Effects of chlordecone on 20-hydroxyecdysone concentration and chitobiase activity in a decapod crustacean, *Macrobrachium rosenbergii*

Anne Lafontaine [a,*], Eric Gismondi [a], Céline Boulangé-Lecomte [b], Perrine Geraudie [c], Nathalie Dodet [a], Fanny Caupos [d,e], Soazig Lemoine [d], Laurent Lagadic [e], Jean-Pierre Thomé [a], Joëlle Forget-Leray [b]

[a] *University of Liège, Laboratory of Animal Ecology and Ecotoxicology (LEAE), Centre of Analytical Research and Technology (CART), 15 Allée du Six Aout, B-4000 Liège, Belgium*
[b] *Normandie University, ULH, UMR I-02, Environmental Stresses and Biomonitoring of Aquatic Ecosystems (SEBIO)—FR CNRS 3730 SCALE, 25 rue Philippe Lebon, F-76600 Le Havre, France*
[c] *Akvaplan-Niva (Norwegian Institute of Water Research) AS, Fram Centre, 9296 Tromsoe, Norway*
[d] *DYNECAR-UMR BOREA (MNHN/CNRS 7208/IRD207/UPMC), University of the French West Indies and Guiana, Campus de Fouillole, Pointe-à-Pitre, Guadeloupe F-97110, France*
[e] *INRA, UMR0985 Ecology and Ecosystem Health Research Unit, Ecotoxicology and Quality of Aquatic Environments Research Group, 65 rue de Saint Brieuc, F-35042 Rennes, France*

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ABSTRACT

Chlordecone (CLD) is an organochlorine insecticide abundant in aquatic environment of the French West Indies. However, few studies have investigated its impact on freshwater invertebrates. Whereas CLD is suspected of inducing endocrine disruption, this work aimed to study the effects of environmentally relevant concentrations of CLD on the 20-hydroxyecdysone (20-HE) hormone concentration and on the chitobiase activity, both having key roles in the molting process of crustaceans. In addition, the bioaccumulation of CLD was measured in the muscle tissue of *Macrobrachium rosenbergii* to underline potential dose-response relationship. The results have shown that CLD was bioaccumulated in exposed organisms according to a trend to a dose-response relationship. Moreover, it was observed that CLD decreased the 20-HE concentration in exposed prawns when compared to control, whatever the duration of exposure, as well as it inhibited the chitobiase activity after 30 days of exposure. The present study indicates that CLD could interfere with molting process of *M. rosenbergii* by disturbing the 20-HE concentration and the activity of chitobiase, suggesting consequences at the long term on the shrimp development. This study also confirmed that CLD could be an endocrine disruptor in decapod crustaceans, as it was already observed in vertebrates.

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1. Introduction

Endocrine disrupting compounds (EDCs) are exogenous substances or mixtures able to interfere with the endocrine system of exposed organisms. They could affect the hormonal signaling pathways through several mechanisms, for example by inhibiting the synthesis of hormones or decreasing the hormone release by endocrine cells (Craig et al., 2011; Tabb and Blumberg, 2006; Rodríguez et al., 2007). EDCs can also disrupt hormone-receptor interactions and act as an agonist or antagonist by binding to the hormone-receptor complex (Rodríguez et al., 2007; Tabb and Blumberg, 2006). Aquatic environments are considered the main sink for contaminants (Kloas et al., 2009; Meyer-Reil and Köster, 2000), and thus aquatic organisms are therefore major potential targets for EDCs (Kloas et al., 2009).

Several studies on the biological effects of EDCs in aquatic vertebrates have led to the development of biomarkers in order to measure the biological responses towards anthropogenic pollutions, such as changes in the reproductive function. For example, Jobling and Sumpter (1993) introduced the concept that the production of vitellogenin (Vg) in male fish indicates an exposure

to estrogenic compound and Vg is now a widely used biomarker of xenoestrogen exposure in vertebrates. Although EDC effects have been extensively described in aquatic vertebrates (Kortenkamp et al., 2011; LeBlanc, 2007), only few studies have examined the effects of EDCs in invertebrates, although they represent the major part of the animal kingdom (DeFur et al., 1999). This lack could be partially due to the fact that the endocrine system of invertebrates is still obscure (Tillmann et al., 2001). It has nevertheless been observed that EDCs can also disrupt invertebrate physiology. For example, Giusti et al. (2013) have shown that tributyltin affected the production of yolk ferritin (i.e. Vg equivalent) in *Lymnaea stagnalis*, and Xuereb et al. (2011) observed that nonylphenol (estrogenic compound) impacted the expression of a vitellogenin-like gene in the amphipod *Gammarus fossarum*. It appears that vitellogenin-like proteins in invertebrates could serve as biomarkers of EDC exposure, but mechanisms involved in Vg production in invertebrates are still unclear (e.g. hormonal receptor).

EDCs can come from different sources such as synthetic hormones (e.g. ethinylestradiol), synthetic substances (e.g. plasticizers such as phthalates, herbicides) and pesticides often used to eradicate insects. In the French West Indies (FWI), the tropical climate promotes the rapid development of pests which exerts significant pressure on crops leading to the use of considerable amounts of pesticides in these regions (Bocquené and Franco, 2005). The use of organochlorine pesticides first started in the 1950's and led to widespread contamination of the environment (Coat et al., 2006). Because of their physicochemical properties, organochlorine pesticides are persistent in the environment and are known to be accumulated along food chains, resulting in pronounced ecotoxicological damage (Cailleaud et al., 2009; Matsumura, 1975). The most worrying organochlorine pesticide residue in Guadeloupe (FWI) is chlordecone (CLD) (Coat et al., 2011). CLD is an insecticide that was commonly employed to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993 under the trade name Kepone® or Curlone® (Cabidoche et al., 2006, 2009). A few years after the use of CLD, widespread pollution of soils, rivers, wild animals and aquatic organisms was reported (Cavelier, 1980; Snegaroff, 1977), which led to the prohibition of the use of this pesticide in Guadeloupe in 1993. Moreover, in 2009, CLD was included in the Stockholm Convention on POPs (Persistent Organic Pollutants) and its production and use were banned worldwide (Zaldívar and Baraibar, 2011). Nowadays, CLD is still present in soils, especially in the densely cultivated areas from south of Basse-Terre (Guadeloupe) (Cabidoche et al., 2009). Although, Fernández-Bayo et al. (2013) showed the existence of CLD degrading organisms in a tropical soil (andosol) microcosm under aerobic conditions, CLD undergoes no significant or quick biotic or abiotic degradation (Dolfing et al., 2012; Levillain et al., 2012). Driven by the water cycle, CLD in soils is progressively transferred to aquatic ecosystems (Coat et al., 2011) and also in the food web because of its high K_{oc} (Soil Organic Carbon Water Partitioning Coefficient) (15849 L/Kg), K_{ow} (Octanol-Water Partition Coefficient) (4.5–6.0) and its affinity for lipids (Cabidoche and Lesueur-Jannoyer, 2012; Clostre et al., 2013; Sterrett and Boss, 1977; UNEP, 2005; US-EPA, 2008). Human contamination has also been detected in FWI population mainly resulting from consumption of contaminated food, seafood and root vegetables (Dubuisson et al., 2007; Gaume et al., 2014; Guldner et al., 2010). Fishing became prohibited when most aquatic species have exceeded the European legal maximal residual limit (LMR) of 20 μg of CLD per kg wet weight (determined by National ordinance, Anon., 2008). Until 2008, one of the most important aquaculture resources in Guadeloupe was the farms of the tropical giant freshwater prawn *Macrobrachium rosenbergii*. Several studies have underlined effects of pesticides on this species (Revathi and Munuswamy, 2010; Satapornvanit et al., 2009), but very few investigations were carried on the CLD impacts on *M. rosenbergii*. However, Gaume et al.

(2014) have observed that the CLD exposure caused the induction of genes involved in defense mechanisms against oxidative stress (e.g. catalase and glutathione peroxidase), or involved in the biotransformation process (i.e. cytochrome P450 and glutathione-S-transferase).

As CLD is suspected of being an endocrine disruptor (Newhouse et al., 2009) and as *M. rosenbergii* molting is hormonally controlled, the present study aims to investigate the CLD impact on the molting process by investigating the effects of CLD on the concentration of the 20-Hydroxyecdysone (20-HE) hormone and on chitobiase activity in tissues of *M. rosenbergii*. The 20-HE hormone is an ecdysteroid hormone, secreted by the Y-organ (Mykles, 2011), which initiates many physiological processes, such as ovarian maturation, growth, molting, and reproduction in crustaceans (LeBlanc, 2007). A few studies have been designed to investigate the effects of exposure to environmental EDCs on the endocrine system of crustaceans through ecdysteroid concentration, or to investigate the endocrine system of invertebrates (Oberdörster et al., 1999; Palma et al., 2009; Soetaert et al., 2007), but no studies have used the 20-HE concentration to investigate the effects of chlordecone.

Chitobiase is a chitinolytic enzyme involved in the exoskeleton degradation in arthropods and thus plays an important role in the molting and growth of crustaceans (Duchet et al., 2011; Zou and Fingerman, 1999a). Several studies have demonstrated that chitinolytic enzymes are induced by the 20-HE hormone (Zou and Fingerman, 1999b). However, many pollutants have been also shown to induce the chitobiase enzyme. Indeed, Zou and Fingerman (1999c) observed that some estrogenic agents, such as Aroclor 1242, diethylstilbestrol or endosulfan, inhibited chitobiase activity of the crustacean *Uca pugilator*. Similarly, Gismondi and Thomé (2014) noted that some pollutants, suspected of being EDCs (i.e. polybromodiphenyl ethers), could disturb the chitobiase activity of the amphipod *Gammarus pulex*.

Finally, in parallel with these two parameters, the bioaccumulation of CLD in *M. rosenbergii* was evaluated to underline potential concentration-response relationships between the CLD concentration in tissues, the 20-HE concentration and the chitobiase activity. These measures ensure assessing the molting disruption, called the invisible endocrine disruption, because of the disruption of the crustacean molting which is not readily seen in the wild (Zou, 2005).

2. Materials and methods

2.1. Tested organisms

The 3-month-old post-larvae of *M. rosenbergii* (approximately 2 g, 7 cm length and sexually undifferentiated) coming from the same berried female, were provided by an aquaculture farm (OCEAN-SA) located at Pointe-Noire (Guadeloupe, FWI) in a geographic area free of CLD contamination. Pretests have previously been carried out to evaluate the presence or absence of CLD in tissues of prawns from Pointe-Noire and results have shown no contamination (concentrations below detection limit) (data not shown). Prawns were then transferred to the laboratory (DYNECAR, University of the French West Indies and Guiana, Guadeloupe), and acclimated for one week in glass aquaria filled with 28 L of tap water prefiltered on activated carbon. Aquaria were under constant aeration with a 12 h light/dark photoperiod. During acclimation, prawns were fed once daily with artificial shrimp pellets (complete food for rearing, Le Gouessant, France) at one pellet per individual. A constant water temperature of $27.6 \pm 0.2\,^{\circ}C$ was maintained, and pH remained at 7.57 ± 0.03 throughout the experiment. These values are in accordance with optimal water temperature and pH commonly used in prawn farms (New, 2002). Survival was

$96.3 \pm 3.8\%$ throughout the experiment, regardless of the conditions.

2.2. Experimental design

The $10\,mg\,mL^{-1}$ stock solution of CLD (100%, Riedel-de-Haën, Sigma-Aldrich, USA) was prepared in acetone as were also its three different dilutions: $10\,\mu g\,mL^{-1}$, $100\,\mu g\,mL^{-1}$ and $1\,mg\,mL^{-1}$. A volume of $56\,\mu L$ of each dilution and of the stock solution was diluted into the 28 L of aquarium water in order to obtain the four nominal concentrations of CLD in water: $0.02\,\mu g\,L^{-1}$, $0.2\,\mu g\,L^{-1}$, $2\,\mu g\,L^{-1}$ and $20\,\mu g\,L^{-1}$. These concentrations were chosen because of their environmental relevance. According to the Guadeloupean DIREN (Regional Department for the Environment), Guadeloupean rivers are contaminated by CLD at concentrations that range from 0.2 to $4\,\mu g\,L^{-1}$ with a maximum of $8.6\,\mu g\,L^{-1}$ measured in the Grande Anse River in 2003 (GREPP, 2004; InVS-Inserm, 2009).

In this experimental design, two different controls were used. The first, called "water control", was tap water prefiltered through activated carbon; and the second, called "solvent control", was obtained by spiking 28 L of prefiltered tap water with $56\,\mu L$ of acetone. Ten aquaria were used for each condition of exposure. During the 30 days of exposure, M. rosenbergii were fed daily with artificial shrimp pellets (complete food for rearing, Le Gouessant, France), with one pellet per individual. Waste was removed every day prior to feeding. Based on the results of a pre-test designed to evaluate the concentration of CLD in water according to the duration of exposure, it was decided to renew the exposure medium every 96 h, over a total exposure time of 30 days. This process allowed to maintain constant concentrations of CLD during the 30 days of exposure.

A total of 360 post-larvae of M. rosenbergii coming from the same berried female and having the same age (see Section 2.1), were exposed (i.e. 36 prawns per condition) for 30 days, which is the duration of two molt cycles (Ross, 2001). At the beginning of the exposure (T0), prawns of M. rosenbergii were in premolt stage, confirmed by the measurement of the 20-HE concentration in 12 prawns randomly sampled before exposure (i.e. $2058.61 \pm 371.66\,pg\,g^{-1}$). This concentration corresponds to the peak reached by the 20-HE hormone during the molt cycle (see below the confirmation by results of 20-HE concentration in both controls from 1 to 15 days and from 15 to 30 days, corresponding to one molt cycle each). Fifteen M. rosenbergii were randomly sampled for each condition, after four durations of exposure: 1, 4, 15 and 30 days. Five prawns (corresponding to 5 replicates) were used for the CLD bioconcentration assessments, and ten prawns (corresponding to 10 replicates) were used to measure the 20-HE concentration as well as the chitobiase activity. After sampling, the prawns were immediately frozen in liquid nitrogen and stored at $-80\,°C$ until analyses. Before analyses, body length was measured and no significant difference (two-way ANOVA test, $p > 0.05$) was observed between groups (Supplementary material 1).

2.3. Chlordecone concentration in exposure water and M. rosenbergii

2.3.1. Chlordecone extraction from water

The CLD concentration was analyzed in aquarium water by liquid-liquid extraction. A volume from 10 mL to 1 L of water (according to the CLD concentration) was collected and 5 mL of dichloromethane (Biosolve-Chimie, France) was added. Then, $50\,\mu L$ of acetonic solution of PCB112 $(100\,pg\,\mu L^{-1})$ (Dr. Ehrenstorfer, Germany), used as surrogate internal standard, were added before the extraction procedure. This internal surrogate was used in order to quantify possible loss of CLD during the extraction and

purification procedures. The organic phase was recovered and then a purification procedure was performed (see Section 2.3.3).

2.3.2. Chlordecone extraction from muscle tissue

The CLD concentration was analyzed in abdominal muscle of M. rosenbergii according to a method derived from Debier et al. (2003), Multigner et al. (2010) and Lagarrigue et al. (2014). Prawns were thawed, and approximately 500 mg of muscle tissue was freeze-dried during 20 h with a Benchtop 3 L Sentry Lyophilisator (VirTis, USA). The lyophilized samples were weighed in order to determine water content. The extraction of CLD was performed with a solvent mixture of n-hexane: dichloromethane (90:10; v:v; Biosolve-Chimie, France) using an Accelerated Solvent Extractor (ASE200) (Dionex, Thermo Scientific, USA) at $80\,°C$ and under a pressure of 1500 Psi. Before the extraction, $100\,\mu L$ of a hexanic solution of PCB congener 112 (Dr. Ehrenstorfer, Germany) was added to the samples as a surrogate internal standard to obtain a final concentration of $50\,pg\,\mu L^{-1}$. Then, 500 mg of anhydrous sodium sulfate were added to avoid any water trace in the extract. The solvent, containing the extracted fat, was collected in pre-weighed vials and evaporated at $40\,°C$ under a gentle nitrogen flow using a Turbovap LV (Zymarck, USA) until a constant weight of residues. Then, the lipid content was determined gravimetrically.

2.3.3. Sample purifications

The purification step was the same for water and biological samples. The residues from the two different extraction were resuspended into 2 mL of n-hexane (Biosolve-Chimie, France) and transferred into a test tube to carry out an acid clean-up. A volume of 2 mL of 98% sulfuric acid (Merck, Germany) was added in the extracts in order to remove organic matter (e.g. lipids, lipoproteins, carbohydrates). The mixture was homogenized by vortexing for 1 min with a Vibramax 110 (Heidolph, Germany), and centrifuged for 3 min at $2160g$, $10\,°C$. The organic phase was collected into a new tube and the sulfuric acid layer was extracted in the same way with another 3 mL of n-hexane to ensure an optimal recovery. The two resulting organic layers were pooled and $5\,\mu L$ of nonane were added as a keeper. This extract was evaporated under a gentle nitrogen stream using a Visidry evaporator (Supelco, Sigma-Aldrich, USA) before being resuspended with $45\,\mu L$ of n-hexane and $50\,\mu L$ of a solution of PCB 209 $(100\,pg\,\mu L^{-1}$ in n-hexane) as an injection volume internal standard (Dr. Ehrenstorfer, Germany).

In parallel with sample extractions, a procedural blank and a Quality Control (QC) were carried out. The procedural blank consisted of the ASE extraction without biological matrix, allowing to control the extraction and the clean-up procedure. The QC was performed to assess the CLD recovery by using a CLD-free water or biological matrix (here, freeze-dried muscle of the decapod Penaeus monodon) spiked with an acetonic solution of CLD in order to obtain a final concentration of $2.5\,ng\,L^{-1}$ for water and $2.5\,ng\,g^{-1}$ wet weight for biological sample. Muscle tissue of P. monodon was used here as biological matrix of decapod because they came from geographic area free of CLD contamination.

2.3.4. Chromatography analysis

The purified extracts, procedural blank and QC were analyzed by high-resolution gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a [63]Ni ECD detector (Thermo Scientific, USA) and an auto-sampler Thermo Quest AS 2000 (Thermo Scientific, USA). The extract was injected in the on-column mode at $60\,°C$. CLD was separated on a 30 m × 0.25 mm (0.25 μm film) DB-XLB capillary column (J&W Scientific, USA). The other analytical parameters were described elsewhere (Lagarrigue et al., 2014; Multigner et al., 2010). Data were recorded with Chromcard 2.8 (Fisons Instruments, Italy) software for Windows. CLD was identified based on its retention time previously determined

Table 1
Chlordecone concentration (mean ± S.D.) measured in water of exposure. LOQ means limit of quantification (0.01 ng L^{-1}).

Conditions:	Watercontrol	Solvent control	0.02 μg L^{-1}	0.2 μg L^{-1}	2 μg L^{-1}	20 μg L^{-1}
Nominal concentration (μg L^{-1})	0	0	0.020	0.20	2.00	20.00
Measured concentration (μg L^{-1})	<LOQ	<LOQ	0.019 ± 0.002	0.20 ± 0.03	1.80 ± 0.13	20.43 ± 2.56

Table 2
CLD bioconcentrations (mean ± S.D.) measured in the muscle tissue of *Macrobrachium rosenbergii* exposed at four concentrations of CLD and sampled at four times of exposure. LOD means limit of detection (0.02 ng g^{-1} wet weight) and LOQ means limit of quantification (0.06 ng g^{-1} wet weight). Different letters indicate significantly different values for each CLD concentration of exposure. Tukey's HSD test was performed with the log-transformed CLD concentration (p-values < 0.05).

Nominal Concentration (μg L^{-1})	Time (days)	Chlordecone concentration in muscle tissue (ng g^{-1})	BCF
Control	1	<LOD	/
	4	<LOQ	/
	15	<LOQ	/
	30	<LOQ	/
Solvent Control	1	<LOD	/
	4	<LOQ	/
	15	<LOQ	/
	30	<LOQ	/
0.02	1	1.58 ± 1.09 [a]	79
	4	4.20 ± 3.34 [b]	210
	15	5.08 ± 1.13 [b]	254
	30	30.80 ± 10.37 [c]	1540
0.2	1	10.56 ± 1.64 [a]	53
	4	7.89 ± 1.68 [a]	39
	15	38.12 ± 13.48 [b]	191
	30	45.96 ± 34.09 [b]	230
2	1	36.06 ± 11.90 [a]	32
	4	27.66 ± 5.01 [a]	14
	15	241.52 ± 127.38 [b]	121
	30	845.15 ± 374.31 [c]	423
20	1	292.75 ± 133.11 [a]	15
	4	547.36 ± 228.74 [a]	27
	15	26637.37 ± 12411.53 [b]	1332
	30	5312.04 ± 2683.82 [c]	266

with a linear calibration curve (1.5 to 200 pg μL^{-1}) established with certified solutions of CLD (Riedel-de Haën, Germany). The CLD concentrations in each sample and in the QC were corrected with the percentage recovery of the surrogate PCB 112. The recovery efficiency based on the CLD recovery in QC and on the recovery of the surrogate internal standard (PCB112) ranged from 88 ± 4% to 115 ± 5%, which was within the limits recommended by SANCO (i.e. range from 60 to 140% – Document No SANCO/12495/2011 European Union, 2011). The limit of detection (LOD) was fixed at three times the background noise of the chromatogram (i.e. 0.005 ng L^{-1} for water sample and 0.02 ng g^{-1} wet weight for muscle tissue). The limit of quantification (LOQ) was determined with CLD-free water or freeze-dried prawn muscle spiked with various concentrations of CLD and was established at 0.01 ng L^{-1} for water sample and 0.06 ng g^{-1} wet weight for muscle tissue, corresponding to about ten times the background noise of the chromatogram. The CLD concentrations in water and in muscle tissue of *M. rosenbergii* were measured in five replicates per condition and the mean calculated. The CLD concentrations expressed in μg L^{-1} for water sample are presented in Table 1 and in ng g^{-1} wet weight for biological sample are presented in Table 2. The bioconcentration factor (BCF) was calculated for each condition using the method of Taylor (1983) described in Duquesne et al. (2000) (Table 2). BCF is the ratio between CLD concentration in muscle tissues of prawns and CLD concentration in tank water.

2.4. 20-Hydroxyecdysone concentration

The concentrations of the 20-HE hormone and its derivatives (called "20-HE" in the following text, figures and tables) were measured by following the manufacturer's instructions of the Enzyme ImmunoAssay (EIA) kit (Cayman Chemical Company, USA) which were adapted to our biological organism (e.g. weight of tissue, solvent of homogenization, standard curve). Muscle tissue was chosen as biological matrix in order to be consistent with the tissue used for the CLD concentration assessment. In addition, a pretest, which investigated the 20HE concentration in different organs, showed that the muscle tissue has a high 20-HE concentration (see Supplementary material 2). A weight of 250 mg of frozen muscle tissue of *M. rosenbergii* was homogenized in 2:1 (w:v) of methanol-water (4:1, v:v) (i.e. 500 μL of methanol-water to 250 mg of tissue) using a Precellys homogenizer (Bertin Technologies, France). Samples were incubated overnight at −20 °C and centrifuged for 10 min at 10,000g and 4 °C. The resulting supernatant was transferred into a glass tube and the solvent was evaporated with a CentriVap Centrifugal Vacuum Concentrator combined with a CentriVap Cold Trap (Labconco, USA) using centrifugal force, vacuum and controlled temperature. After evaporation, samples were resuspended in EIA Buffer (1 M phosphate, containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide).

A 96-well microplate precoated with mouse anti-rabbit IgG (Cayman Chemical Company, USA) was used. Each microplate contained blanks (i.e. appropriated substrate solution), diluted samples in EIA Buffer in duplicate, and an eight point 20-HE (Sigma-Aldrich, USA) standard curve (of 7.8125–1000 pg mL^{-1}). Each sample received a solution of 20-HE EIA Antiserum (Cayman Chemical Company, USA) containing anti-20-HE rabbit IgG. Then, a solution of 20-HE Acetylcholinesterase (AChE) EIA Tracer (Cayman Chemical Company, USA) containing a covalent conjugate of 20-HE and electric eel AChE was added to each sample. After an

overnight incubation at 4 °C, the plate was washed with Wash Buffer (4 M phosphate, pH 7.4). Next, Ellman's Reagent (Cayman Chemical Company, USA) was added and the plate was incubated for 2 h in the dark with gentle shaking. The absorbance was measured at 420 nm and the average of duplicates of each sample was calculated. The 20-HE concentrations were analyzed in 10 replicates per condition (i.e. 10 samples per condition), and the results were expressed in $pg\,g^{-1}$ of fresh weight of *M. rosenbergii* muscle tissue.

2.5. Chitobiase activity

A weight of 50 mg of frozen muscle tissue of *M. rosenbergii* was homogenized in 500 μL of citrate phosphate buffer (0.15 M, pH 5.5) using a Precellys homogeneizer (Bertin technologies, France). Samples were centrifuged for 10 min at 10 000*g* and 4 °C, and the resulting supernatants were recovered and used to analyze the chitobiase activity. Chitobiase activity was measured using the method described by Espie and Roff (1996). The chitobiase reaction used 4-methylumbelliferyl-*N*-acetyl-ß-D-glucosaminide (MUFNAG) as a substrate and generated the fluorescent 4-methylumbelliferone (MUF), which was evaluated. Measurements were carried out in 96-well microplates containing a blank (i.e. appropriated substrate solution), an eight-point MUF (Sigma-Aldrich, USA) standard curve (0–20 μM), and samples in duplicate. Every sample was incubated in the dark with the substrate MUFNAG (Sigma-Aldrich, USA) for 30 min at 25 °C. The reaction was stopped by addition of 0.25 N NaOH (pH 14.1). The liberated MUF was measured fluorometrically at the excitation wavelength of 360 nm and emission wavelength of 450 nm. The chitobiase activity was measured in 10 replicates per conditions. Each replicate was analyzed in duplicate and the mean was calculated. In general, enzyme activities are normalized against the total protein content in sample extracts. However, as natural variation of protein contents related to physiological changes could constitute a source of variability, several studies recommended to express enzyme activity in $\mu mol\,h^{-1}$ (Owen et al., 2002; Radenac et al., 2008; Xuereb et al., 2009). Therefore, the chitobiase activity was expressed here in μmol of MUF formed per hour $^{-1}$.

2.6. Statistical analysis

After a log-transformation of the CLD concentrations in muscle tissue, all data met normality and homogeneity of variance assumptions (Shapiro and Bartlett tests, $p > 0.05$). For each measured parameter (i.e CLD bioaccumulation, 20-HE concentration and chitobiase activity), comparisons were performed by two-way ANOVA followed by Tukey HSD post-hoc tests. A probability value of less than 0.05 was regarded as significant.

The correlations between measured parameters were analyzed using the Pearson correlation coefficient. All tests were performed with STATISTICA 10 Software (StatSoft, 2012, Belgium).

3. Results

3.1. Chlordecone concentrations in muscle tissue of M. rosenbergii

The CLD concentrations in the muscle tissue of *M. rosenbergii* were significantly influenced by the CLD concentrations of exposure, duration of exposure and their interaction ($p < 0.001$). Results shown that the CLD concentrations were below the limit of quantification in prawns from control and solvent control conditions. Moreover, the CLD bioconcentration in prawns was concentration-dependent, since prawns exposed to higher concentrations of CLD accumulated greater amounts of CLD (Table 2). The bioaccumulation of CLD in *M. rosenbergii* was also time-dependent. Indeed,
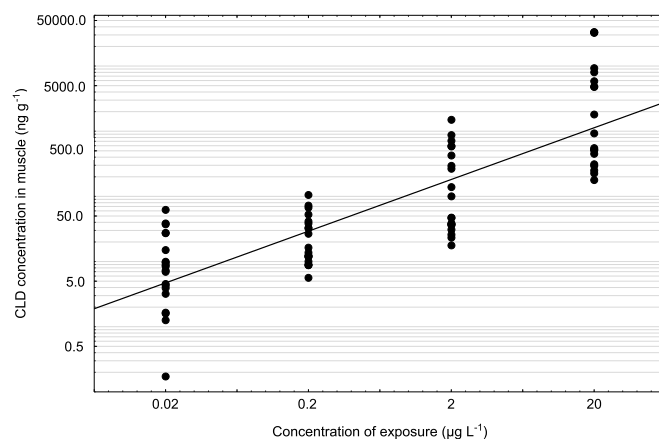

Fig. 1. Positive correlation between CLD bioconcentrations measured in the muscle tissue of *Macrobrachium rosenbergii* and CLD concentrations in water of exposure ($p < 0.001$, $r = 0.49$, $n = 100$), taking into account all duration of exposure.

the longer the prawns were exposed, the higher was the CLD bioconcentration. These observations were underlined by a strong significant positive correlation between concentrations of CLD in water and the CLD bioconcentration in muscle tissue of *M. rosenbergii* ($p < 0.001$, $r = 0.49$, $n = 100$; Fig. 1).

Generally, whatever the CLD concentration of exposure, results showed that a bioconcentration of CLD was measured from the first day of exposure (Table 2), following a trend towards a concentration-response relationship. However, no significant differences were observed in the CLD bioconcentrations after 1 and 4 days of exposure, except for exposure to 0.02 μg L^{-1}. In addition, for each condition of exposure, the highest CLD bioconcentration measured was observed after 30 days of exposure; except for exposure to 20 μg L^{-1} where the highest CLD bioconcentration was measured after 15 days of exposure (Table 2).

The bioconcentration factor (BCF) was calculated for the four CLD concentrations and results showed that BCF increased with the duration of exposure. Indeed, BCF were higher in prawns exposed for 30 days that those exposed for 1 or 4 day. Moreover, BCF appeared higher in *M. rosenbergii* exposed to lowest CLD concentration (i.e. 0.02 μg L^{-1}) than to the other CLD concentrations.

3.2. 20-Hydroxyecdysone concentration

Results highlighted a natural variation of the 20-HE concentration in control prawns according to the time. Indeed, the 20-HE concentration decreased between the 1st and the 4th day of exposure, and increased between the 4th and the 15th day (Fig. 2). In addition, no significant differences were observed between 20-HE concentrations measured in water control and in solvent control ($p > 0.05$) regardless of the duration of the exposure.

Results revealed that 20-HE concentration was significantly influenced by the CLD concentration, the duration of exposure and their interaction. Generally, lower 20-HE concentrations were seen in exposed prawns compared to the respective control, regardless of the duration of exposure, except after 4 days of exposure. Results also showed that 20-HE concentrations seemed generally lower after 4 days of exposure compared to exposures lasting for 1, 15 or 30 days. For each duration of exposure, the largest decrease of 20-HE concentration was measured in prawns exposed to 0.2 μg L^{-1} (except after 30 days of exposure where it was the second lowest). Indeed, the 20-HE concentration was on average twice lower in this CLD concentration than in the respective controls for each duration of exposure.
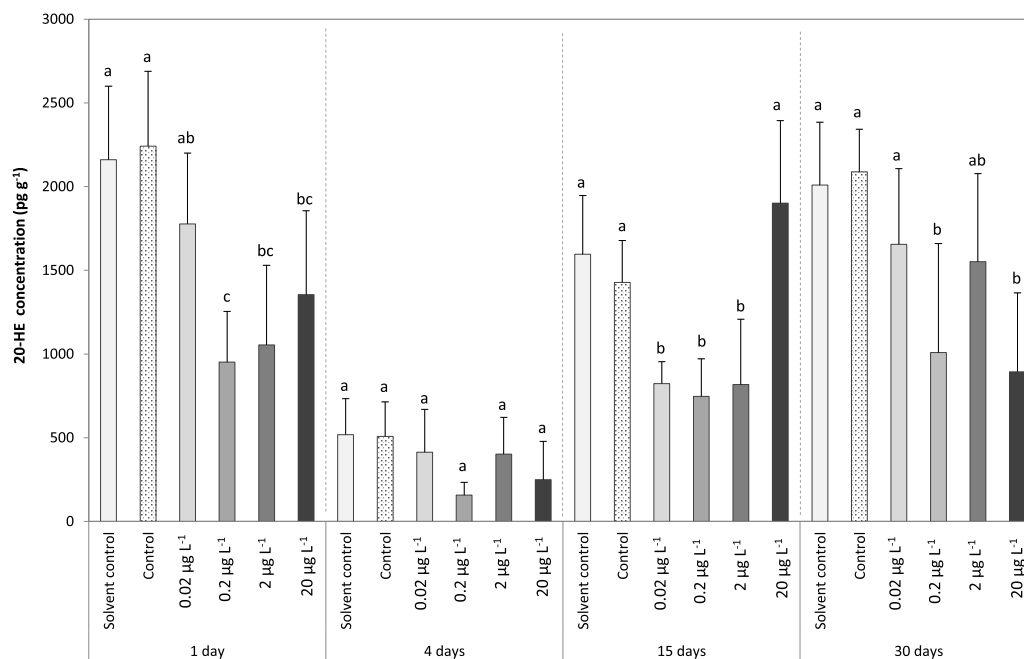
Fig. 2. 20-HE concentrations (pg g^{-1}; mean ± S.D.) in the muscle tissue of *Macrobrachium rosenbergii* exposed at four concentrations of CLD and sampled at four times of exposure. Different letters above the bars indicate significantly different values for each sampling time (Tukey's HSD test, *p*-values <0.05).
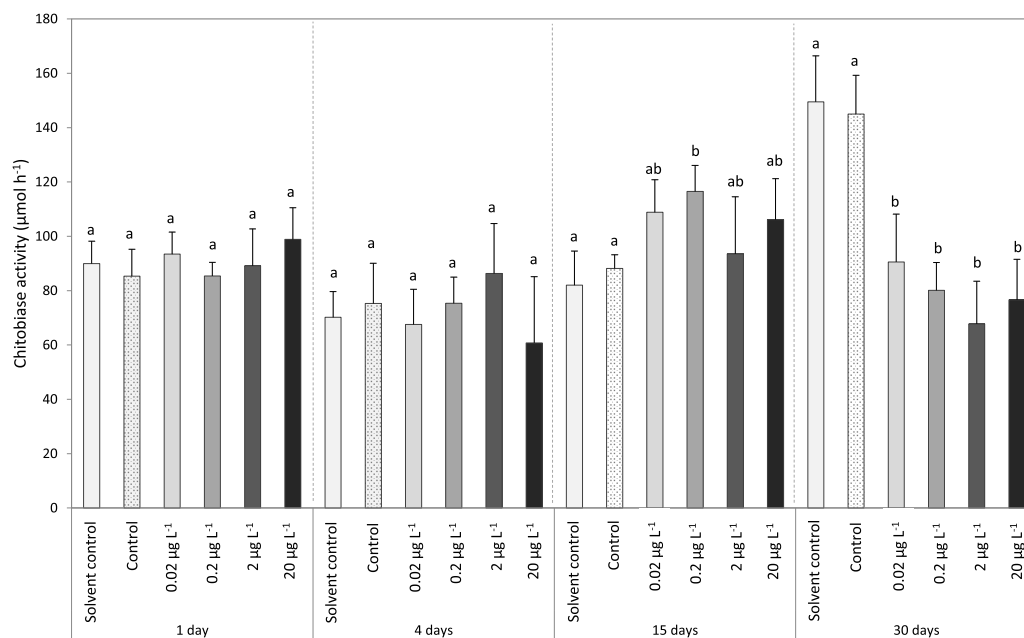

Fig. 3. Chitobiase activity (μmol MUF hydrolysed h^{-1}; mean ± S.D.) in the muscle tissue of *Macrobrachium rosenbergii* exposed at four concentrations of CLD and sampled at four times of exposure. Different letters above the bars indicate significantly different values for each sampling time (Tukey's HSD test, *p*-values <0.05).

After 1 day of exposure, the 20-HE concentration was significantly from 1.5-fold to 1.8-fold lower in *M. rosenbergii* exposed to 0.2, 2 and 20 μg L^{-1} of CLD compared to the control. After 4 days of exposure, although the 20-HE concentrations seemed to be lower in exposed prawns compared to the control, no significant differences were observed. However, a significant reduction of the 20-HE concentration (on average 1.8-fold lower) was measured after 15 days of exposure in all exposure conditions compared to the control, except for 20 μg L^{-1}. Finally, after 30 days of exposure, prawns exposed to 0.2 and 20 μg L^{-1} CLD had twice smaller 20-HE concentration than the control.

Moreover, a significant negative correlation between 20-HE concentration and the log-transformed CLD bioconcentrations was observed regardless of the duration of the exposure (1 day: p < 0.001, r = −0.87, n = 19; 4 days: p < 0.001, r = −0.83, n = 20; 15 days: p < 0.001, r = −0.76, n = 24; 30 days: p < 0.001, r = −0.72, n = 21) (Fig. 4).

3.3. Chitobiase activity

The activity of chitobiase in muscle tissue of *M. rosenbergii* was significantly influenced by the CLD concentration, the duration of exposure and their interaction (p < 0.001; Fig. 3). The chitobiase
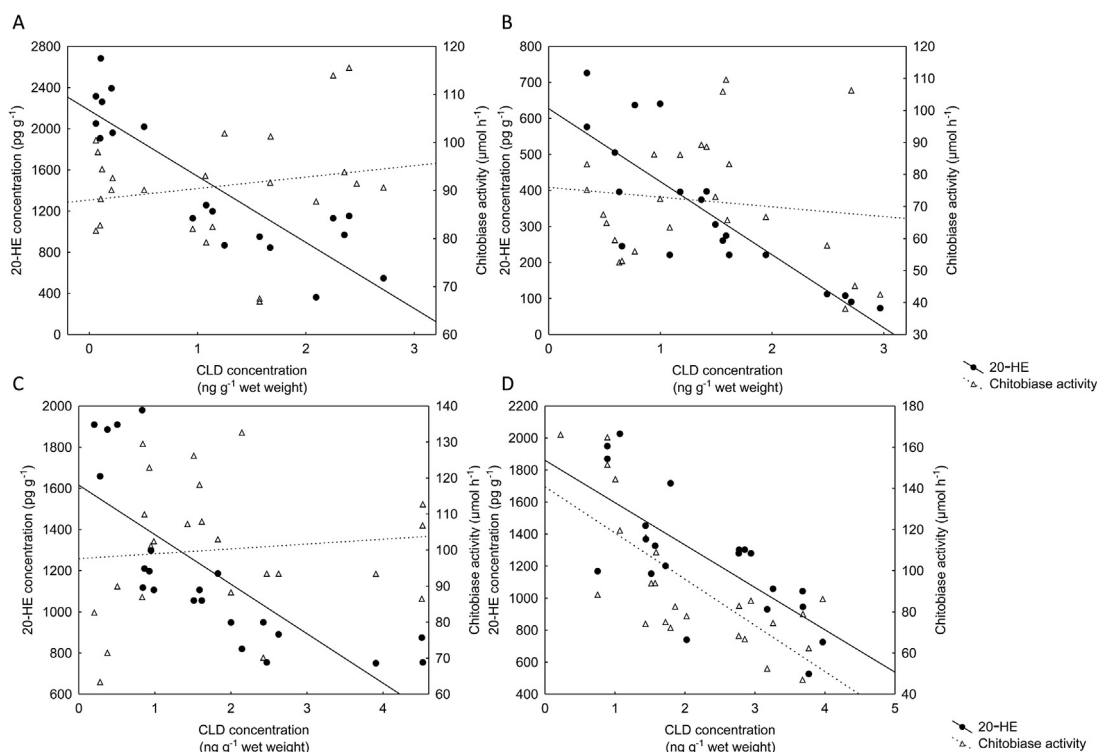
Fig. 4. Logarithmic correlation between log-transformed CLD bioconcentrations and the 20-HE concentration represented by circles, as well as the correlation between the log-transformed CLD bioconcentrations and chitobiase activity represented by triangles in the muscle tissue of *Macrobrachium rosenbergii* sampled after 1 day (A), 4 days (B), 15 days (C) and 30 days (D) of exposure.

activity measured in water control and solvent control were not significantly different (p > 0.05) regardless of the duration of the exposure (Fig. 3). Moreover, no significant difference was observed in the chitobiase activity measured in prawns exposed to CLD for 1 and 4 days, regardless of the CLD concentration compared to the respective controls (Fig. 3). After 15 days of CLD exposure, a significant increase in the chitobiase activity was only observed in prawns exposed to 0.2 μg L^{-1} compared to the control. The chitobiase activity was 1.5-fold higher than in the control prawns. On the contrary, a significant inhibition of the chitobiase activity was observed after 30 days of exposure, whatever the CLD concentration. Indeed, the chitobiase activity was in average 1.8-fold lower in exposed prawns that in the control. No correlation was observed between the chitobiase activity and the log-transformed CLD bioconcentration in prawns, expect for in prawns exposed to CLD for 30 days (1 day: p = 0.38; 4 day: p = 0.85; 15 days: p = 0.65; 30 days: p < 0.001, r = −0.74, n = 25) (Fig. 4).

4. Discussion

This work aimed to study the impacts of CLD in the crustacean *M. rosenbergii* by measuring CLD effects on the 20-HE hormone concentration and the chitobiase activity, both involved in the molting process and thus in the growth and the development, as well as the CLD bioaccumulation in the muscle tissue of prawns.

4.1. Chlordecone bioaccumulation

This study highlighted that chlordecone was accumulated in tissues of *M. rosenbergii* following a trend towards a concentration-response relationship. The prawn survival was not affected by the CLD exposure, indicating that exposure concentrations were below lethal concentrations. Indeed, Schimmel and Wilson (1977) reported that the 96-h LC50 of CLD was 121 μg L^{-1} for grass shrimp

Palaemonetes pugio and no significant mortality in blue crabs *Callinectes sapidus* at measured concentrations as high as 210 μg L^{-1} CLD was observed.

The bioaccumulation observed in *M. rosenbergii* is consistent with the fact that CLD has a high potential of bioaccumulation in aquatic organisms, based on its physicochemical properties (ATSDR et al., 1995). This result is in agreement with previous studies highlighting the CLD concentration in different organisms. Indeed, Van Veld et al. (1984) demonstrated that CLD could be accumulated in the channel catfish when exposed for 30 days, and Coat et al. (2011) observed the CLD bioaccumulation in tropical food web including various species of mussels, shrimps and fishes. The CLD concentrations measured in muscle tissue of *M. rosenbergii* were somewhat lower than those measured in other decapods exposed to similar CLD concentrations (Bahner et al., 1977; Gaume et al., 2014). This observation could be explained by the distribution of CLD in tissues of prawns. Indeed, because of its structure and binding properties, CLD binds preferentially to serum proteins such as lipoproteins or albumin and therefore, its distribution in the whole organism is quite different compared to other organochlorines (ATSDR et al., 1995; Newhouse et al., 2009). For example, the accumulation of CLD is highest in the liver (Newhouse et al., 2009) while other organochlorine pesticides such as HCB or p,p′-DDE are preferentially distributed in the adipose tissue (Gomez-Catalan et al., 1991; Soine et al., 1982). In addition, Roberts (1981) observed higher CLD residuals in reproductive tissues than in the hepatopancreas of the blue crabs *C. sapidus*.

Our results suggest that an accumulation of CLD was observed from the 1st day of exposure and that this increase of CLD concentrations in prawns was time-dependent from the 4th day of exposure, whatever the CLD concentration of exposure. However, in the highest CLD condition (i.e. 20 μg L^{-1}), the accumulation of CLD decreased between the 15th and 30th day of exposure. This observation suggests that organisms have reached a stage beyond

which the accumulation is no longer exponential at this concentration of exposure. Several studies have highlighted the ability of crustaceans to depurate CLD slowly (Bahner et al., 1977; Roberts, 1981; Schimmel et al., 1979). This depuration of CLD could explain the difference of CLD concentrations in *M. rosenbergii* between 15 and 30 days of exposure. This decrease could also be explained by a toxicity of CLD on the organism physiology, resulting in lower CLD concentration in prawns (e.g. gill destruction reducing exchange between the organism and its environment Hebel et al., 1997). Indeed, once in the organism, as most pesticides, chlordecone can exert adverse toxic effects (Sanders et al., 1981). Several studies have demonstrated that CLD could impact the crustacean development and growth through disturbance of the molting process. Indeed, Schimmel et al. (1979) showed that CLD interferes with the molting of *C. sapidus*. Similarly, Bookhout et al. (1980) and Oberdörster and Cheek (2001) have shown that CLD impacted the molting and the metamorphosis of *P. pugio* and *Rhithropanopeus harrisii*, respectively. In addition, Nimmo et al. (1977) and Sanders et al. (1981) reported that the growth of *Mysidopis bahia* and *Gammarus pseudolimnaeus,* respectively, closely linked to the molting process, was reduced by exposure to CLD. CLD could also affect the reproduction process (e.g. duration of embryonic development, the juvenile period, the reproductive and post-reproductive periods, etc.) of the rotifers of *Brachionus calyciflorus* as observed by Zha et al. (2007). All these results suggest that CLD accumulated in organisms could disturb critical physiological processes controlled by the hormonal system.

BCF results showed that the bioconcentration of CLD was more efficient with lowest concentration in water. Prawns exposed to $0.02 \, \mu g \, L^{-1}$ had higher BCF than prawns exposed to other CLD concentrations. Here, we can hypothesize that the higher BCF observed in the lowest CLD concentration could be explained by the potential EDC effect of CLD. Indeed, it is well known that endocrine disruption occurs with low EDC concentrations by their binding to hormonal receptors (Vandenberg et al., 2012). At an exposure concentration of $0.02 \, \mu g \, L^{-1}$, CLD could be sequestered in tissues as many pollutants but in addition CLD could be sequestered by receptors, resulting in higher CLD concentration in organisms. Moreover, BCF results indicated that no plateau level was reached after 30 days of exposure, especially when *M. rosenbergii* were exposed to $0.02 \, \mu g \, L^{-1}$, suggesting a strong accumulation of CLD in organisms exposed on the long term, which could have deleterious effects.

4.2. Chlordecone effects on the 20-HE concentration

In control conditions (i.e. unexposed prawns), results shown lower 20-HE concentration after 4 days of exposure compared to the other controls (i.e. 1, 15 and 30 days). This difference may be explained by the premolt stage of prawns at the beginning of the experiment and the molt cycle occurring during the experiment. Indeed, it was established that the molt cycle of *M. rosenbergii* lasts an average of 15 days (Ross, 2001). Moreover, several studies have highlighted the variation of the 20-HE concentration during the different stages of the molt cycle of crustaceans (Hyne, 2011; Okumura and Aida, 2000; Zou and Bonvillain, 2004; Zou, 2005). The variation of 20-HE concentration observed here could be due to variations of ecdysteroid synthesis according to the developmental stage of prawns. In crustaceans, the endocrine system regulates many processes that differentiates males from females, larvae from juveniles, and reproductively active from reproductively senescent organisms (LeBlanc, 2007). Chang and Bruce (1980) attested that ecdysteroid composition is affected by the developmental stage as well as the physiological and reproductive status of the organism. Indeed, the authors showed that the total ecdysteroid concentration in hemolymph of *Homarus americanus* can fluctuate during

the molt cycle from less than $10 \, pg \, \mu L^{-1}$ in postmolt to more than $350 \, pg \, \mu L^{-1}$ in premolt. Generally, ecdysteroid concentration is low during intermolt and postmolt, rising during premolt and reaching a peak shortly before molting (Mykles, 2011). This increase in ecdysteroid concentration is not only related to ecdysteroid synthesis but could also be explained by increased conversion of ecdysone or inactive ecdysteroids to the active form of ecdysteroids (i.e. 20-HE) (Mykles, 2011).

After exposure to CLD, results revealed an effect of CLD on the 20-HE concentration. Indeed, a decrease of 20-HE in *M. rosenbergii* exposed to CLD was seen regardless of the concentration and the duration of exposure (except for prawns exposed to $20 \, \mu g \, L^{-1}$ of CLD for 15 days). This observation was supported by the fact that the 20-HE concentration and the CLD bioconcentration were significantly negatively correlated regardless of the duration of the exposure. The 20-HE disruption corroborates the hypothesis that CLD could be endocrine disruptor in invertebrates as already observed in vertebrates. Indeed, several previous studies have demonstrated that CLD could be an endocrine disruptor in vertebrates such as fish (Ankley et al., 1998; Donohoe and Curtis, 1996; Nimrod and Benson, 1997; Sumpter and Jobling, 1995), birds and mammals (Eroschenko, 1981), by having estrogenic properties through interaction with the estrogen-receptor system (Donohoe and Curtis, 1996; Hammond et al., 1979; Rodríguez et al., 2007). In crustaceans, various estrogenic compounds and estrogen receptor agonists (e.g. bisphenol A; nonylphenol) have been shown to act as ecdysteroid synthesis inhibitors or ecdysteroid receptor (EcR) antagonists (LeBlanc, 2007), leading to a possible decrease of ecdysteroid concentrations (Forget-Leray et al., 2005; LeBlanc, 2007; Rodríguez et al., 2007). Thus, it could be hypothesized that CLD decreased the 20-HE concentration by binding directly to ecdysteroid receptor, as a consequence of its anti-ecdysteroidal activity (Zou and Fingerman, 1999d; Zou, 2005). However, as other EDCs, CLD can also interfere the endocrine system indirectly by several mechanisms, at any step of the transduction pathway of the hormones (Hyne, 2011; Rodríguez et al., 2007). The 20-HE decrease observed here could also be explained by a modification of the excretion rate of the hormone or an inactivation of the enzyme involved in the ecdysteroid biotransformation. Because of the fact that CLD impacts the 20-HE concentration, it could therefore in the long-term disturb the molting of organisms which is induced by this hormone (Hyne, 2011; Rodríguez et al., 2007). This hypothesis is supported by the studies of Hirano et al. (2009) which have shown that low 20-HE concentration in the shrimp *Americamysis bahia* exposed to nonylphenol (also known to be an inhibitor of ecdysteroid activity) affected the growth of organisms. In similar way, a decrease of the 20-HE concentrations in juveniles of *Daphnia magna* exposed to the fungicide fenarimol was associated with abnormal development (LeBlanc, 2007; Mu and Leblanc, 2002). Snyder and Mulder (2001) have also suggested that the delay in the beginning of molting of larvae in the lobster *H. americanus* when exposed to heptachlor, probably correlated to a drop in the concentrations of circulating ecdysteroids. Moreover, knowing that crustacean reproduction is linked to the molt cycle (Hyne, 2011), disruption in the molting process could also cause disturbances in reproduction as hypothesized by Gismondi and Thomé (2014) in the amphipod, *G. pulex*. Indeed, it was observed that the anti-ecdysteroidal activity of nonylphenol caused a reduction in fecundity of many insects (LeBlanc et al., 2000).

Results also highlighted stronger decrease (or tendency to stronger decrease) of the 20-HE concentration in prawns exposed to $0.2 \, \mu g \, L^{-1}$ of CLD with time (except after 30 days of exposure), while this decrease was less obvious in prawns exposed to high CLD concentrations (i.e. 2 and $20 \, \mu g \, L^{-1}$). This observation could be explained by the fact that all receptors, through which CLD

may exert its effects, were already occupied by CLD molecules at the exposure concentration of $0.2\,\mu g\,L^{-1}$. With higher CLD concentrations required to increase the response, no additional binding can occur, and thus, the maximal effect was observed in prawns exposed to $0.2\,\mu g\,L^{-1}$ (Welshons et al., 2003). Moreover, it is now well established that EDCs act at low levels (Vandenberg et al., 2012). Therefore, it can also be hypothesized that $0.02\,\mu g\,L^{-1}$ of CLD is too weak to cause significant disturbances, while the $0.2\,\mu g\,L^{-1}$ seems to be the lowest sufficient CLD concentration to induce effects on endocrine hormones.

4.3. Chlordecone effects on chitobiase activity

With regard to chitobiase activity, results highlighted no significant effects of CLD in prawns exposed for 1 or 4 days. However, after 15 days of exposure, a significant increase of the chitobiase activity was observed, but only for prawns exposed to $0.2\,\mu g\,L^{-1}$ of CLD, while a significant inhibition was observed in all CLD conditions after 30 days of exposure. In addition, unlike 20-HE results, chitobiase activity was not strongly correlated with the CLD exposure. This result is surprising due to the fact that chitinolytic enzymes are regulated by ecdysteroids in crustaceans (Zou and Fingerman, 1999d). Indeed, Zou and Fingerman (1999b) underlined that a decrease in the chitobiase activity in the fiddler crab, U. pugilator, was correlated to a reduction of ecdysteroid concentration during the molting cycle, suggesting that the chitobiase activity is regulated at least in part by 20-HE. In the present study, based on the decrease of the 20-HE concentration, an inhibition of the chitobiase activity was therefore expected. However, the precise mechanisms linking the 20-HE concentration and the chitobiase activity are not well established yet. CLD may affect the chitobiase activity not through the effect on 20-HE concentration but maybe by disrupting several pathways and interactions with a few key receptors. This result is in line with those of Snyder and Mulder (2001). Indeed, although Snyder (1998) reported, in the lobster H. americanus, that although an increase in 20-HE concentration can induce the expression of cytochrome P450-dependent detoxifying enzymes (CYP45), an upregulation of the expression of this enzyme in larvae of H. americanus exposed to heptachlor (organochlorine pesticide) occurred together with a decrease of the 20-HE concentration. In the present study, the major absence of modification of the chitobiase activity, while a decrease of the 20-HE concentration was observed, could be explained by a direct effect of CLD on chitobiase activity through other receptors as hypothesized by Rodríguez et al. (2007). Indeed, it could be hypothesized that the inhibition of the chitobiase activity due to the decrease of the 20-HE concentration could be offset by an induction of the chitobiase activity through a direct effect of CLD. Thus, these two opposite effects could result in a lack of the chitobiase activity disruption. However, results showed a decrease of the chitobiase activity after 30 days of exposure. This observation could be understood by a difference in the time of response. Indeed, as explained above, a decrease of the 20-HE concentration was observed after 15 days of exposure. 20-HE is known to induce the molt cycle, which involves the chitobiase enzyme to break down the old cuticle (Hyne, 2011). Nevertheless, no information is available on the duration of the molt cycle and the different pathway cascades in M. rosenbergii (i.e. from 20-HE synthesis to chitobiase activity induction). Therefore, it could be hypothesized that the consequence of the 20-HE decrease can appear later on the chitobiase activity (here, after 30 days of exposure).

The disturbance of the chitobiase activity observed here suggests a disruption of the molt cycle of M. rosenbergii which could have serious consequences on the individual development, due to the fact that the decapod growth is strongly linked to molt (LeBlanc, 2007). It could be hypothesized that chlordecone could affect the embryonic and juvenile development of M. rosenbergii, as has been observed by Zha et al. (2007) in the rotiferia B. calyciflorus where CLD could affect the reproduction process (e.g. duration of embryonic development, the juvenile period, the reproductive and post-reproductive periods).

5. Conclusion

This study is the first to highlight the impacts of a CLD exposure on the hormonal system of the commercial prawn, M. rosenbergii, by measuring the 20-HE concentration and the chitobiase activity. The results underscored that CLD is bioaccumulated in M. rosenbergii causing the decrease of the 20-HE concentration. In addition, an inhibition of the chitobiase activity was measured after 30 days of exposure. Although it has been documented a few times that CLD can disrupt the molting process of invertebrates, the mechanisms involved in this effect remain unknown. This work suggests that CLD could disrupt the molting process through the disturbance of the 20-HE concentration, as well as the chitobiase activity. Results allowed us to conclude that CLD could have an anti-ecdysteroidal activity, as suggested for other estrogeno-mimetic EDCs. Nevertheless, further studies are needed to better understand the mechanism involved in this endocrine disruption and to describe the chlordecone effects on M. rosenbergii population. It could be especially interesting to focus research on the synthesis of the 20-HE and the ecdysteroid metabolism pathways, as well as the effects of CLD on ecdysteroid receptors to confirm the anti-ecdysteroidal activity of CLD, and the designation of CLD as an EDC in invertebrates. Finally, the lack of information on the endocrine system of invertebrates makes it difficult to explain the results and their expected consequences. Therefore, future investigations should focus on the invertebrate endocrine system to refine its understanding, and to improve the assessment of effects of endocrine disruptors. Moreover, future studies might also include an assessment of the influence of molt stage on the response of organisms to xenobiotic exposure.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.04.006.

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