression of gene of the nervous system

The effects of environmental doses of CLD on the expression of some genes involved in the nervous system are summarized in Figure 4. The expression of AChe was repressed at low dose (2-fold at 0.5 µg/L (p=0.0165)), and induced at higher dose (2-fold at 5 µg/L (p=0.0038)). Hym-355 expression was decreased by 5-fold at 1 µg/L (p=0.0002), and induced by 4-fold at 0.1 µg/L (p=0.0001), 2-fold at 0.5 µg/L (p=0.0281), 1.5-fold at 5 µg/L, by 3-fold at 10 µg/L (p<0.0001), and by 6-fold at 20 µg/L (p=0.0002).

Asexual reproduction rates of Hydra

The Kruskal-Wallis test has shown, that there is an overall difference in the budding rate (Ktot) between the different groups (P= 0.008) at day 14. Ktot was significantly lower in polyps exposed to 5 µg/L of CLD as compared to controls (0.093 ± 0.003, and 0.103 ± 0.002, respectively, P< 0.05) (Figure 5A). All other budding rate values, whatever the CLD concentration and the exposure period, were not different from controls.

The ANOVA has shown that there is an overall difference in the reproductive *Hydra* rate (RHR) between the different groups at day 14 (P< 0.0001). RHR was significantly lower in polyps exposed to 5 µg/L of CLD as compared to controls (152.2 ± 28.4 and 223.3 ± 15.1, respectively, P= 0.001) (Figure 5B). All other RHR values, whatever the CLD concentration and the exposure period, were not different from controls.

Morphological stages after CLD exposures

The Kruskal-Wallis test has shown that there is an overall difference in the morphological stage occurrences between the different tested concentrations (P<0.0001). Further comparisons have revealed that after 14 days, significant morphological changes were observed in polyps exposed to 1, 10, and 20 µg/L CLD (Figure 6). Indeed, the percentage of *a* stage was significantly lower in polyps exposed to 1 µg/L (89.8 ± 7.0), 10 µg/L (87.7 ± 7.3) and 20 µg/L (71.7 ± 7.7), as compared with controls (100%). The percentage of *b* stage was significantly higher in polyps exposed to 1 µg/L (10.3 ± 6.7), 10 µg/L (11.7 ±6.9), and 20 µg/L (22.4 ± 6.9), as compared with controls (0%). The percentage of *c* stage was significantly higher only in polyps exposed to 20 µg/L (5.9 ± 6.6), as compared with controls (0%).

Discussion

The aim of this work was to evaluate, in the cnidarian *Hydra circumcincta*, the effects of chronic exposure (14 days) to low concentrations of CLD, compatible with an environmental exposure (0.1 to 20 µg/L), on several biological parameters: the expression of various target genes involved in oxidative stress, in detoxification and in

neurobiological processes, the morphology and the reproductive rates. The overall results showed that the animal model, *Hydra circumcincta*, is a pertinent and sensitive model to study the biological effects of low and environmentally relevant freshwater concentrations of CLD, since impacts were observed from the molecular to the population level.

The significant effects were numerous at the level of stress genes expression (Figures 2-4), and at first, could appear stochastic because not concentration dependent. Furthermore, for a given gene, and in the range of tested CLD concentrations, it can be observed: increased, as well as decreased, or unchanged mRNA levels. Indeed, our results showed effects that were not linearly dependent of concentrations that are illustrated by non-monotonic dose-response curves (NMDRCs). These types of curves are frequently encountered when studying the effects of xenobiotics that affect the endocrine system of animals. Although *Hydra* species do not possess an endocrine system or organs, these organisms possess neurosecretory and gland cells that are able to secrete peptides such that stimulates budding, regeneration, and growth (Galliot, 2013).EDCs have challenged traditional concepts in toxicology, notably the Paracelse principle "the dose makes the poison". EDCs can have highest effects at low doses rather than at higher doses (Gore et al. 2015; Vanderberg et al. 2012). Moreover, the effects of EDCs are often not linearly dependent of concentrations and are characterized by NMDRCs. Generally, NMDRCs have a U- or inverted U-shape (Conolly and Lutz 2004); these NMDRCs are thus also referred to as biphasic dose-response curves.

Oxidative stress induced by pollutants is the consequence of an imbalance between the production of reactive oxygen species (ROS), and their detoxification by antioxidant systems. ROS are involved in intracellular signaling cascades and redox regulation (Adler et al. 1999; Nordberg and Arnér 2001; Bonello et al. 2007). Oxidative stress, induced by xenobiotics or inflammation, can produce lipid peroxidation and oxidative damage of proteins and DNA, leading to membrane disruption, apoptosis, and mutagenesis respectively (Simon et al. 2000). Therefore, ROS levels are tightly controlled. Primarily, SOD catalyzes the conversion of $O_2^{\bullet-}$ in H_2O_2. H_2O_2 is further converted into H_2O by CAT or GPx. GPx requires GRed to reduce the oxidized glutathione, and the active form of CAT requires $NADPH_2$ that is also associated to thioredoxins, glutaredoxins and peroxiredoxins antioxidant systems. These different systems are linked to G6PD that produces $NADPH_2$ during pentose phosphate pathway (Nóbrega-Pereira et al. 2016). GST is involved in the detoxification of hydroperoxides (Veal et al. 2002), despite its main role is the conjugation of xenobiotics, previously bioactivated into electrophile metabolites, mainly by cytochromes P450 (CYPs).

Our data showed, that the expression of most of the studied genes involved in oxidative stress is modified by the treatments, even at the lowest concentration tested (2-fold induction of GRed at 0.1 µg/L). G6PD expression was only slightly modified while SOD and GRed expressions were more markedly modified. Interestingly, we observed NMDRCs which lets suggest that CLD can also produce adverse effects that have a similar pattern than those EDCs. Biphasic dose-response curves were observed in our study with SOD, GPx and GRed. GST also shows a tendency to the increase associated to a biphasic dose response curve, despite the non-significance of the results, probably due to the variability of the results. Taken together, our results demonstrated that even at low environmental concentrations, CLD is able to modulate the response of *Hydra circumcincta* to ROS, while such modulations were already described in this freshwater cnidarian, but only after exposure to high concentration of toxaphene (Woo et al. 2012). Concerning genes involved in detoxification processes, we have observed for CYP1A an inverted U shape dose response. CYPs are the main phase I enzyme involved in xenobiotic degradation, they are generally described as detoxification enzymes, since by increasing the polarity of their substrate, they enhance their elimination. However, CYPs can also catalyze the bioactivation of procarcinogens into electrophilic metabolites, which are mutagenic compounds that are further detoxified by GST (Reed et al. 2018). CYP1A was largely studied in rodents and human, since it is involved in the bioactivation of numerous environmental procarcinogens, such as polycyclic aromatic hydrocarbons and arylamines (Villard et al. 1998; Ma and Lu 2007). Moreover, CYP1A is also involved in the production of ROS (Knerr et al. 2006). CYP1A and its enzymatic activity is highly conserved in vertebrates and have been well documented (Nelson et al. 1996). Concerning the functions of CYP1A in freshwater invertebrates, a role for CYP1A was suggested for the detoxification of toxaphene and acclimation to toxaphene in the crustacean *Daphnia magna* (Kashian 2004), and little is known concerning CYPs in *Hydra* species. UBI was either induced at very low concentration (0.5 µg/L) or repressed at higher concentrations (1 and 10 µg/L). Ubiquitination signals for the degradation by cellular proteasomes of short-lived, or unnecessary denatured proteins, (Hershko and Ciechanover 1998). UBI was proposed as a general molecular biomarker for various types of environmental stressors in marine fish medaka (Woo et al. 2009). Therefore, our result also suggested more pronounced side effects at low concentrations, rather than at high concentrations, which lets suggest that CLD can also produce adverse effects that have a similar pattern than those of EDCs. Inversely to UBI, AChe was repressed at very low concentration (0.5 µg/L) and enhanced at higher concentrations (5 and 20 µg/L). AChe catalyzes the hydrolysis of the neurotransmitter acetylcholine to choline, in the synaptic cleft. The expression of the neuropeptide Hym-355, which specifically induces differentiation of neurons (Takahashi et al. 2000), was also modulated, and exhibited a U shape dose-responses. Woo et al. (2012) did not observed any

modification of either AChe, or Hym-335 expression in *Hydra*, after exposure to toxaphene, but the studied concentrations were far much higher (0.3 and 3 mg/L), as compared to our study.

Taken together, our data demonstrated that it is possible to observe the modulation of the expression of various target genes related to oxidative stress, detoxification processes, and neurobiological processes, when *Hydra circumcincta* is exposed to low doses of CLD, that are compatible with environmental pollution. Indeed, CLD concentration in Martinique freshwater, generally ranges between 0.2 and 1.0 µg/L, but can reach up to 20 µg/L. We have observed NMDRCs that are similar to those illustrating EDC effects. SOD, GRed and Hym-355 and potentially GST exhibited U shape dose responses, and GPx and CYP1A exhibited an inverted one in the range of the low environmental concentrations tested. The studied genes in our study are not directly involved in endocrine response. However, studies performed notably in mammals, show that EDC exert their detrimental effect generally by targeting the immune system (chronic low-grade inflammation, autoimmune diseases) or the hypothalamic-pituitary axis (Gore et al. 2015). Some of our studied genes are indirectly related to those pathways. In the other hand, our data demonstrated that very low concentrations can exert higher effects than high concentrations, suggesting that the level of studied concentrations should be based on environmental data rather than LC50 values for environmental monitoring.

Changes in gene expression are not always followed by ecotoxicological deleterious effects but, in the present study, morphological changes, reflecting toxicity, and asexual reproduction of *H. circumcincta* were also impacted. These effects have occurred in the same range of CLD environmental concentrations than those that have led to changes in the stress genes expressions. Interestingly, as for stress genes expressions, the effects at the organism and population levels were not concentration dependent. Concerning the morphological endpoint (Figure 6), the significant effects seemed to follow a biphasic dose-response curve, since one event of intoxication was observed at 1 µg/L of CLD and then at 10 and 20 µg/L, but not at 0.1, 0.5 and 5 µg/L. Our results suggested that the increase in SOD gene expression together with the decrease in CAT gene expression could participate to an enhanced ROS production leading, at least in part, to morphological damages. Such relationships between impairment in anti-oxidative systems, lipid peroxidation and morphological damages were also suggested in a previous ecotoxicological study using the hydra animal model (de Jong et al. 2016). Concerning asexual reproduction (Figure 5), our results have shown that the significant decrease in reproductive *Hydra* rate (RHR), which is observed only at 5 µg/L, can be related to the decrease in the budding rate (Ktot). These demographic responses follow a typically inverted "U-shaped" dose-response curve. The Ktot is a reproductive rate that takes into account the number of polyps AND the number of buds that are attached to the individuals. The RHR takes into account

the numbers polyps only. It is obvious that the two parameters are related, however, we have previously shown that bud detachment could be retarded without changes in the number of polyps after xenobiotic exposure (de Jong et al. 2016). Here, the biological pathways that controls of buds detachment may not be impacted by CLD since both the Ktot AND the RHR decrease when polyps were exposed to 5 µg/L CLD. As it was stated above, *Hydra* do not possess endocrine system or organs. However, morphogenesis and bud formation which are under the control of epithelial cells were clearly affected by CLD. The epithelial cells produce and secrete signaling molecules produced by the interstitial cells, suggesting that the differentiation program of epithelial cells is modified only when hydra was exposed to a particular CLD concentration (i.e. 5µg/L in the present study). Here, an EDC effect of CLD is suspected to explain the impact of CLD on reproduction rate of *H. circumcincta.*

Currently, the EDC properties of chemical compounds are poorly evaluated during their development. The evaluation being generally limited to the use of gene reporter studies, where luciferase expression is regulated by estrogen receptor (ER), androgen receptor (AR), or thyroid hormone receptor (TR). EDCs exert their effects mainly by means of epigenetic mechanisms, which have been insufficiently studied (Gore et al. 2015). Organochlorine pesticides are well known EDCs, and their detrimental effects were at least in part mediated by epigenetic mechanisms (Hou et al. 2012; Song et al. 2014). Furthermore, it was now clearly established that molecular mechanisms and signal transduction pathways described in invertebrate species, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, are comparable in vertebrates, although the complexity is higher in vertebrates (i.e. ecdysone receptors described in Drosophila have evolved in mammals into the family of steroid hormone receptors). Although *Hydra sp*. do not possess endocrine system or organs, our data strongly suggested that this cnidarian could be a useful model to evaluate environmental EDC effects of new chemicals during their development and opens the way for further investigations to characterize the epigenetic modifications.

Moreover, The European Union drinking water quality standard for CLD is less than 0.1 µg/L. Our results showed that at this concentration, CLD can significantly induce GRed, CYP1A, and Hym355 expression. Water quality standard are defined from a risk analysis performed on chronic toxicological studies, which are generally performed in rodents (mice or rats), and in dogs. Therefore, it will be of interest to evaluate the effect of very low doses of CLD in these mammal species, since toxicological studies are undertaken with high doses relatively to EDC properties.

To conclude, in *Hydra circumcincta*, exposure to very low concentrations of CLD led to *i*) a modulation of the expression of target genes involved in oxidative stress, detoxification, and neurobiological processes, and *ii*) morphological damages and asexual reproduction impairment. We have observed NMDRCs similar to that

observed when studying EDC effects. Thus, “U-shaped” dose-response curves were observed for SOD, GRed, Hym355 and potentially GST gene expression; inverted “U-shaped” curves for GPx and CYP1A gene expression and reproductive rates; a biphasic dose-response curve for morphological damages. Therefore, in the here tested environmental range of CLD concentrations, very low concentrations can produce equally or more important deleterious effects than higher concentrations. Finally, to our knowledge, this study is the first one to fill the lack of knowledge concerning the effects of CLD in *Hydra circumcincta,* and confirms that this diploblastic organism is a pertinent freshwater model in the risk assessment.

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Gene	Nucleotide sequence	Tm (°C)
TUBA1	Forward 5'-TTGATGAAATACGCACAGGAACA-3'	52
	Reverse 5'-CCACCAAAGGAATGAAAAAT-3'	46
CAT	Forward 5'-GCTCCAAACTACTTCCCTAACAG-3'	55
	Reverse 5'-GCTCATCTATCGCTTCATTT-3'	48
G6PD	Forward 5'-GCATTGCCACCATCTGTATTCA-3'	53
	Reverse 5'-GCAAACCTTAGCACCATTAT-3'	48
GPx	Forward 5'-TCGATATCTGGAACCAATGACAAA-3'	52
	Reverse 5'-CGAGGCGCCCACTATGACTT-3'	56
GRed	Forward 5'-GAGGAGCGATATTTTGGGTAT-3'	50
	Reverse 5'-GTTTAACCTCAGCAACCAGT-3'	50
GST	Forward 5'-CGAGGCAGCTAAGTTAAAGT-3'	58
	Reverse 5'-ACTTAAGGTAATGGGGGATG-3'	58
SOD	Forward 5'-TCAGTTTGGGGATTATTCAGGTG-3'	58.4
	Reverse 5'-TCCAGCATTTCCGGTAGTTTTG-3'	47.9
CYP1A	Forward 5'-GCTGGCGATCATGTTGCTGTTT-3'	49.7
	Reverse 5'-AGTTTCTGCTGTAGTGTATTGA-3'	44.1
AChe	Forward 5'-GGTTATAGTCCTGACAGCGAGTTT-3'	50.6
	Reverse 5'-AGGCAGTGAAGCAAGAAGACC-3'	49.2
Hym-355	Forward 5'-ATGCCTAACCGGTGATGCT-3'	54
	Reverse 5'-TTGCCTCCTCTTGGTAAAAA-3'	56
UBI	Forward 5'-CACCAGACCAGCAACGACTTAT-3'	49.7
	Reverse 5'-TCTCTTGAGGCGGGTTATTTTA-3'	46

Table 1 : Sequences of primers used in qRT-PCR analyses

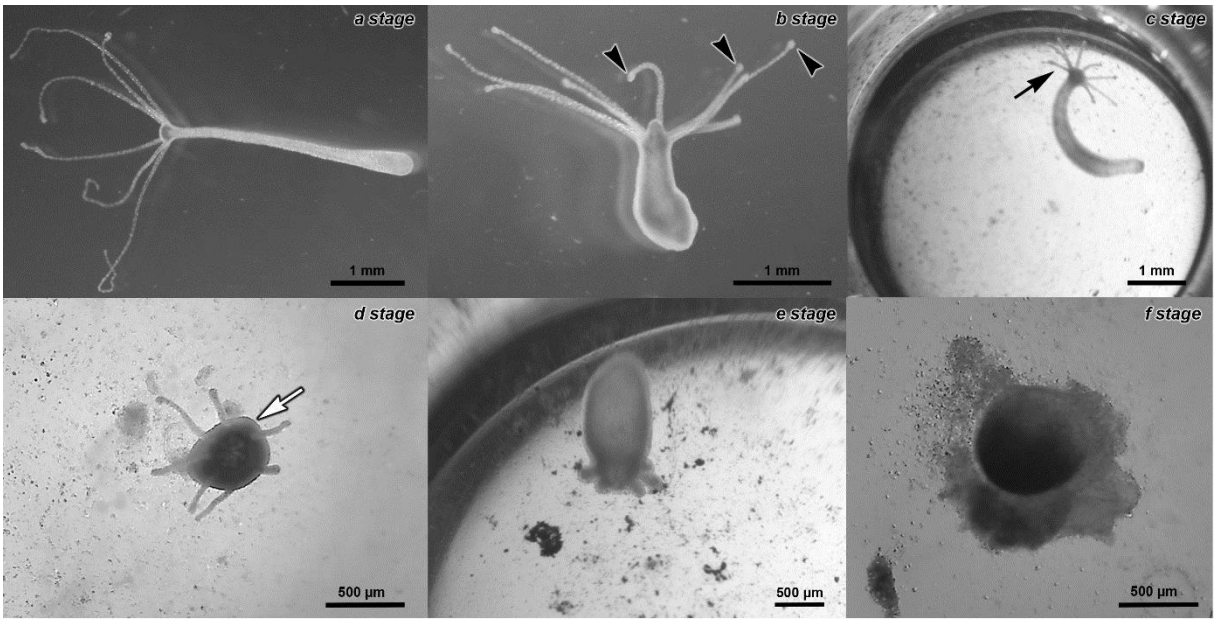

Figure 1: Morphological stages of *Hydra circumcincta* showing progressive intoxication (from *a* stage to *f* stage). *a* stage, normal healthy polyps with extended body and tentacles; *b* stage, appearance of bulbs at tentacle tips (arrowheads); *c* stage, slightly shortened body and tentacles (black arrow); *d* stage, very shortened body (white arrow) and tentacles; *e* stage, body and tentacles drastically shortened; *f* stage, disintegrated polyp.

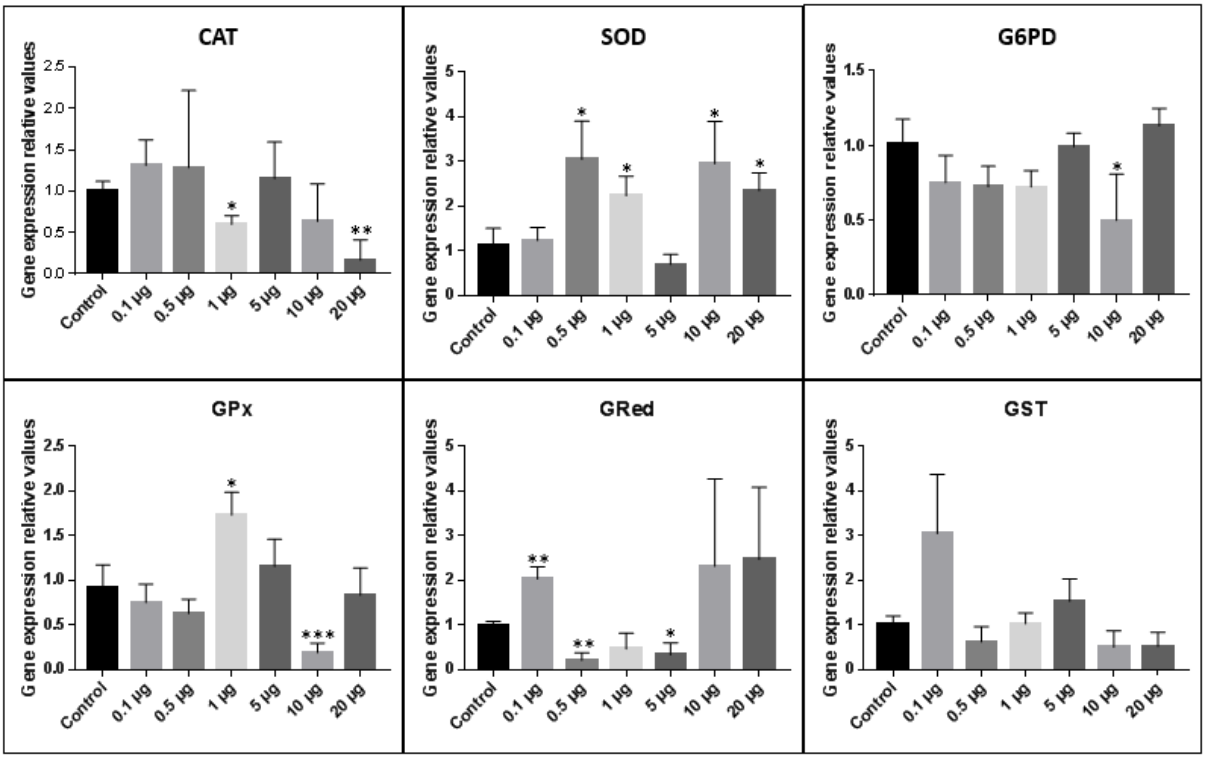

Figure 2: Relative expression of genes involved in oxidative stress response. Briefly, *Hydra circumcincta* were exposed for 14 days to CLD at different doses, ranging from 0.1 to 20 µg/L. After extraction of total RNA, the expression of the genes of interest was evaluated by qRT-PCR. CAT: catalase. SOD: superoxide dismutase. G6PD: glucose 6-phosphate dehydrogenase. GPx: glutathione peroxidase. GRed: glutathione reductase. GST: glutathione S-transferase. The values are represented as mean ± SD. n = 10 per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly different from controls.

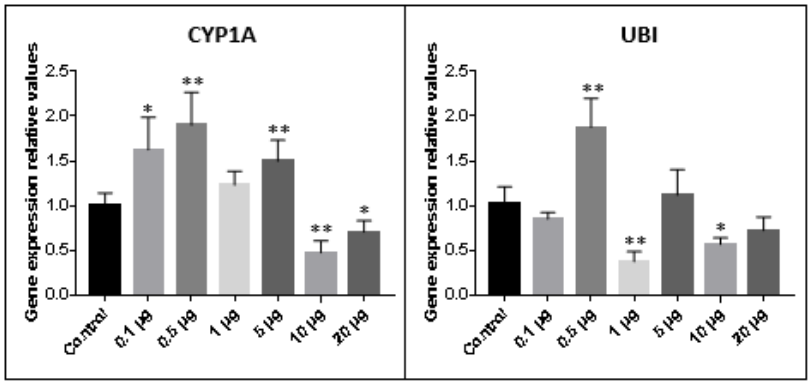

Figure 3: Relative expression of genes involved in detoxification. Briefly, *Hydra circumcincta* were exposed for 14 days to CLD at different doses, ranging from 0.1 to 20 µg.L-1. After extraction of total RNA, the expression of the genes of interest was evaluated by qRT-PCR. CYP1A: cytochrome P450 1A. UBI: ubiquitin. The values are represented as mean ± SD. n = 10 per group. The level of significance is given by comparison of treated trials with controls: * $p < 0.05$; ** $p < 0.01$ significantly different from controls.

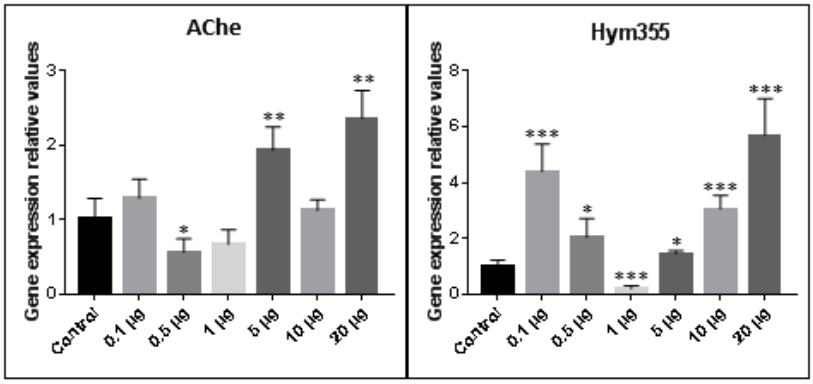

Figure 4: Relative expression of genes involved in the nervous system functioning. Briefly, *Hydra circumcincta* were exposed for 14 days to CLD at different doses, ranging from 0.1 to 20 µg.L-1. After extraction of total RNA, the expression of the genes of interest was evaluated by qRT-PCR. AChe: acetylcholinesterase. Hym355: Hym355 precursor. The values are represented as mean ± SD. n = 10 per group. The level of significance is given by comparison of treated trials with controls: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly different from controls.

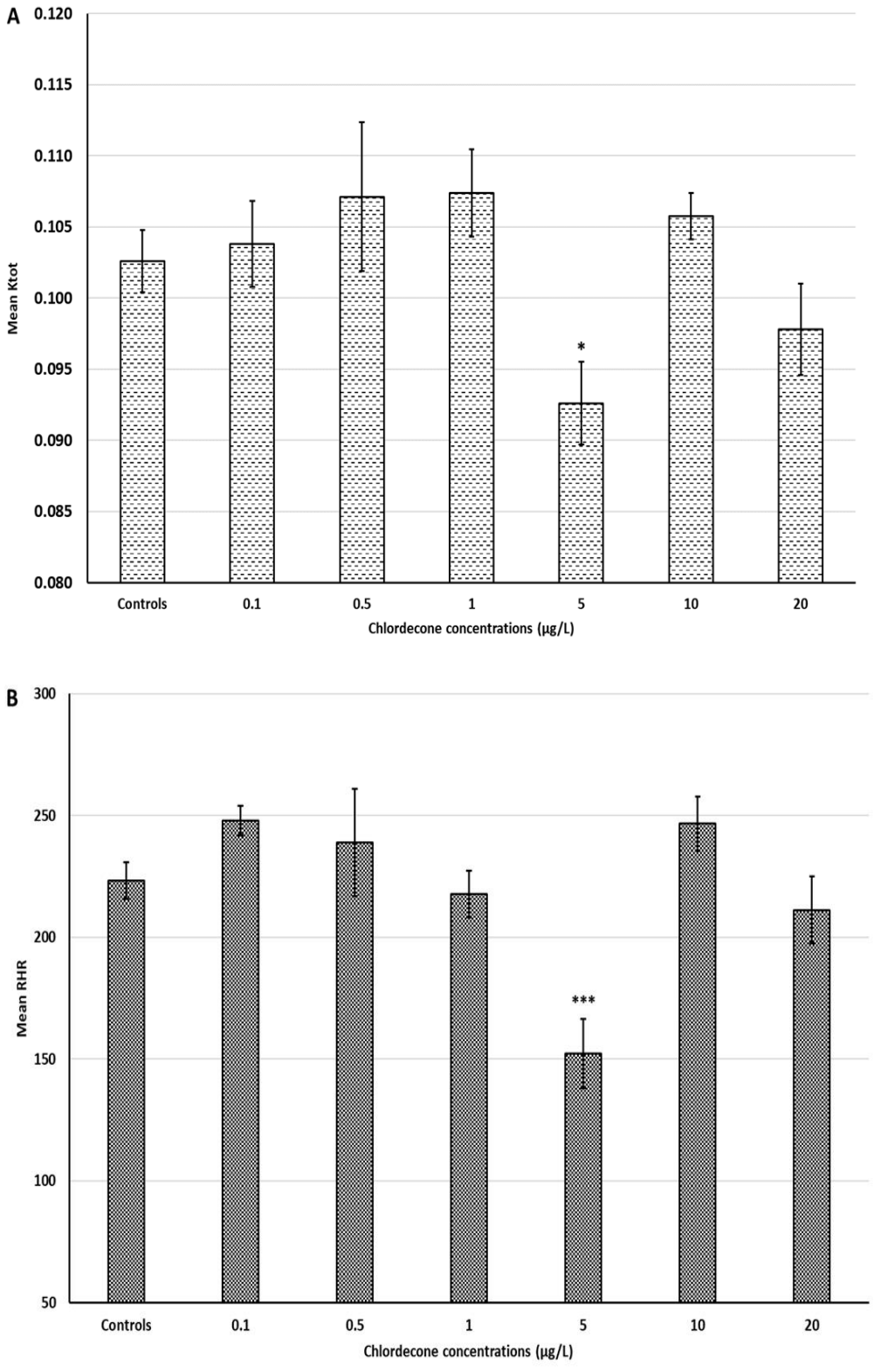

Figure 5: Asexual reproduction expressed as mean ± SD budding rate (Ktot) (A), and as mean ± SD reproductive *Hydra* rate (RHR) (B) in *Hydra circumcincta* exposed 14 days to chlordecone. * P<0.05; *** P=0.01: significantly different from controls.

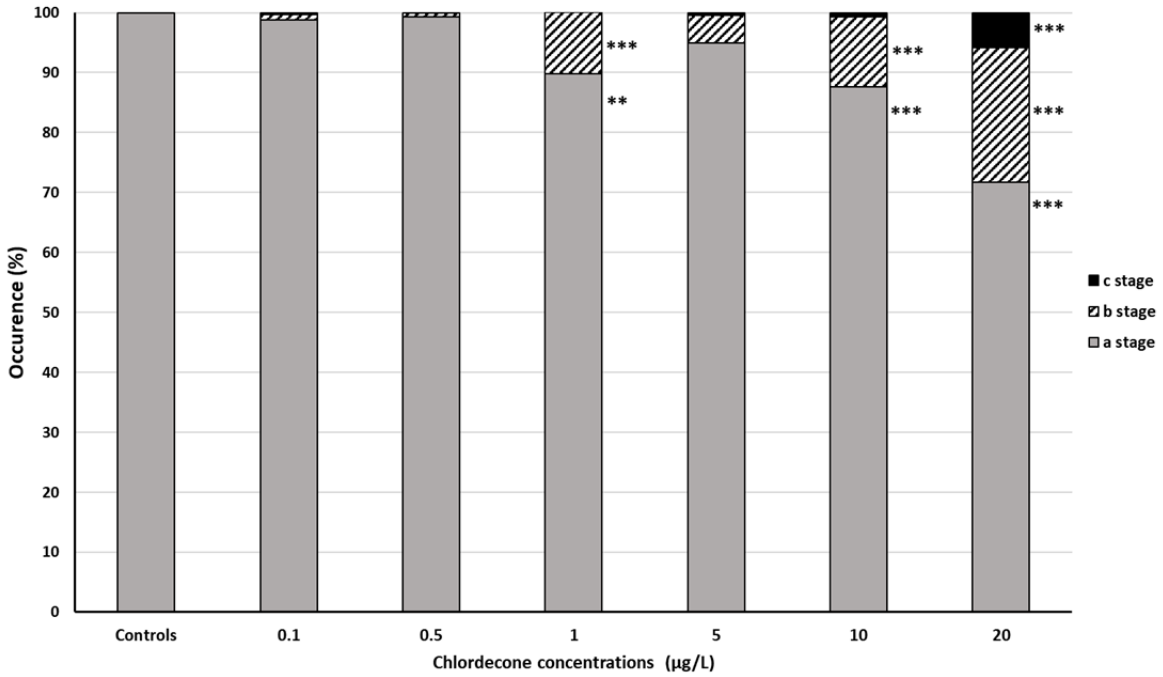

Figure 6: *Hydra circumcincta* morphological stage occurrence (in percent) reflecting toxicity, after 14 days of CLD exposure. For better readability, standard errors are not presented. ** P<0.01; ***: P<0.001 significantly different from controls.