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Effects of chlordecone on *Macrobrachium rosenbergii* : Bioaccumulation, elimination and perturbation of biological processes related to the endocrine system

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Abstract

Endocrine disrupting compounds are chemicals that interfere with the endocrine system of exposed organisms, at very low levels. They may act through various mechanisms and at different stages of signaling pathways of hormones. Chlordecone is an organochlorine compound considered as a strong endocrine disruptor due to its ability to bind to estrogen receptors in vertebrates. Some recent studies also report the impact of chlordecone on the endocrine system in invertebrates. Chlordecone is an organochlorine insecticide widely used in Guadeloupe (French West Indies), from 1972 to 1993, to control the banana weevil *Cosmopolites sordidus* (Germar, 1824).. In the late 70's, widespread pollution of soils, rivers, wildlife and aquatic organisms was observed. The use of chlordecone has been prohibited in 1993 because of its toxicity and persistence in the environment. A number of restrictions were established to limit the exposure of the human population. The Maximum Residue Limit (MRL) of 20 micrograms of chlordecone per kilogram of fresh weight in food of animal and plant origin was adopted. The adoption of this norm has resulted in the closing of many aquaculture units due to the presence of chlordecone in excess of the MRL in fish products. This contamination of aquaculture products, has a serious impact on farming of the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), which represents an economically important resource in Guadeloupe.

The aim of this work was to improve the understanding of the effects of chlordecone, and more widely of endocrine disrupting compounds in the decapod crustacean *Macrobrachium rosenbergii*, through laboratory and *in situ* exposures. Mechanisms of accumulation and elimination of chlordecone as well as its distribution in different anatomical compartments of *M. rosenbergii* were assessed through measures of the concentration in tissues of exposed organisms. The effects of chlordecone on 20-hydroxyecdysone hormone (20-HE), the molting hormone, and on chitobiase, one of the enzymes involved in the degradation of the cuticle were then. The effects of chlordecone on vitellogenin, a molecule involved in the reproductive process, were also evaluated through the analysis of its impact on gene expression of vitellogenin as well as that of its receptor. Finally, the effect of chlordecone on the proteome of *M. rosenbergii* was studied through the analysis of modifications of protein expression in organisms exposed to chlordecone.

Results showed that chlordecone in the aquatic environment was rapidly accumulated in organisms. Chlordecone is then distributed in various organs and anatomical compartments of exposed individuals. In addition, a decrease in chlordecone concentrations in *M. rosenbergii* tissues was highlighted in depuration experiment. Results also suggest that chlordecone could interfere with the growth and development of crustaceans, through disruption of two key molecules (20-HE and chitobiase) involved in the molting process. Chlordecone could have an anti-ecdysteroidal activity. Moreover, chlordecone could also interact with the reproduction of *M. rosenbergii*, through disruption of the expression of the vitellogenin gene and that of its receptor, which are key molecules involved in this process. Finally, proteomic analysis allowed to highlight the impact of chlordecone on the expression of protein involved in important physiological processes, such as detoxification, but also reproduction and growth.

This work allowed to show the important accumulation of chlordecone in tissues of M. rosenbergii, but also the depuration capacity of organisms contaminated with chlordecone. Further studies could focus on the crustacean depuration capacity in order to determine the mechanisms by which organisms are able to eliminate chlordecone. In addition, laboratory and in situ results were similar which shows that the laboratory experiment carried out in this study reflected the natural conditions found in situ. This work also allowed to highlight the potential interference of chlordecone with the endocrine system of crustaceans through its effects on key molecules involved in the molting and reproduction process. However, the approaches used in our work did not allow us to describe the mechanism of action of chlordecone. Further investigations are needed to highlight the metabolic pathways disrupted by exposure to chlordecone of M. rosenbergii, focusing on key molecules such as peptide hormones (MIH, GIH ...) or key receptors such as EcRs.

Chapter 3 – Analytical Approach

Publication 1

Bioaccumulation, distribution and depuration of chlordecone in the giant freshwater prawn *Macrobrachium rosenbergii*: field and laboratory studies.

Prepared for submission in Chemosphere

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Abstract

Chlordecone is a persistent organochlorine pesticide that has been widely used in Guadeloupe (French West Indies) to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993. A few years after its introduction, widespread contamination of soils, rivers, wild animals and aquatic organisms was reported. Although high chlordecone concentrations have been reported in several crustacean species, the uptake, internal distribution, and elimination have been poorly described. This study therefore aimed at investigating the accumulation and tissue distribution of chlordecone in the giant freshwater prawn *Macrobrachium rosenbergii*, using both laboratory and field approaches. In addition, depuration in chlordecone-free water was highlighted. In the laboratory study, the bioaccumulation of chlordecone was investigated in prawns exposed for 30 days at four environmentally relevant concentrations. In the field study, chlordecone concentrations were analysed in prawns collected after different exposure periods up to 8 months in an aquaculture pond supplied by a chlordecone-contaminated river. Results showed that chlordecone concentration in exposed prawns was dose-dependent and time-dependent. Moreover, females appeared to be less contaminated than males after 5 and 7 months of exposure. This could be due to successive spawning resulting in the elimination of chlordecone through the eggs. In addition, chlordecone distribution in tissues of exposed prawns showed that chlordecone was mostly found in cephalothorax organs, mainly represented by the hepatopancreas. Moreover, results showed that chlordecone was also accumulated in cuticle and this accumulation can represent up to 40 % of chlordecone body burden. Accumulation in the cuticle could be considered as a depuration mechanism since chlordecone is eliminated with the exuviae during successive molts.

Keywords: Chlordecone, bioaccumulation, crustaceans

1. Introduction

The tropical climate of the French West Indies (FWI) promotes the rapid development of pests which exert 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). The most worrying organochlorine pesticide residue in Guadeloupe (FWI) is chlordecone ($C_{10}Cl_{10}O$; CAS number 143-50-0) (Coat et al., 2011). Chlordecone (CLD) is an insecticide which production started in 1966 under the trade name Kepone® (Allied Chemical Corporation, Hopewell, VA, USA) mainly for the export to tropical countries, where it was used against a wide range of pests (Dolfing et al., 2012; Sterrett and Boss, 1977). In 1975, due to poor working conditions and failure to respect the safety measures, the Hopewell plant workers underwent acute toxicity and developed toxicity symptoms, primarily in the nervous system, liver and testes (Cannon et al., 1978). CLD was also associated with estrogenic effects that lead to reproductive dysfunction (Guzelian, 1982; Taylor, 1982; Tilson et al., 1982). Moreover, nearby the Hopewell plant, CLD was detected in the air and soil, as well as in the River James causing extensive contamination of surface water, sediment and aquatic organisms (Huggett and Bender, 1980). This widespread contamination therefore led to the prohibition of CLD in 1976 in the US (ATSDR et al., 1995). In Guadeloupe, CLD was commonly employed to control the banana weevil *Cosmopolites sordidus* from 1972 to 1978 (Kepone®) and from 1982 to 1993 (Curlone®) (Cabidoche et al., 2009, 2006).

A few years after the introduction and use of CLD in Guadeloupe, widespread pollution of soils, rivers, wild animals and aquatic organisms was reported (Cavelier, 1980; Snegaroff, 1977). The use of CLD was finally prohibited in 1993 (Cabidoche et al., 2009). Moreover, CLD was included in the Stockholm Convention on POPs (Persistent Organic Pollutants) in 2009, and its production and use were banned worldwide (Zaldívar and Baraibar, 2011). CLD can exert toxic effects in the organism, like most pesticides, and could disturb critical physiological processes and also those controlled by the hormonal system (Giusti et al., 2013a; Guzelian, 1982).

Indeed, several studies have demonstrated that CLD could impact the crustacean development, growth and reproduction through disturbance of the hormonal process (Bookhout et al., 1980; Lafontaine et al., 2016a, 2016b; Nimmo et al., 1977; Oberdörster and Cheek, 2001; Sanders et al., 1981; Schimmel et al., 1979; Zha et al., 2007).

Nowadays, CLD is still present in soils, especially in andosols which are rich in organic matter, in the densely cultivated areas from the south of the Basse-Terre Island (Guadeloupe). It was estimated that CLD could persist for several centuries because of its resistance to degradation in the environment (Cabidoche et al., 2009). Although Fernández-Bayo et al. (2013) showed the existence of CLD-degrading microorganisms in andosols under aerobic conditions, CLD undergoes no significant or quick biotic or abiotic degradation (Dolfing et al., 2012; Levillain et al., 2012). In nitisols and ferralsols, CLD is more easily released, and driven by the water cycle, it is progressively transferred to aquatic ecosystems (Coat et al., 2011). Because of its high K_{oc} (Soil Organic Carbon Water Partitioning Coefficient) (K_{oc} = 15849 L Kg^{-1}), K_{ow} (Octanol-Water Partition Coefficient) (K_{ow} = 4.5 – 6.0), and its affinity for lipids, CLD is persistent in the environment and accumulates in food webs (Cabidoche and Lesueur-Jannoyer, 2012; Clostre et al., 2013; Sterrett and Boss, 1977; UNEP, 2005; US-EPA, 2008). According to the Guadeloupean DIREN (Regional Department for the Environment), rivers in Guadeloupe are contaminated by CLD at concentrations that range from 0.2 to 4 µg L^{-1} with a maximum of 8.6 µg L^{-1} measured in the River Grande Anse in 2003 (GREPP, 2004; InVS-Inserm, 2009).

CLD contamination is widespread and affects all food web levels (Bahner et al., 1977). Freshwater and coastal fishes, molluscs and freshwater crustaceans are the most contaminated organisms, due to their very high CLD storage capacity, especially in crustaceans (Bertrand et al., 2010; Cabidoche et al., 2006; Coat et al., 2011; Dromard et al., 2016; Kermarrec, 1980). Human contamination by CLD has also been detected in the FWI population mainly as a result of consumption of contaminated food, seafood and root vegetables (Dubuisson et al., 2007; Guldner et al., 2010). CLD has been detected in blood samples of about 70 % of the population in Guadeloupe (Guldner et al., 2010; Multigner et al., 2007, 2006), causing an abnormally high rate of prostate cancer (Multigner et al., 2010) and of children with impaired development (Boucher et al., 2013; Dallaire et al., 2012).

In such a context, the European legal Maximal Residue Limit (MRL) of 20 µg of CLD per kg wet weight for food of plant and animal origin has been adopted in 2008 (Ministère de l'Écologie du Développement durable et de l'Énergie, 2015a). As a consequence, fishing and the consumption and distribution of fish have been severely restricted. Furthermore, CLD contamination was detected in farms of the tropical giant freshwater prawn *Macrobrachium rosenbergii*, resulting in the closing of many aquaculture units in 2008. This had serious consequences because prawn farming was an important economic resource in Guadeloupe. Although, studies showed toxic effects of pesticides and biocides in *M. rosenbergii* (Revathi and Munuswamy, 2010; Satapornvanit et al., 2009), very few investigations were carried out with CLD. It is known that the uptake of CLD occurs through the gills of prawns in contaminated water (Coat et al., 2011). However, much less is known on CLD uptake kinetics, and its variation with water concentration and exposure time. In addition, once internalized, the distribution of CLD within prawn tissues is unknown. Finally, it is not known whether *M. rosenbergii* is able to depurate CLD.

The present study therefore aimed at investigating the bioaccumulation and tissue distribution of CLD in the giant freshwater prawn *M. rosenbergii*, (*i*) in laboratory conditions (short-term exposure) and (*ii*) in the field (*in situ*) conditions (long-term exposure), in order to understand CLD behaviour in a crustacean decapod which can be considered as a good model for the wild *Macrobrachium spp*. living in freshwater ecosystems of these regions. In addition, remaining prawns from the long-term exposure were then placed in CLD-free water for 45 days to investigate possible depuration processes. As it is well known that animal gender can interfered with chemical uptake, distribution and CLD concentrations were measured separately in males and females when sexual differentiation was possible. Besides filling a knowledge gap on the fate of CLD in tropical prawns, which may help to understand its toxic effects in these organisms, the results of this study may have practical implications in e.g., the modelling of wild freshwater *Macrobrachium spp* CLD contamination for river biomonitoring, or in the regulatory control of the quality of marketed food products.

2. Materials and methods

2.1 Tested organisms

Three-month-old *Macrobrachium rosenbergii* (De Man, 1879) were obtained from post-larvae (approx. 2 g, 1.4 cm cephalothorax length), provided by an aquaculture farm (OCEAN-SA) located in a geographic area free of CLD contamination. This was confirmed by pre-tests previously carried out to evaluate the presence of CLD in tissues of prawns from the hatchery. The results showed no contamination of the prawns (concentrations below detection limit; data not shown).

2.2 Short-term chlordecone exposure: laboratory experimental design

A total of 1540 post-larvae of *M. rosenbergii* were transferred to the laboratory (UMR BOREA, University of the French West Indies, Guadeloupe). Prawns were acclimated for one week in 55 glass aquaria filled with 28 L of tap water filtered through activated charcoal. The loading was 28 prawns per aquarium. Aquaria were randomly distributed in the laboratory and were under constant aeration with a 12 hours light / dark photoperiod. During acclimation, the prawns were fed daily with one artificial shrimp food pellet per individual (complete food for rearing, Le Gouessant, France).

The 10 g L^{-1} stock solution of CLD (100 %, Riedel-de-Haën, Sigma-Aldrich, USA), as well as three successive dilutions (1000, 100 and 10 mg L^{-1}) were prepared in acetone (Biosolve-Chimie, France). A volume of 56 µL of each dilution and of the stock solutions was added into 28 L of aquarium water in order to obtain four nominal concentrations of CLD in water: 0.02 µg L^{-1}, 0.2 µg L^{-1}, 2 µg L^{-1} and 20 µg L^{-1}, respectively. These concentrations were chosen for their environmental relevance and because they are typically found in surface waters in Guadeloupe. In parallel to these four CLD concentrations, two controls were used. The control water consisted of tap water filtered through activated charcoal. The solvent control was obtained by spiking 28 L of filtered tap water with 56 µL of acetone. Ten aquaria were used for each condition of exposure, except for the solvent controls where only five aquaria were fed.

During the 30 days of exposure, *M. rosenbergii* were fed daily with one pellet of artificial shrimp food per individual. The food pellets were entirely consumed in less than fifteen minutes by the prawns, thus limiting degradation of water quality by decaying food pellets as well as contamination of food by CLD. Based on the results of a pretest designed to evaluate the decrease with time of CLD concentrations in water, it was decided to renew the exposure medium every 96 hours, in order to maintain constan concentrations of CLD during the 30 days of exposure. A constant water temperature of 27.6 ± 0.2°C was maintained, and pH remained at 7.57 ± 0.03 throughout the experiment. These measured values were in accordance with optimal water temperature and pH commonly used in prawn farms (New, 2002). The mortality rate was about 4 % throughout the experiment, whatever the conditions.

In order to measure the CLD contamination levels, five *M. rosenbergii* (corresponding to 5 replicates) were randomly collected in each condition, after eight durations of exposure: 6 h, 12 h, 1 day, 2 d, 4 d, 8 d, 15 d and 30 d. After sampling, the prawns were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Other prawns were used for immuno-enzymatic tests or proteomic and transcriptomic analyses.

2.3 Long-term chlordecone exposure: *in situ* experimental design

Like for the laboratory experiment, post-larvae of *M. rosenbergii* were provided by the aquaculture farm (OCEAN-SA). A total of 7000 post-larvae were transferred into two farming ponds. The first one, called "control site", was located in Pointe-Noire (north of the Basse-Terre Island, Guadeloupe), and was supplied by the River Petite Plaine, where CLD concentration was below the detection limit (i.e. 0.01 µg L^{-1}) (Bonan and Prime, 2001). The second one, called "contaminated site", was located in Saint-Claude (south of the Basse-Terre Island, Guadeloupe), and was supplied by the River Rivière-aux-Herbes, a CLD-contaminated river (average water concentration of 0.33 µg L^{-1} measured between 2003 and 2008 by the DIREN Guadeloupe). The farm based in Pointe-Noire is still in operation, whereas the farm in Saint-Claude had to cease prawn farming because the CLD concentration in prawns was higher than the French and European legal maximal residual limit (MRL) of 20 ng g^{-1} wet weight (Anon., 2008; Ministère de l'Écologie du Développement durable et de l'Énergie, 2015a).

During the 8 months of the experiment, prawns were fed daily with artificial shrimp pellet (complete food for rearing, Le Gouessant, France). A constant water temperature was measured in Pointe Noire (27.6 ± 1.5°C) and Saint-Claude ponds (27.9 ± 1.6°C) whereas, the pH remained at 8.47 ± 0.43 in the Pointe-Noire pond and 9.00 ± 0.44 in the Saint-Claude pond throughout the experiment. The pH values were slightly higher in Saint-Claude than in Pointe Noire due to the higher photosynthesis activity of phytoplankton in the contaminated pond (data not shown).

Six *M. rosenbergii* (corresponding to 6 replicates) were sampled in each pond, after seven durations of exposure: 8 days, 15 d, 30 d, 3 months, 5 m, 7 m and 8 m. Sex can only be differentiated for prawns sampled after 5, 7 and 8 months of exposure and was determined during sampling. After sampling, the prawns were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3.1. Depuration

After 8 months of exposure in the Saint-Claude pond, some remaining prawns were placed in aquaria filled with 28 L of tap water filtered through activated charcoal, in order to evaluate CLD depuration. Natural photoperiod was kept (12 hours light / dark) and the prawns were fed once daily with one artificial shrimp pellets. Eight *M. rosenbergii* (corresponding to 8 replicates) were collected after 1, 2, 4, 8, 14, 21, 35 and 45 days of depuration. After sampling, prawns were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

2.4 Chlordecone concentrations in *M. rosenbergii*

2.4.1. Chlordecone extraction from *M. rosenbergii*

The prawns were dissected into four parts: internal organs within the cephalothorax, mainly represented by the hepatopancreas (and thereafter referred to as “the hepatopancreas”), cephalothorax cuticle, abdominal muscle and abdominal cuticle. However, prawns sampled in the ponds after 8, 15 and 30 days of exposure were too small to be dissected into four parts, and concentrations were thus measured in the whole body.

CLD concentration in each tissue was analyzed according to a method adapted from Debier et al. (2003) and Multigner et al. (2010). Each of the four anatomic parts was thawed separately, and a minimum of 200 mg of tissue was freeze-dried during 20 hours 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 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 µL of a hexanic solution of PCB congener 112 (Dr. Ehrenstorfer, Germany) was added to the samples as an internal surrogate standard to obtain a final concentration of 50 pg μL^{-1}. Then, 500 mg of anhydrous sodium sulfate were added to avoid any water trace in the extract. The solvent, containing the extracted fat and CLD, 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.

2.4.2. Sample purification

After evaporation of the extracted fat fraction, residues were resuspended in 2 mL n-hexane (Biosolve-Chimie, France) and transferred to a test tube for acid clean-up. A volume of 2 mL 98 % sulfuric acid (Merck, Germany) was added to the extracts, in order to remove organic matter (e.g. lipids, lipoproteins, carbohydrates). The mixture was homogenized by vortex-mixing for 1 min using a Vibramax 110 (Heidolph, Germany), and centrifuged for 3 min at 2160 g and 10°C. The organic phase was collected in a new tube and the sulfuric acid layer was extracted a second time in the same way with another 3 mL n-hexane, to ensure an optimal recovery. The two resulting organic layers were pooled and 5 µL 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 µL n-hexane and 50 µL of a solution of PCB 209 (100 pg μL^{-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 was obtained by ASE extraction without biological matrix, allowing the control of the extraction and the clean-up procedure. The QC was performed to control CLD recovery by using a biological matrix (here, freeze-dried *Penaeus monodon*) spiked with an acetonic solution of CLD in order to obtain a final concentration of 2.5 ng g^{-1} wet weight for biological sample. Tissues of *P. monodon* were used in order to have a biological matrix of decapod free from CLD contamination.

2.4.3.Chromatography analysis

The purified extracts, procedural blank and QC were analyzed by high-resolution gas chromatography using a ThermoQuest Trace 2000 gas chromatograph equipped with a ^{63}Ni ECD detector (Thermo Scientific, USA) and an auto-sampler ThermoQuest AS 2000 (Thermo Scientific, USA). The extract was injected into the on-column mode at 60°C. CLD was separated on a 30 m x 0.25 mm (0.25 µm film) DB-XLB capillary column (J&W Scientific, USA). The carrier gas was hydrogen and the make-up gas was Ar/CH_4 (95/5 v/v). The injector was at room temperature and the detector was kept at 300°C. The temperature program was as follows: 2 min at 60°C, 4 min of gradual heating until 180°C at the rate of 30°C/min, 3 min at 180°C, 36 min of gradual heating from 180°C to 270°C at the rate of 2.5°C/min, and 12 min at 270°C. The other analytical parameters are 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 with a linear calibration curve (1.5 to 200 pg μL^{-1}) established with CLD certified solutions (Riedel-de Haën, Germany). CLD concentration in each sample and in the QC was corrected by the percentage recovery of the internal surrogate PCB 112. The recovery efficiency based on CLD recovery in QC and on the recovery of the surrogate internal standard (PCB112) ranged from 88 ± 4 % to 115 ± 5 % respectively, which was within the limits recommended by SANCO, i.e. range from 60 to 140 % (SANCO/12571/2013 European commission, 2013).

The limit of detection (LOD) was fixed at three times the background noise of the chromatogram (i.e. 0.02 ng g^{-1} wet weight). The limit of quantification (LOQ) was determined with freeze-dried tissues of *P. monodon* spiked with various CLD concentrations and was established at 0.06 ng g^{-1} wet weight. CLD concentrations in *M. rosenbergii* were measured in several replicates (see Sections 2.2 and 2.3) per condition and the average was calculated for each condition. CLD concentration was expressed as ng g^{-1} wet weight.

The bioaccumulation factor (BAF) was also calculated at the end of the two experiments for each condition using the method of Arnot and Gobas (2006). According to these authors, BAF is the ratio between CLD concentration in tissues of prawns and CLD concentration in water of exposure, including chemical exposure in the diet. As a consequence, the BAF is typically measured under field conditions and we have also chosen to characterise the bioaccumulation process in the laboratory experiment, in order to take account of the contamination that could occur through food pellet and through cannibalism.

2.5 Statistical analysis

All data met normality and homogeneity of variance assumptions (Shapiro and Bartlett tests, $p > 0.05$). To investigate the effects of the exposure duration, exposure concentration, prawn gender and exposure site on CLD concentrations in prawn tissues, data were analysed using a two- or three-way ANOVA. Then, Tukey HSD post-hoc tests were performed to describe significant differences. A probability value of less than 0.05 was regarded as significant. Correlations between CLD concentrations in *M. rosenbergii* and CLD concentrations in water were analysed using the Pearson correlation coefficient. All tests were performed with STATISTICA 10 Software (StatSoft, 2012, USA).

3. Results

3.1 Laboratory chlordecone exposure

Total CLD concentrations in whole *M. rosenbergii* body were obtained by summing the amounts of CLD in each compartment (i.e. the CLD concentration of each compartment multiplied by its weight) dividing this total by the total body weight. Total CLD concentration was significantly influenced by both the exposure concentration and the exposure duration (two-way ANOVA test, $p < 0.001$). Moreover, CLD concentrations in exposed prawns were concentration-dependent, since prawns exposed to higher CLD concentrations, accumulated greater amounts of CLD (Figure 3.1). The relations between CLD concentrations and exposure durations are presented in Figure 3.1 and show significant logarithmic correlations (Table 3.1). CLD was detected in prawns from water and solvent controls, at concentrations below 10 ng g_{-1} except in control prawns exposed for 30 days. Moreover, logarithmic correlations between CLD concentrations and exposure durations were not significant for prawns from control and solvent conditions.

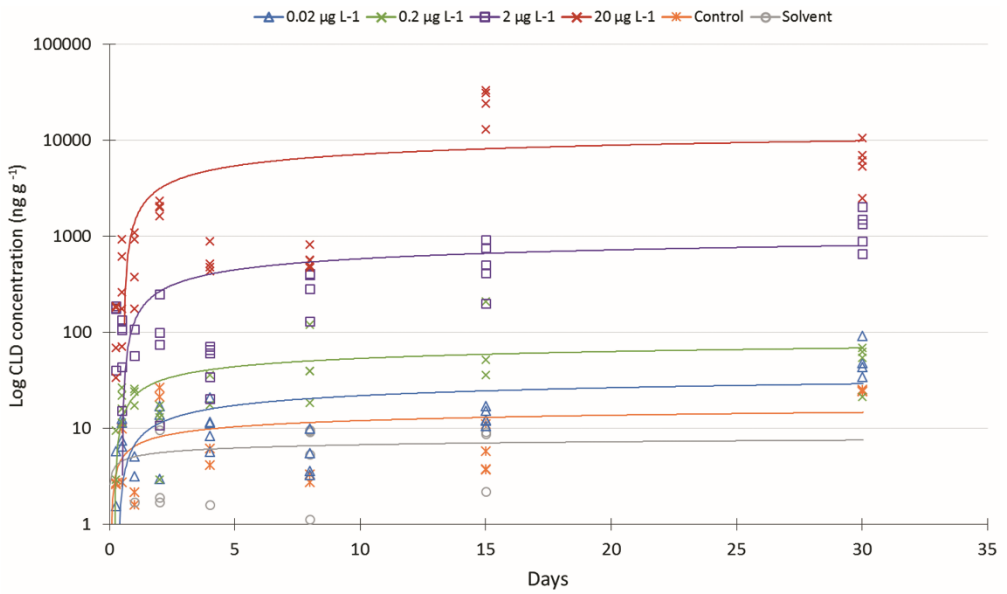

Figure 3.1 - Chlordecone concentrations (ng g^{-1} wet weight, mean + S.D.) measured in whole *Macrobrachium rosenbergii* exposed to four chlordecone concentrations (i.e. 0.02, 0.2, 2 and 20 µg L^{-1}) in laboratory experiment and sampled after eight exposure durations.

Table 3.1 – Parameters of the logarithmic regressions which express relation between CLD concentrations and exposure durations.

	r	n	*p*	Equation
Control	0.17	20	> 0.05	
Solvent	0.44	16	> 0.05	
0.02 µg L^{-1}	0.55	29	< 0.01	y = 6.58 ln (x) + 6.72
0.2 µg L^{-1}	0.51	27	< 0.01	y = 13.86 ln (x) + 21.54
2 µg L^{-1}	0.67	35	< 0.01	y = 197.8 ln (x) + 128.8
20 µg L^{-1}	0.48	37	< 0.01	y = 2501.3 ln (x) + 1397.1

Generally, whatever the exposure concentration, CLD concentration was measurable as early as 6 hours of exposure. The relation between exposure time and accumulation was assessed through correlation analysis. It appeared that CLD accumulation in *M. rosenbergii* was time-dependent in prawns exposed to 0.02 and 2 µg L^{-1}. Indeed, the longer the prawns were exposed, the higher the CLD accumulated was. This observation is supported by a significant positive correlation (r = 0.84, $p < 0.001$, n = 40) between CLD concentration in *M. rosenbergii* and the concentration of exposure.

The bioaccumulation factor (BAF) was calculated at the end of the experiment for the four CLD concentrations (Table 3.2A) and results showed that BAF was highestin *M. rosenbergii* exposed to the lowest CLD concentration (i.e. 0.02 µg L^{-1}) and conversely.

Table 3.2 - Bioaccumulation factor (L Kg^{-1}) calculated at the end of experiment for the four concentrations of exposure (i.e. 0.02, 0.2, 2 and 20 µg L^{-1}) during the laboratory experiment (A) and for the contaminated pond during the *in situ* experiment (B). CLD concentrations were not measured in the water of control tanks, nor in the water of Pointe-Noire pond.

	***In lab* (A)**				***In situ* (B)**
	0.02 µg L^{-1}	0.2 µg L^{-1}	2 µg L^{-1}	20 µg L^{-1}	Saint-Claude
30 days	2708	368	642	317	
8 months					3701

3.3.1.Chlordecone in anatomical compartments

CLD quantity in the different anatomical compartments of *M. rosenbergii* was obtained by multiplying the concentrations in each compartment by the weight of each respective compartment. CLD quantity in the different anatomical compartments of *M. rosenbergii* exposed to 0.2 µg L^{-1} are presented in Figure 3.2A. The distribution profiles were similar for the other exposure concentrations (i.e. 0.02, 2 and 20 µg L^{-1}) and are therefore not shown. Results showed that after 6 hours of exposure, CLD was mostly (i.e. more than 90 % of the total body burden) found in cephalothorax organs, mainly represented by the hepatopancreas. In prawns sampled at the other durations of exposure, CLD was also mainly found in the hepatopancreas (from 40 % to 70 % of the total quantity). Muscle and abdominal cuticle are only contaminated after 12 hours of exposure. The proportion of CLD in muscle then varies between 10 and 30 %, between 8 and 15 % in the abdominal cuticle and between 5 to 32 % in the cephalothorax cuticle. The proportion of CLD in both types of cuticle ranges from 12 to 42 %.

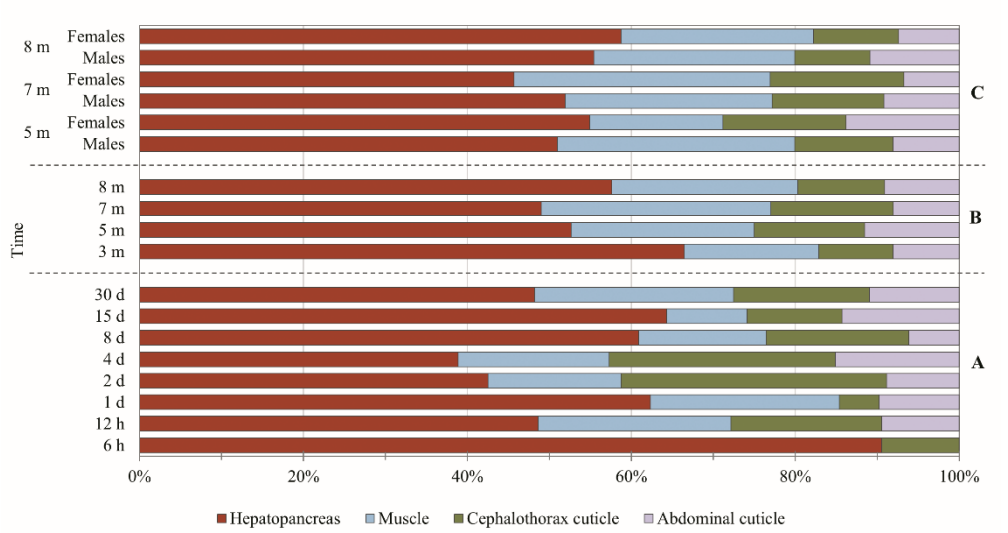

Figure 3.2 - Distribution of chlordecone in the four anatomical compartments studied (internal organs within the cephalothorax, mainly the hepatopancreas, cephalothorax cuticle, abdominal muscle and abdominal cuticle) related to CLD body burden of *M. rosenbergii*. The CLD quantity measured in each anatomical compartment were represented by a percentage of the total quantity (i.e. CLD body burden) measured in all the anatomical compartments of *M. rosenbergii.* (A) Prawns exposed to 0.2 µg L^{-1} of chlordecone during the laboratory experiment and sampled after eight exposure durations. The distribution profiles were similar for prawns exposed to 0.02, 2 and 20 µg L^{-1} of CLD.

(B) Prawns exposed to chlordecone during the field experiment in the contaminated site (Saint-Claude pond) and sampled after four exposure durations. (C) Male and female prawns exposed to chlordecone during the field experiment in the contaminated site (Saint-Claude pond) and sampled after 5, 7 and 8 months of exposure.

The CLD concentrations, expressed relative to the fresh weight of prawns, were measured in the anatomical compartments of *M. rosenbergii* exposed for 6 h, 12 h, 1 d, 2 d, 4 d, 8 d, 15 d and 30 d and were presented in Table 3.3A. CLD concentrations measured in each anatomical compartments of *M. rosenbergii* showed a preferential accumulation of CLD in hepatopancreas as accumulation levels are highest in this compartment.

Table 3.3 - CLD concentrations (ng g^{-1} wet weight) measured in the four anatomical compartments (internal organs within the cephalothorax, mainly the hepatopancreas, cephalothorax cuticle, abdominal muscle abdominal cuticle, and the sum of concentrations from both types of cuticle) of *M. rosenbergii* exposed to 0.2 µg L^{-1} of chlordecone during the laboratory experiment (A) and during the field experiment in the contaminated site (Saint-Claude pond) (B)

		Hepatopancreas	**Muscle**	**Cephalothorax cuticle**	**Abdominal Cuticle**	**Cuticle (Cephalo. Cuticle + Abd. Cuticle)**
A	**6 h**	**12.7 ± 10.6**	1.2 ± 0.0	2.4 ± 3.3	< LOD	1.3 ± 2.8
	12 h	**43.7 ± 30.8**	14.6 ± 8.7	13.3 ± 8.4	17.7 ± 8.4	15.1 ± 7.2
	1 d	**36.8 ± 19.3**	12.0 ± 10.6	5.2 ± 5.5	11.5 ± 9.3	7.1 ± 6.3
	2 d	**21.0 ± 9.7**	5.1 ± 3.6	13.2 ± 10.0	8.0 ± 3.3	11.5 ± 7.5
	4 d	**73.4 ± 61.2**	39.2 ± 30.0	43.4 ± 35.4	42.5 ± 38.8	39.2 ± 29.3
	8 d	**125.4 ± 116.5**	21.7 ± 11.1	38.7 ± 19.4	25.5 ± 17.7	36.2 ± 17.8
	15 d	**367.2 ± 306.2**	41.3 ± 18.0	53.9 ± 37.7	128.8 ± 124.5	80.8 ± 43.0
	30 d	**126.9 ± 114.3**	53.8 ± 37.9	32.6 ± 11.6	45.5 ± 24.4	40.22 ± 9.9
B	**3 m**	1070.7± 182.2	246.2 ± 84.1	**1286.7 ± 277.4**	622.2 ± 173.5	976.2 ± 384.4
	5 m	611.1 ± 126.6	290.1 ± 167.9	605.6 ± 191.9	478.0 ± 96.5	**638.0 ± 163.3**
	7 m	612.2 ± 93.2	359.5 ± 73.9	**715.5 ± 126.7**	408.2 ± 139.7	561.8 ± 152.5
	8 m	**1007.2 ± 156.8**	490.7 ± 134.4	896.3 ± 438.3	504.3 ± 57.8	773.0 ± 346.2

3.2 *In situ* chlordecone exposure

Total CLD concentrations in *M. rosenbergii* were significantly influenced by the exposure site (i.e. Pointe-Noire vs. Saint-Claude) and the exposure duration (two-way ANOVA test, < 0.001). An important accumulation of CLD was observed in the prawns sampled in the Saint-Claude pond throughout the experiment (Figure 3.3). Results showed that CLD concentrations were significantly higher in prawns exposed for more than 3 months, than in prawns exposed for 1 month or less. In prawns sampled in the Pointe-Noire pond, low contamination of CLD was found but concentrations were below the MRL of 20 ng g^{-1}.

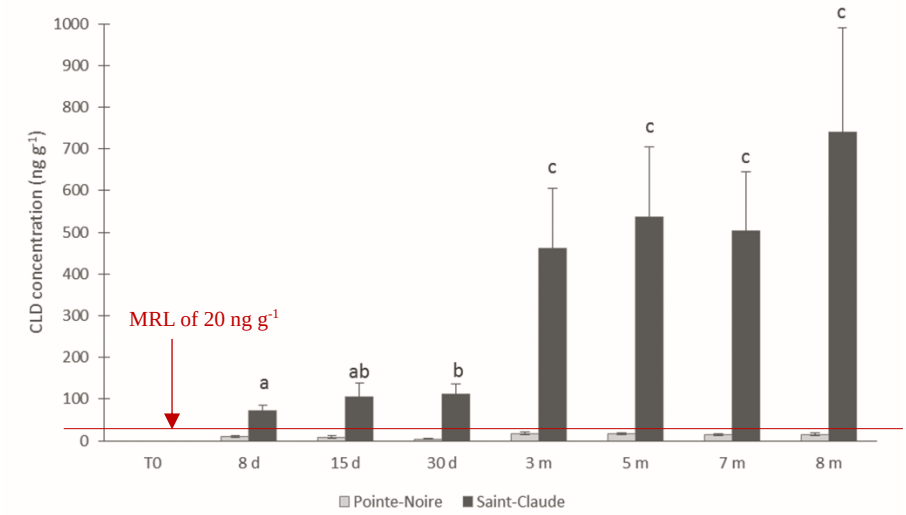

Figure 3.3 - Chlordecone concentrations (ng g^{-1} wet weight, mean + S.D.) measured in whole *Macrobrachium rosenbergii* sampled in the control site in Pointe-Noire and in the contaminated site in Saint-Claude after seven exposure durations. Different letters above the bars indicate significantly different values for the concentrations measured in Saint-Claude (Tukey's HSD test, *p*-values < 0.05).

Results obtained in the field experiment were compared to results obtained in prawns exposed to 0.2 µg L^{-1} of CLD in the laboratory experiment, since the concentration of exposure in the Saint-Claude pond was about 0.3 µg L^{-1}. CLD concentrations obtained in both field and laboratory experiment, and measured in prawns exposed for the same duration of exposure (i.e. 8, 15 and 30 days), were not significantly different ($p > 0.05$) (Table 3.4).

Table 3.4 - Chlordecone concentrations (ng g^{-1} wet weight) measured in whole *M. rosenbergii* exposed in the field and in the laboratory (0.2 µg L^{-1}) and sampled after 8, 15 and 30 days of exposure.

	In lab **0.2 µg L^{-1}**	***In situ*** **Saint-Claude**
8 days	55.3 ± 10.2	72.5 ± 12.9
15 days	98.6 ± 32.5	104.9 ± 33.0
30 days	78.6 ± 34.8	112.7 ± 23.0

3.2.1.Anatomical compartment

CLD quantity in the different anatomical compartments of *M. rosenbergii* exposed for 3, 5, 7 and 8 months are presented in Figure 3.2B p 94. CLD distribution in the prawns exposed in the Saint-Claude pond was similar as compared to the prawns exposed in the laboratory. The accumulation of CLD in the hepatopancreas represented 50 to 65 % of the total body burden, while the accumulation in both types of cuticle represented 15 to 25 % of the total body burden.**B**

CLD concentrations, measured in the anatomical compartments of *M. rosenbergii* exposed for 3, 5, 7 and 8 months, were presented in Table 3.3B p 95. Results shows that after the short-term exposure in laboratory, the CLD concentrations were higher in hepatopancreas of exposed prawns. However, after the long-term exposure *in situ*, cephalothorax cuticle and hepatopancreas accumulated CLD at similar levels, levels that were higher than those of abdominal compartments.

3.2.2.Level of contamination according to the gender

Total CLD concentrations, expressed relative to the fresh weight of prawns, were measured in males and females *M. rosenbergii* exposed in the Saint-Claude pond (Figure 3.4). Females that carried eggs (ovigerous females) were sampled, so that CLD concentration could also be measured separately in egg masses. CLD concentration in ovigerous females and nonovigerous females are presented separately.

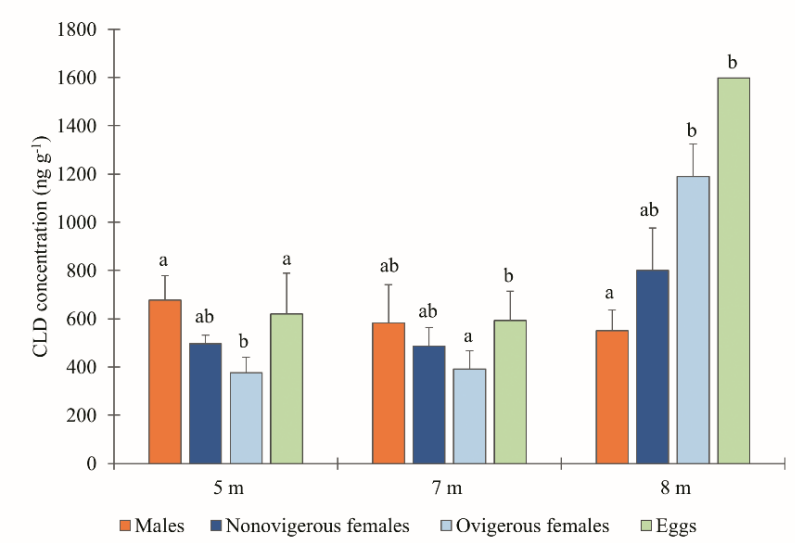

Figure 3.4 - Chlordecone concentrations (ng g^{-1} wet weight, mean + S.D.) measured in whole males, nonovigerous females, and ovigerous females sampled in the contaminated site in Saint-Claude after 5, 7 and 8 months of exposure. In addition, chlordecone concentration was measured in mass eggs of ovigerous females. Different letters above the bars indicate significantly different values for each sampling time (Tukey's HSD test, *p*-values < 0.05).

CLD concentrations were significantly influenced by the prawn gender (p = 0.006, two-way ANOVA test). Results showed that males were more contaminated than females except after 8 months of exposure. Moreover, CLD concentrations in males decreased with the exposure duration whereas it increased with time in females. CLD concentrations were higher in nonovigerous females than in ovigerous females sampled after 5 and 7 months of exposure. In addition, CLD concentrations measured in eggs were significantly higher than in the females that carried these eggs, except in females exposed for 8 months, for which the difference was not significant.

The distribution of CLD in the four anatomical compartments showed that most of CLD was accumulated in the hepatopancreas of exposed males and females (Figure 3.2C p 94). The accumulation of CLD in both types of cuticle represented 20 to 30 % of the total contamination.

3.3 Chlordecone depuration

After 8 months of CLD exposure in the Saint-Claude pond, prawns were placed in water free of CLD contamination in order to evaluate the CLD depuration. Figure 3.5 shows CLD accumulation curve measured in *M. rosenbergii* exposed in the *in situ* experiment and CLD depuration curve measured in the decontamination experiment. Results showed a decrease of CLD concentration in whole *M. rosenbergii* from the 2nd day of depuration. After 15 days in CLD-free water, the concentration in prawns was halved, and at the end of experiment (i.e. 45 days), there were only 15 % of the initial body concentration of CLD.

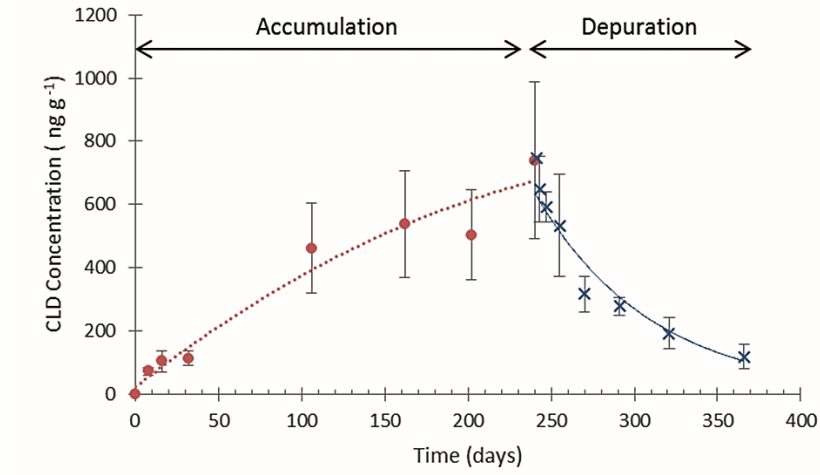

Figure 3.5 - Accumulation and depuration of chlordecone (ng g $^{-1}$ wet weight ± S.D.) in whole *Macrobrachium rosenbergii*, placed in water free from contamination after 8 months (240 days) of exposure in the Saint-Claude pond.

4. Discussion

4.1 Accumulation of chlordecone in whole *M. rosenbergii*

The laboratory experiment highlighted that CLD accumulation in *M. rosenbergii* was significantly influenced by both the exposure concentration and the exposure duration. The longer prawns were exposed, the higher the CLD concentrations accumulated were.

Results also showed that the accumulation of CLD in prawns was measurable as early as after 6 hours of exposure. The results obtained for control prawns showed a slight accumulation of CLD. This accumulation of CLD in theoretically unexposed prawns could be partly explained the presence of CLD at levels in tap water lower than the LOD (i.e. 0.01 µg L^{-1}) (Agence de Santé de Guadeloupe, personnal communication, 2015). Although tap water was filtered over active charcoal, CLD elimination was not 100 % effective, and trace amounts of CLD were still present (Observatoire des Résidus en Pesticides, 2010).

The field experiment allowed to confirm that CLD has a high potential of bioaccumulation in aquatic organisms based on its physicochemical properties, even when concentrations are very low (ATSDR et al., 1995). This result is in agreement with previous studies highlighting CLD concentration in different organisms. Indeed, Bahner et al. (1977) showed the accumulation of CLD in estuarine shrimp, *Palaemonetes pugio*, exposed to 0.026 and 0.41 µg CLD L^{-1}, as well as Coat et al. (2011) who observed CLD bioaccumulation in a tropical food web including in various species of mussels, shrimps and fishes. Moreover, Monti (2007) reported the CLD contamination in crustacean species from the River Rivière aux Herbes, which is the river that supplies the Saint-Claude pond. CLD concentrations of 2204 ng g^{-1} in *Macrobrachium carcinus*, 1134 ng g^{-1} in *Macrobrachium faustinum* and 770 ng g^{-1} in *Atya scabra* sampled in this river were measured. These concentrations were of the same order than those measured in our study in *M. rosenbergii* sampled in the Saint-Claude pond (i.e. 774 ng g^{-1}). In prawns collected in the Pointe-Noire pond very low concentrations were nevertheless detected. The presence of CLD in these individuals, that also constituted the controls for the Sainte-Claude pond experiment, could be explained by the bioaccumulation process of trace amounts of CLD (lower than the LOQ in water, i.e. 0.01 µg L^{-1}) detected in the River Petite Plaine, that supplies the Pointe-Noire farming pond (Bonan and Prime, 2001; Ministère de l'Écologie du Développement durable et de l'Énergie, 2015b). However, this contamination in prawns from the control pond was not alarming since the measured concentrations were always under the MRL of 20 ng g^{-1} (Ministère de l'Écologie du Développement durable et de l'Énergie, 2015a).

Prawn survival was measured throughout the laboratory experiment and was not affected by the four exposure concentrations, probably due to the fact that exposure concentrations were below lethal concentration. Indeed, Schimmel and Wilson (1977) reported that the 96 h LC_{50} of CLD was 121 µg L^{-1} for grass shrimp *P. pugio* and no significant mortality in blue crabs *Callinectes sapidus* at measured concentrations as high as 210 µg L^{-1} was observed.

BAF results from the laboratory study showed that the bioaccumulation of CLD was more efficient with lowest concentration in water. Indeed, prawns exposed to 0.02 µg L^{-1} had higher BAF than prawns exposed to other CLD concentrations. The higher BAF in prawns exposed to low CLD concentration suggests that in the field, lower CLD concentration in water could result in high CLD contamination in the aquatic species of the food chain. This observation was confirmed by the significant accumulation of CLD in the field experiment. The higher BAF in prawns from *in situ* study when compared to the BAF from the laboratory experiment could be due to the indirect uptake of CLD with potentially contaminated food, a process which was likely more important in the field experiment than in the laboratory experiment, where precautions have been taken to limit the extent of food contamination. The higher BAFs could also be due to the exposure duration, since the field study lasted 8 months while the laboratory study lasted only 1 month.

Results from the field experiment can be compared to results obtained in prawns exposed to 0.2 µg L^{-1} of CLD in the laboratory experiment, since the concentrations of exposure were similar. CLD concentrations in both field and laboratory approaches were similar for prawns exposed for the same duration of exposure (i.e. 8, 15 and 30 days). This observation showed that prawn contamination occurred in the same way in both experiments, and that the contamination pathway is mainly the bioconcentration process in our experiments. The experiment led in laboratory could thus reflect what it takes place in the natural environment, regarding accumulation of CLD in organisms in the short-term.

Contaminant concentrations in exposed organisms from laboratory experiments are frequently different than in the field, due to exposure conditions which can vary between both approaches (Burton et al., 2005; Hill et al., 2011; Mann et al., 2010). Results obtained from field studies are more representative and ecologically meaningful than laboratory tests (Connon et al., 2012; Crane and Babut, 2007), but are often difficult to analyse because of several confounding factors (e.g. season, temperature, dissolved oxygen, turbidity, predation, synergistic or antagonist effects of various toxic substances in the environment…) (Rotchell and Ostrander, 2003). Therefore, the relationship between laboratory and field exposures has always been the topic of ongoing debate.

4.1.1.Chlordecone in males and females *M. rosenbergii*

Male and female results highlighted that females appeared less contaminated than males in prawns exposed for 5 and 7 months. This result is consistent with those of Roberts (1981) and Schimmel et al. (1979) who observed higher level of CLD contamination in males than in females *C. sapidus*. This difference could be explained by the fact that most collected females carried on eggs. Indeed, after 5 and 7 months of CLD exposure, females which carried on eggs were less contaminated than nonovigerous females. Moreover, CLD concentrations in eggs were higher than in females which carried these eggs, whatever the duration of exposure. Our results are in agreement with those of Roberts and Leggett (1980) and Roberts (1981) who showed that egg productions was a major way of CLD depuration in the blue crab, *C. sapidus*, collected from the River James and Lower Chesapeake Bay (Virginia, USA), since CLD concentration in the egg mass was higher than in corresponding females. However, high CLD concentrations in eggs could also be partly due to the CLD adsorption on the egg mass. In males, CLD concentration appeared more stable than in females. Moreover, after 8 months of exposure, CLD concentrations were higher in ovigerous and nonovigerous females than in males. This observation could be due to the cycle of ovarian maturation of females, during which lipid reserves were stored along with CLD before spawning and CLD elimination in eggs, resulting in a fluctuation of CLD concentrations (see Section 4.2). However, reproductive stages have not been studied in this work and future investigations could focus on the impact of reproductive stages on CLD concentrations in exposed *M. rosenbergii*.

4.2 Chlordecone in the anatomical compartments of *M. rosenbergii*

Our results showed that CLD was mainly accumulated in the hepatopancreas as early as after 6 hours of exposure and then, was redistributed into other anatomical compartments over time. This initial storage of CLD in the hepatopancreas could be explained by its structure and binding properties. Indeed, 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). Accumulation of CLD is highest in the organs characterized by a high lipoprotein content such as liver or hepatopancreas (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). Moreover, the accumulation of CLD mainly in hepatopancreas in prawns exposed for 6 h showed that the main way of prawn contamination could be through gills (direct uptake), since prawns had not been fed yet.

Results also showed that CLD was accumulated in both types of cuticle. Indeed, CLD concentrations in cuticle were high in exposed prawns. Moreover, CLD quantities may represented up to 40 % of the total body burden of exposed prawns. The exoskeleton of crustaceans consists of a calcified cuticle containing chitin. During the moult cycle, specific metabolic processes occur that involve an exchange of calcium between body fluids of crustaceans and their cuticle (Greenaway, 1985; Jung and Zauke, 2008). CLD could be simultaneously transferred through the ectoderm via body fluids to the cuticle (Dittman and Buchwalter, 2010). This accumulation of CLD in cuticle could be considered as a depuration mechanism, since prawns could get rid of CLD during successive molts (Dittman and Buchwalter, 2010). The amount of 15 to 40 % of the total contamination of CLD measured in cuticle of exposed prawns could be explained by this depuration mechanism, but also partly by CLD adsorbed onto the exoskeleton of prawns (Dittman and Buchwalter, 2010; Jung and Zauke, 2008). In the laboratory study, CLD distribution in prawn tissues varied in prawns exposed to CLD for 4 and 8 days. Indeed, the accumulation of CLD in hepatopancreas was only 40 % of the total quantity in prawns exposed for 4 days, probably due the fact that the sampling occurred just prior the first water renewal.

On the contrary, for prawns exposed for 8 days, the sampling occurred few hours after the renewal of exposure water and, as in the beginning of exposure, CLD was preferentially accumulated in hepatopancreas, resulting in higher accumulation (about 60 % of the total contamination) in this compartment for prawns exposed for 8 days than for prawns exposed for 4 days.

4.3 Depuration of chlordecone

Our results highlighted a decrease of CLD concentrations in *M. rosenbergii* during the decontamination experiment. At the end of this experiment, there were only 15 % of the initial concentration. Several studies showed invertebrates and crustaceans slowly eliminate CLD (Bahner et al., 1977; Roberts, 1981; Schimmel et al., 1979). Indeed, Bahner et al. (1997) showed the presence of CLD at 1.78 µg g^{-1} (dry weight) in faeces from oysters, *Crassostrea virginica*, which were contaminated at 0.21 µg CLD g^{-1} (wet weight). Moreover, Schimmel et al. (1979) observed the loss of CLD in blue crabs, *C. sapidus*, fed with contaminated oysters in a CLD-free environment beyond 28 days. The depuration rate observed in our study can be considered slow compared to other invertebrate species. Bahner et al. (1977) showed a rapid depuration of CLD from *C. virginica* at a rate of 35 % loss in 24 h, while our results revealed a loss of 35 % in 10 days. This depuration rate was similar in *P. pugio* exposed at 0.023 and 0.4 µg L^{-1} of CLD for 28 days and collected from River Lafayette (Norfolk, Virginia), then held in clean seawater (Bahner et al., 1977).

Moreover, using the equation of the depuration curve, MRL value of 20 ng g^{-1} could be reached in tissues of *M. rosenbergii* after 85 days of depuration in CLD-free water. This elimination of CLD, decreasing CLD concentration in *M. rosenbergii*, could be due to the detoxification and/or biotransformation processes. Indeed, Gaume et al. (2014) showed that CLD induced expression of enzymes involved in biotransformation and detoxification processes, such as cytochrome P450 enzymes or GST (glutathione-S-transferase), in *M. rosenbergii*. These detoxifying enzymes allow oxidation or reduction of exogenous molecules in order to increase their water solubility and facilitate their excretion by the organism (Koenig et al., 2012; Snyder, 1998).

These processes occur in the hepatopancreas, which implies that hepatopancreas considered the detoxifying organ in invertebrates, could play an important role in the accumulation, storage and detoxification of CLD in *M. rosenbergii* (Sreeram and Menon, 2005; Zeng et al., 2010). However, biotransformation of CLD is slow, and cannot explain alone the important decrease of CLD concentrations in prawn tissues observed (Newhouse et al., 2009; Toppari et al., 1996). As explain above, this decrease of CLD concentration could also be due to the elimination of CLD during successive moults of prawns. Indeed, prawns of *M. rosenbergii* moulted every 9 to 22 days, and during intermolt stage, CLD could be accumulated in prawns and partly stored in their cuticle before being eliminated from organism with the old cuticle during ecdysis. Moreover, this decrease of CLD concentration could also be explained by another excretion process. Indeed, a cytosolic aldo-keto reductase enzyme (CLD reductase) is responsible for the conversion of CLD to CLD alcohol in some species of mammals and thus, would increase the excretion of CLD (Molowa et al., 1986). However, CLD reductase activity was detected only in the liver cytosol of rabbits, gerbils, and humans but was absent in rats, mice, hamsters, and guinea pigs and no study has been carried on invertebrates (Fariss et al., 1980; Houston et al., 1981). Further investigations could focus on this CLD reductase in order to determine if it can be detected in invertebrates and establish its possible role in detoxification of CLD.

5. Conclusion

In this work on bioaccumulation of chlordecone in the giant freshwater prawn, *Macrobrachium rosenbergii*, we used both laboratory and field approaches. Results highlighted a rapid accumulation of CLD in prawn tissues as well as its high accumulation in crustaceans. Moreover, our results allowed to improve the knowledge on CLD distribution in prawn tissues. Indeed, results showed that CLD was mainly accumulated in the hepatopancreas as early as 6 hours of exposure and then, was redistributed into other anatomical compartments over time. Results also showed that CLD was accumulated in the cuticle. This accumulation in the cuticle could be a depuration mechanism through which prawns could get rid of CLD during successive moults. A decrease of CLD concentration in prawn tissues was also highlighted in the depuration experiment.

CLD contamination of environment in Guadeloupe is expected to last several years, decades or centuries and its rapid accumulation from water and slow depuration rate indicate that CLD may be transferred through food chains. In addition to the toxic effects observed in many vertebrates and invertebrates, CLD has been found to disrupt several hormonally regulated biological processes such as reproduction or development. Further investigations are necessary in order to provide information on the effects of CLD in invertebrates but also on the detoxification mechanisms involved in the depuration process and the impacts of reproductive and moulting stages on bioaccumulation of xenobiotics.

Moreover, since prawn farming was an important economic resource in Guadeloupe and *M. rosenbergii* can be considered a good model for the wild *Macrobrachium spp.* living in these regions, results of this study filled a knowledge gap on the fate of CLD in tropical prawns, and may have practical implications in e.g., the modelling of CLD contamination of freshwater *Macrobrachium spp*. in the river contamination biomonitoring or in the regulatory control of the quality of marketed food products.

Acknowledgments

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Chapter 4 – Immuno-enzymatic Approach

Publication 2

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

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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* in order to underline a potential dose-response relationship. Results showed that CLD was bioaccumulated in exposed organisms according to a trend to a dose-response relationship. Moreover, it was observed that CLD decreased 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 20-HE concentration and activity of chitobiase, suggesting consequences in the long term on the shrimp development. This study confirmed that CLD could be an endocrine disruptor in decapod crustaceans, as it was already observed in vertebrates.

Keywords: Chlordecone; *Macrobrachium rosenbergii*; 20-Hydroxyecdysone; Chitobiase; Endocrine disruptors

1. Introduction

Endocrine disrupting compounds (EDCs) are exogenous substances or mixtures that 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 (Rodriguez 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 a major potential targets for EDCs (Kloas et al., 2009). Several studies on the biological effects of EDCs in aquatic vertebrates led to the development of biomarkers in order to measure 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 EDCs effects have been extensively described in aquatic vertebrates (Kortenkamp et al., 2011; LeBlanc, 2007), only few studies examined the effects of EDCs in invertebrates, while 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 disrupt the invertebrate physiology. For example, (Giusti et al., 2013b) showed that tributyltin affected the production of yolk ferritin (i.e. Vg equivalent) in *Lymnaea stagnalis*, as well as Xuereb et al. (2011) observed that nonylphenol (estrogenic compound) impacted the expression of the 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 may 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 much ecotoxicological damages (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 used 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 were reported (Cavelier, 1980; Snegaroff, 1977), which led to 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 the Basse-Terre Island (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 (MRL) 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 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 peroxydase), or involved in the biotransformation process (i.e. cytochrome P450 enzymes and glutathione-S-transferase).

As CLD is suspected of to be an endocrine disruptor in invertebrates (Newhouse et al., 2009) and as *M. rosenbergii* molting is hormonally controlled, the present study aims to investigate the potential impacts of CLD the molting process by investigating the effects of CLD on 20-Hydroxyecdysone (20-HE) hormone concentration and on chitobiase activity in tissues of *M. rosenbergii*. 20-HE hormone is an ecdysteroid hormone, secreted by the Y-organ (Mykles, 2010), 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 CLD. 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, some studies showed that pollutants increase chitobiase enzyme activity. 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*.

In the same way, Gismondi and Thomé (2014) underlined that some pollutants, suspected to be EDCs (i.e. polybromodiphenyl ethers), may disturb the chitobiase activity of the amphipod *Gammarus pulex*.

Finally, in parallel with these two parameters, CLD concentration in *M. rosenbergii* was evaluated to underline potential dose-response relationships between the CLD concentration in tissues, the 20-HE concentration and the chitobiase activity. These measures ensure to assess 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, 1.4 cm cephalothorax length, 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 of CLD in tissues of prawns from Pointe-Noire and results showed no contamination (concentrations below detection limit) (data not shown). Prawns were then transferred to the laboratory (UMR BOREA, University of the French West Indies, 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 ± 0.2°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 about 96 % throughout the experiment, whatever the conditions.

2.2. Experimental design

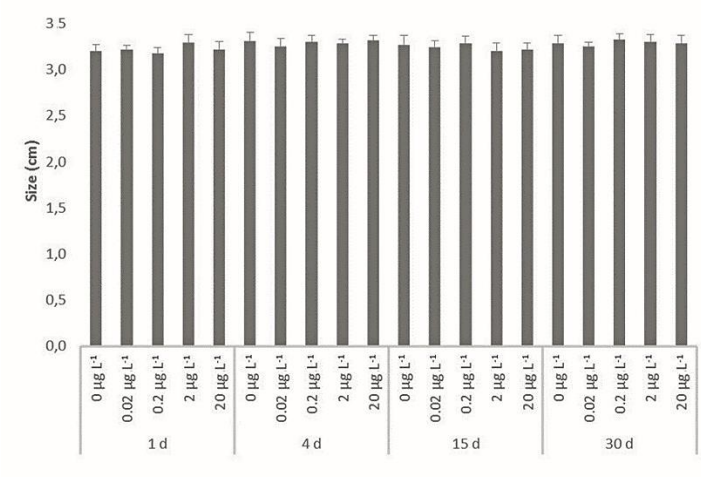
The 10 mg mL^{-1} stock solution of CLD (100 %, Riedel-de-Haën, Sigma-Aldrich, USA) was prepared in acetone as well as three different dilutions: 10 µg mL^{-1}, 100 µg mL^{-1} and 1 mg mL^{-1}. A volume of 56 µL of each dilution and of the stock solution was diluted into the 28 L of aquarium water in order to obtain the four nominal concentrations of CLD in water: 0.02, 0.2, 2 and 20 µg L^{-1}.

These concentrations were chosen for their environmental relevance and because they are typically found in surface water in Guadeloupe. Indeed, according to the Guadeloupean DIREN (Regional Department for the Environment), Guadeloupean rivers are contaminated by CLD at concentrations that ranged from 0.2 to 4 µg L^{-1} with a maximum of 8.6 µg L^{-1} measured in the Grande Anse River in 2003 (GREPP, 2004; InVS-Inserm, 2009).

In this experiment 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 µL 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. 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 hours, 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 1540 post-larvae of *M. rosenbergii* coming from the same berried female and having the same age (see 2.1.), were exposed for 30 days, which is the duration of two molt cycle (Ross, 2001). At the beginning of the exposure, 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 ± 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 each to one molt cycle).

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 concentration 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 were measured and no significant difference (two-way ANOVA test, $p > 0.05$) was observed (supplementary material 1).



Supplementary material 1 - Body length (cm) of *M. rosenbergii* exposed at four concentrations of CLD and sampled at four times of exposure (ANOVA test, $p > 0.05$)

2.3. Chlordecone concentration in exposure water and *Macrobrachium rosenbergii*

2.3.1. Chlordecone extraction from water

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 µl of acetonic solution of PCB112 (100 pg μL^{-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

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 hours 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 µL 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 μL^{-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

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 2160 g, 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 µL 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 µL of n-hexane and 50 µL of a solution of PCB 209 (100 pg μL^{-1} in n-hexane) as an injection volume internal standard (Dr. Ehrenstorfer, Germany).

In parallel of sample extractions, a procedural blank and a Quality Control (QC) were carried out. The procedural blank consisted to perform the ASE extraction without biological matrix, allowing to control the extraction and the clean-up procedure. The QC was performed to control 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 x 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 with a linear calibration curve (1.5 to 200 pg μL^{-1}) established with CLD certified solutions (Riedel-de Haën, Germany).

CLD concentrations in each sample and in 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.

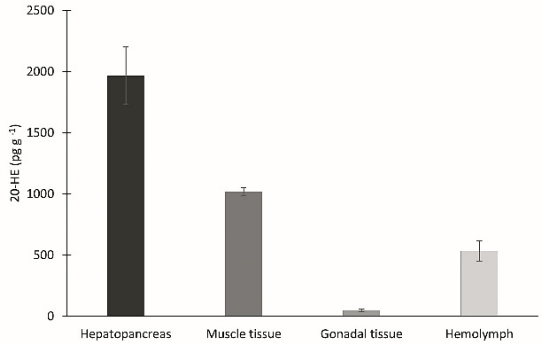
CLD concentrations in water and in muscle tissue of *M. rosenbergii* were measured in five replicates per condition and the average was calculated. The CLD concentrations expressed in µg L^{-1} for water sample are presented in Table 4.1. The CLD concentrations were expressed in ng g^{-1} wet weight for biological sample. The bioaccumulation factor (BAF) was calculated for each condition using the method of Arnot and Gobas (2006). According to these authors, BAF is the ratio between CLD concentration in tissues of prawns and CLD concentration in water of exposure, including chemical exposure in the diet. As a consequence, the BAF is typically measured under field conditions and we have also chosen to characterise the bioaccumulation process in the laboratory experiment, in order to take account of the contamination that could occur through food pellet and through cannibalism.

Table 4.1 - Chlordecone concentration (mean ± S.D.) measured in water of exposure. LOQ means limit of quantification (0.01 ng L^{-1}).

Conditions:	**Water control**	**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

2.4. 20-Hydroxyecdysone concentration

Concentrations of 20-HE hormone and its derivatives (called "20-HE" in the following text, figures and tables) were measured by following 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 because 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).



Supplementary material 2 - Comparison of 20-HE concentration in different tissues collected from non-contaminated *Macrobrachium rosenbergii* (n=10).

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 10000 g 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 to 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 hours 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 to 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 µmol 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 concentration, 20-HE concentration and chitobiase activity), comparisons were performed by using two-way ANOVA tests, 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*

CLD concentrations in the muscle tissue of *M. rosenbergii* were significantly influenced by the CLD concentrations of exposure, duration of exposure and their interaction ($p < 0.001$). Moreover, CLD concentration in prawns was dose-dependent, since prawns exposed to higher concentrations of CLD accumulated greater amounts of CLD (Table 4.2). The bconcentration of CLD in *M. rosenbergii* was also time-dependent. Indeed, the more the prawns were exposed, the higher were the CLD concentration. These observations were underlined by a strong significant positive correlation between concentrations of CLD in water and the CLD concentration in muscle tissue of *M. rosenbergii* ($p < 0.001$, $r = 0.49$, $n = 100$; Figure 4.1). Generally, whatever the CLD concentration of exposure, results showed that a bioconcentration of CLD was measured from the first day of exposure (Table 4.2), following a trend towards a dose-response relationship. However, no significant difference was observed in the CLD concentrations after 1 and 4 days of exposure, except for exposure to 0.02 µg L^{-1}.

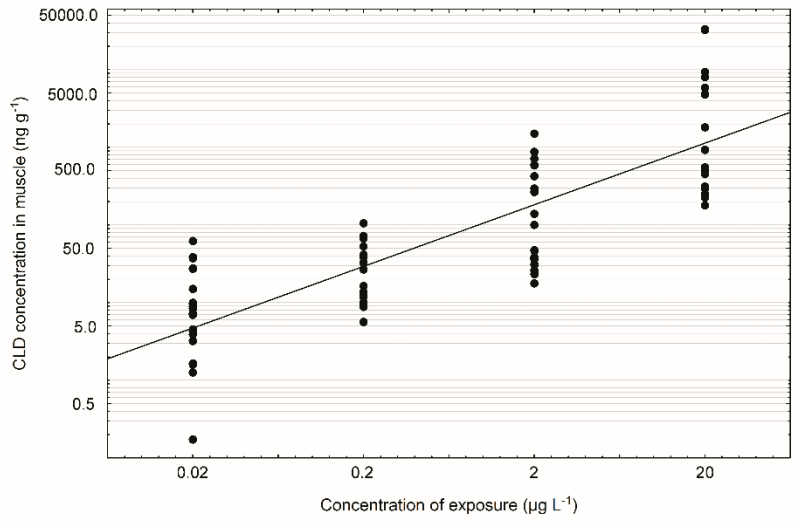


Figure 4.1 - Positive correlation between CLD concentration measured in the muscle tissue of *M. rosenbergii* and CLD concentrations in water of exposure ($p < 0.001$, $r = 0.49$, $n = 100$), taking into account all duration of exposure.

In addition, for each condition of exposure, the highest CLD concentration measured was observed after 30 days of exposure; except for exposure to 20 µg L^{-1} where the highest CLD concentration was measured after 15 days of exposure (Table 4.2).

Table 4.2 - CLD concentration (mean ± S.D.) measured in the muscle tissue of *M. 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). 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). BAF was also calculated for prawns exposed to CLD.

Nominal Concentration (µg L^{-1})	Time (days)	Chlordecone concentration in muscle tissue (ng g^{-1})	BCF
Control	1	0.65 ± 0.74	/
	4	1.03 ± 0.77	/
	15	1.96 ± 0.36	/
	30	1.67 ± 2.46	/
Solvent Control	1	0.26 ± 0.51	/
	4	≤ LOD	/
	15	1.02 ± 0.25	/
	30	≤ LOD	/
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

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

3.2. 20-Hydroxyecdysone concentration

Results highlighted a natural variation of 20-HE concentration in control prawns according to the time. Indeed, 20-HE concentration decreased between the 1st and the 4th day of exposure, and increased between the 4th and the 15th day (Figure 4.2). In addition, no significant difference were observed between 20-HE concentrations measured in water and solvent control ($p > 0.05$) whatever the duration of exposure.

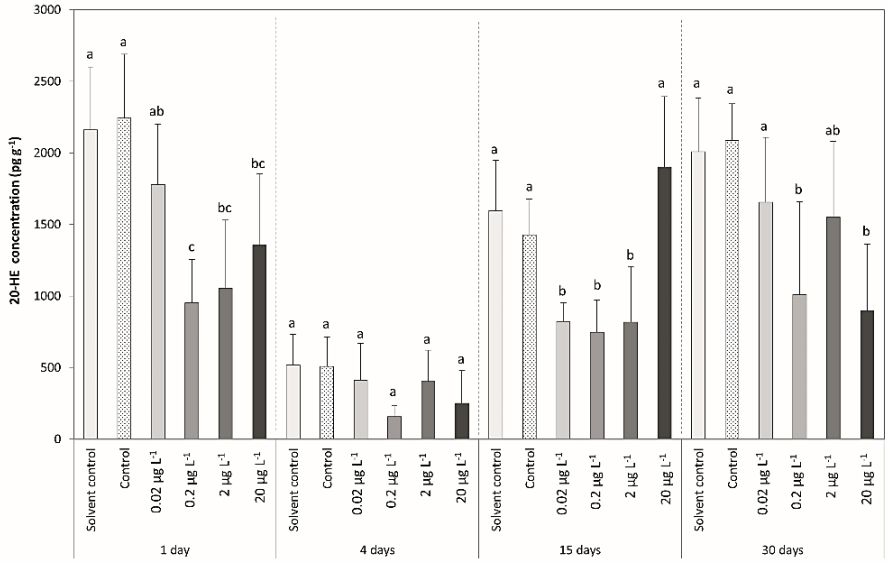


Figure 4.2 - 20-HE concentrations (pg g-1; mean ± S.D.) in the muscle tissue of *M. rosenbergii* exposed at four concentrations of CLD and sampled after four exposure durations. Different letters above the bars indicate significantly different values for each sampling time (Tukey's HSD test, p-values < 0.05).

Results revealed that 20-HE concentration was significantly influenced by CLD concentration, duration of exposure and their interaction. Generally, it was observed lower 20-HE concentrations in exposed prawns compared to the respective control, whatever 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 the prawns exposed for 1, 15 or 30 days. In the same way, 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. 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 control. After 4 days of exposure, although the 20-HE concentrations seemed to be lower in exposed prawns compared to control, no significant difference was observed. However, a significant reduction of 20-HE concentration (on average 1.8-fold lower) was measured after 15 days of exposure in all exposure conditions compared to control, except for 20 µg L^{-1}. Finally, after 30 days of exposure, prawns exposed to 0.2 and 20 µg L^{-1} had twice less 20-HE concentration than the control. Moreover, a significant negative correlation between 20-HE concentration and the log-transformed CLD concentration was observed whatever the duration of exposure (1 day: $p < 0.001$, r = - 0.87, n = 19; 4 day: $p < 0.001$, 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) (Figure 4.3).

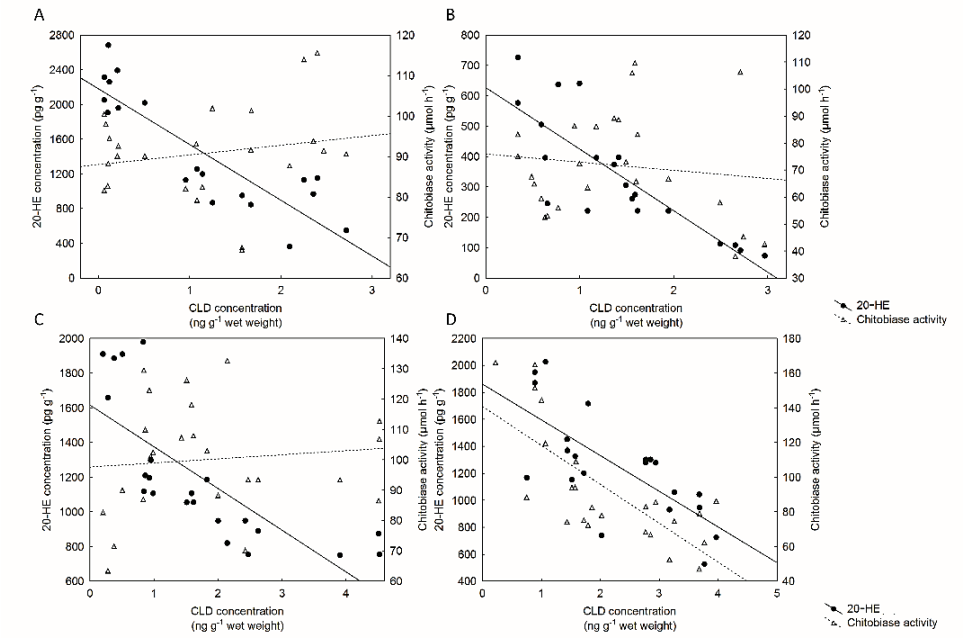


Figure 4.3 - Logarithmic correlation between log-transformed CLD concentration and 20-HE concentration represented by circles, as well as the correlation between the log-transformed CLD concentration and chitobiase activity represented by triangles in the muscle tissue of *M. rosenbergii* sampled after 1 day (A), 4 days (B), 15 days (C) and 30 days (D) of exposure.

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 ($p < 0.001$; Figure 4.4). The chitobiase activity measured in water control and solvent control were not significantly different ($p > 0.05$) whatever the duration of exposure (Figure 4.4).

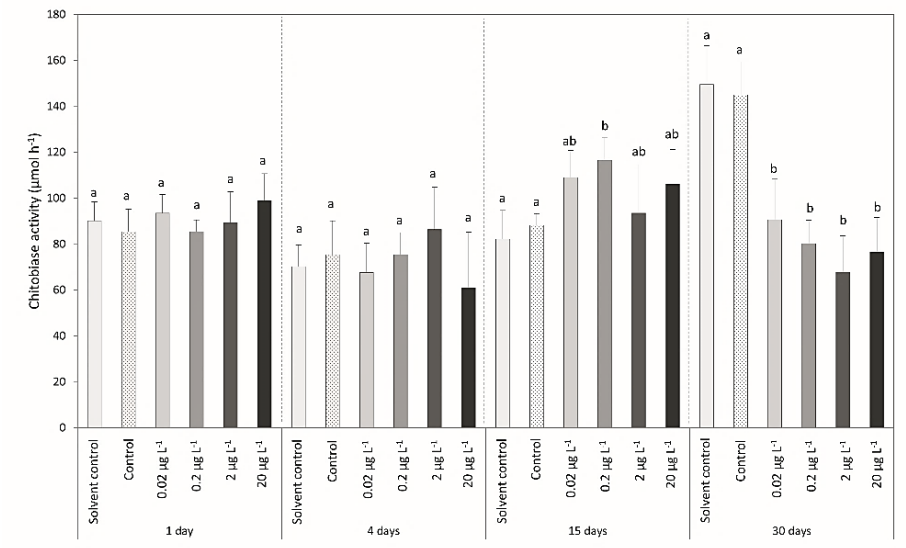


Figure 4.4 - Chitobiase activity (µmol MUF hydrolysed.h-1; mean ± S.D.) in muscle tissue of *M. rosenbergii* exposed at four concentrations of CLD and sampled after four exposure durations. Different letters above the bars indicate significantly different values for each sampling time (Tukey's HSD test, p-values < 0.05).

Moreover, no significant difference was observed in chitobiase activity measured in prawns exposed to CLD for 1 and 4 days compared to the respective controls. After 15 days of CLD exposure, a significant increase in chitobiase activity was only observed in prawns exposed to 0.2 µg L^{-1} compared to controls. Chitobiase activity was 1.5-fold higher than in the control prawns. On the contrary, a significant inhibition of chitobiase activity was observed after 30 days of exposure, whatever the CLD concentrations. Indeed, the chitobiase activity was average 1.8-fold lower in exposed prawns that in controls. No correlation was observed between chitobiase activity and log-transformed CLD concentrations in prawns, expect 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) (Figure 4.3).

4. Discussion

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

4.1 Chlordecone concentration

This study highlighted that chlordecone was accumulated in tissues of *M. rosenbergii* following a trend towards a dose-response relationship. Prawn survival was not affected by the CLD exposure, probably due to the fact that exposure concentrations were below lethal concentrations. Indeed, Schimmel and Wilson (1977) reported that the 96-h LC_{50} 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} was observed.

CLD accumulation 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, as well as Coat et al. (2011) observed the CLD bioaccumulation in tropical food web including various species of mussels, shrimps and fishes. 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 *Callinectes sapidus*. Our results suggest that an accumulation of CLD was observed since the 1st day of exposure and that increase of CLD concentrations in prawns was time-dependent, 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 could suggest that organisms have reached a stage beyond which the accumulation is no longer exponential at this concentration of exposure. Moreover, several studies have highlighted the ability of crustaceans to depurate slowly the CLD (Bahner et al., 1977; Roberts, 1981; Schimmel et al., 1979). This depuration of CLD could aslo explain the difference in CLD concentrations in *M. rosenbergii* between 15 and 30 days of exposure.

BAF results indicated that no plateau level was reached after 30 days of exposure, especially when *M. rosenbergii* were exposed to 0.02 µg L^{-1}, suggesting a strong accumulation of CLD in organisms exposed on the long term. Moreover, BAF results showed that CLD bioaccumulation was more efficient with lowest concentration in water. Prawns exposed to 0.02 µg L^{-1} had higher BAF than prawns exposed to others CLD concentrations. The higher BAF observed in the lowest CLD concentration could induce a potential effect of endocrine disruption in exposed prawns. Indeed, it is well know that endocrine disruption occurs with low doses of EDCs by binding to hormonal receptors (Vandenberg et al., 2012). In a 0.02 µg L^{-1}-exposure, CLD could be sequestered in tissues as many pollutants but in addition CLD could bind to receptors and could result in disruption of metabolic pathway of endocrine control.

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 *Callinectes sapidus*. In the same way, Bookhout et al. (1980) and Oberdörster and Cheek (2001) have shown that CLD impacted the molting and the metamorphosis of *Palaemonetes 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.

4.2 Chlordecone effects on 20-HE concentration

In control conditions (i.e. unexposed prawns), results showed lower 20-HE concentrations 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 highlighted the variation of 20-HE concentration during different stages of 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 ecdysteroids 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, authors showed that the total ecdysteroid concentration in hemolymph of *Homarus americanus* can fluctuate during the molt cycle from less than 10 pg μL^{-1} in postmolt to more than 350 pg μL^{-1} in premolt. Generally, ecdysteroid concentration is low during intermolt and postmolt, rising during premolt and reaches a peak shortly before molting (Mykles, 2010). This increase in ecdysteroid concentration is not only related to ecdysteroid synthesis but could also be explained by the conversion increase of ecdysone or inactive ecdysteroids to the active form of ecdysteroids (i.e. 20-HE) (Mykles, 2010).

After exposure to CLD, results revealed an effect of CLD on the 20-HE concentration. Indeed, it was measured a decrease of 20-HE in *M. rosenbergii* exposed to CLD whatever the concentration and the duration of exposure (except for prawns exposed to 20 µg L^{-1} of CLD for 15 days). This observation was supported by the fact that 20-HE concentration and CLD concentration in prawns were significantly negatively correlated whatever the duration of exposure.

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)(Rodriguez et al., 2007).

Thus, it could be hypothesized that CLD decreased 20-HE concentration by binding to ecdysteroid receptor, as a consequence of its anti-ecdysteroid activity (Zou and Fingerman, 1999d ; Zou, 2005). However, as other EDCs, CLD can also interfere indirectly with the endocrine system by several mechanisms, at any step of the transduction pathway of the hormones (Hyne, 2011; Rodriguez et al., 2007). 20-HE decrease observed here could also be explained by a modification of excretion rate of the hormone or an inactivation of the enzyme involved in ecdysteroid biotransformation.

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 *Callinectes sapidus*. In the same way, Bookhout et al. (1980) and Oberdörster and Cheek (2001) have shown that CLD impacted the molting and the metamorphosis of *Palaemonetes 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.

Because of the fact that CLD could have an impact on 20-HE concentration, it could therefore disturb in the long-term the molting of organisms which is induced by this hormone (Hyne, 2011; Rodriguez et al., 2007). Hirano et al. (2009) showed that low 20-HE concentration in the shrimp *Americamysis bahia* exposed to nonylphenol (also known to be an inhibitor of ecdysteroid activity) affected the organism growth. In the same 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 related to a delay in the beginning of larvae molting in the lobster *Homarus 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 induce disturbances in reproduction as hypothesized by Gismondi and Thomé (2014) in the amphipod, *Gammarus pulex*. It was also observed that the anti-ecdysteroid 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 20-HE concentration in prawns exposed to 0.2 µg L^{-1} of CLD (except after 30 days of exposure), while this decrease was less obvious in prawns exposed to high CLD concentrations (i.e. 2 and 20 µg 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 µg 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 µg L^{-1} (Welshons et al., 2003). Moreover, it is now well established that EDCs act at low doses (Vandenberg et al., 2012). Therefore, it can also be hypothesized that 0.02 µg L^{-1} of CLD is too low to cause significant disturbances, while the 0.2 µg L^{-1} seems to be the lowest sufficient concentration to induce effects on endocrine hormones.

4.3 Chlordecone effects on chitobiase activity

In regards to chitobiase activity, results highlighted no significant effect of CLD in prawns exposed for 1 and 4 days. However, after 15 days of exposure, a significant increase of chitobiase activity was observed, but only in prawns exposed to 0.2 µg 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, 1999c). Indeed, Zou and Fingerman (1999b) underlined that a decrease in chitobiase activity in the fiddler crab, *Uca pugilator*, was correlated with 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 20-HE concentration, an inhibition of chitobiase activity was therefore expected. However, the precise mechanisms linking the 20-HE concentration and the chitobiase activity is not well established yet. CLD may affect the chitobiase activity not through the effect on 20-HE concentration but maybe by disrupting several pathways. This result was in line with those of Snyder and Mulder (2001). Although Snyder (1998) reported, in the lobster *Homarus americanus*, that an increase in 20-HE concentration can induce the expression of cytochrome P450-dependent detoxifying enzymes (CYP45), Snyder and Mulder (2001) observed an upregulation of the expression of this enzyme in larvae of *Homarus americanus* exposed to heptachlor (organochlorine pesticide), while a decrease of the 20-HE concentration was measured.

In the present study, the major absence of modification of the chitobiase activity, while a decrease of the 20-HE concentration was observed, could also be explained by a direct effect of CLD on chitobiase activity, through other receptors as hypothesized by Rodriguez et al. (2007). Indeed, it could be hypothesized that the inhibition of chitobiase activity due to the decrease of 20-HE concentration, could be offset by an induction of chitobiase activity through a direct effect of CLD. Thus, these two opposite effects could result in a lack of chitobiase activity disruption. However, results showed a decrease of chitobiase activity after 30 days of exposure.

This observation could be understood by a difference in the time of response. Indeed, as explain above, a decrease of 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 degrade the old cuticle (Hyne, 2011). Nevertheless, no information is available on the duration of the molt cycle and the different cascade pathways 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 molting (LeBlanc, 2007).

5. Conclusion

This study was the first to highlight the impacts of a CLD exposure on the hormonal system of the commercial prawn, *Macrobrachium rosenbergii*, by measuring 20-HE concentration and chitobiase activity. 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 was few times documented that CLD can disrupt the molting process of invertebrates, the mechanisms involved in this effect remain unknown. This work goes further by underlying that CLD could disrupt the molting process through the disturbance of 20-HE concentration, as well as chitobiase activity. Results allowed to conclude that CLD could have an anti-ecdysteroid activity, as suggested for others EDCs estrogeno-mimetics. 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 on 20-HE synthesis and ecdysteroid metabolism pathways disrupted by a CLD exposure, as well as the effects of CLD on ecdysteroid receptors to confirm its potential anti-ecdysteroid activity, and the designation of CLD as an EDC in invertebrates. Finally, the lack of information on the endocrine system of invertebrates makes difficult to explain 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 influence of molt stage on response of organisms to xenobiotic exposure.

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Chapter 5 – Transcriptomic Approach

Publication 3

Vitellogenin and vitellogenin receptor gene expressions and 20-hydroxyecdysone concentration in *Macrobrachium rosenbergii* exposed to chlordecone.

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Abstract

Chlordecone is a persistent organochlorine pesticide widely used in Guadeloupe (French West Indies) to control the banana weevil *Cosmopolites sordidus*. Although it was previously highlighted that chlordecone could affect the reproduction and growth of vertebrate species, little information is available on the chlordecone effects in invertebrates. The present study investigated the effects of chlordecone on a hormone and a protein having key roles in reproduction and growth of the decapod crustacean *Macrobrachium rosenbergii*, by measuring the 20-hydroxyecdysone concentration, vitellogenin and vitellogenin receptor gene expressions, as well as the bioaccumulation of chlordecone in exposed prawns. First, results revealed that chlordecone was accumulated in *M. rosenbergii*. Then, it was found that Vg and VgR gene expressions were increased in males and females *M. rosenbergii* exposed to chlordecone for 90 and 240 days, while 20-hydroxyecdysone concentrations decreased. This work suggested that chlordecone accumulates in tissues and could affect key molecules involved in reproduction and growth of the invertebrate *M. rosenbergii*. However, many questions remain unresolved regarding the impacts of chlordecone on growth and reproduction, and the signaling pathways responsible of these effects, as well as the potential role of confounding factors present in *in-situ* studies.

Keywords: *Macrobrachium rosenbergii*; chlordecone; vitellogenin; vitellogenin receptor; 20-hydroxyecdysone

1. Introduction

Studies on the impacts of endocrine disrupting compounds (EDCs) on wildlife have focused mainly on hormonal regulation related to reproduction and development processes which have critical roles in population dynamics (Arukwe and Goksøyr, 2003; Gismondi and Thomé, 2014; Jubeaux et al., 2012). Indeed, many pollutants present in the aquatic environment are able to interfere with the endocrine system of exposed crustaceans which could lead to dysfunctions of biological processes (e.g. growth, reproduction) (Hyne, 2011). Endocrine disruption has been extensively described in aquatic vertebrates (Kortenkamp et al., 2011; LeBlanc, 2007), and the best known marker is the vitellogenin (Vg) which has been used as exposure biomarker in several vertebrate species due to its key role in the reproduction and its induction via the control of the estrogen receptor pathway (Jones et al., 2000; Sumpter and Jobling, 1995; Tyler et al., 1996; Zhong et al., 2014). However, Thornton et al. (2003) suggested that ecdysozoans lost the steroid receptor family, and Thomson et al. (2009) underlined the absence of receptors of the 3A group (estrogen receptor) and the 3C group (androgen, progestogen receptors) in *Daphnia pulex*. Therefore, although invertebrates can also be exposed to known EDCs of vertebrates, it's still difficult to make assumptions about the impacts of EDCs on invertebrates using knowledge from vertebrate species (Scott, 2013). Investigations are thus necessary to improve our understanding of the metabolic pathways involved in the disruption of the endocrine functions in invertebrates.

Chlordecone (CLD) was an insecticide commonly used in the French West Indies (FWI) in Guadeloupe to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993. Chlordecone is an organochlorine compound that acts by altering the sodium channels, essential for the transmission of nerve impulses in organisms (Guzelian, 1982; Newhouse et al., 2009). This inhibition of the sodium channels induces an increase of the intracellular concentration of calcium which activates the contractile proteins and causes convulsions and death of the target insect. As CLD interferes with the nervous system, it is considered neurotoxic substance.

A few years after its introduction, widespread pollution of soils, rivers, wild animals and aquatic organisms was reported (Cavelier, 1980; Snegaroff, 1977). Indeed, because of its high K_{oc} (soil organic carbon water partitioning coefficient), K_{ow} (octanol-water partition coefficient) and its affinity for lipids, CLD is persistent in the environment and accumulates in the food web (Cabidoche and Lesueur-Jannoyer, 2012). Since Hammond et al. (1979) demonstrated that CLD can bind to estrogen receptor in rat, many studies investigated the endocrine effects of CLD in various biological models. However, in aquatic ecosystems, endocrine effects of CLD were mainly studied in vertebrate models. For example, Donohoe and Curtis (1996) observed an increase of the Vg concentration in juvenile rainbow trout *Oncorhynchus mykiss* exposed for 33 weeks to CLD. Moreover, Curtis and Beyers (1978) observed a decrease of the oviposition of the teleost *Oryzias latipes* after 252 h of CLD exposure. Until now, few studies have been carried out in invertebrate models to investigate the toxic effects of CLD. Nevertheless, Gaume et al. (2014) highlighted that a CLD exposure caused the induction of genes involved in the antioxidative defense (e.g. catalase and glutathione peroxidase), or in the biotransformation process (i.e. cytochrome P450 enzymes and glutathione-S-transferase). Giusti et al. (2014) showed the reduction of the oviposition and the fecundity of the gastropod *Lymnaea stagnalis* exposed to CLD at environmental relevant concentrations. Finally, our previous study showed that CLD exposure in laboratory affected the 20-hydroxyecdysone concentration (i.e. molting hormone) and the chitobiase activity (i.e. molting enzyme), both controlled by the endocrine system, in the decapod *Macrobrachium rosenbergii*, suggesting that CLD could be an EDC in invertebrates (Lafontaine et al., 2016).

According to previously cited studies, CLD is suspected to be an EDC in invertebrates and could thus affect the reproduction and development. The present *in-situ* study aimed to investigate the effects of an environmental concentration of CLD on endocrine system of the decapod *Macrobrachium rosenbergii*, which is one of the most important resources in Guadeloupe and which can be considered a good model for the wild *Macrobrachium spp*. living in freshwater ecosystems of these regions, by evaluating the relative expression of vitellogenin protein (Vg) gene. As Vg need receptors to cross cell membrane and exert its role in the gonadal tissue, the relative expression of vitellogenin receptor gene (VgR) was also assessed.

In parallel, since the 20-hydroxyecdysone (20-HE) synthesis is linked to the induction of the Vg synthesis (Hyne, 2011), the 20-HE concentration was also assessed. Finally, to incriminate the observed effects to the CLD exposure, CLD concentrations were measured in exposed and non-exposed prawns.

2. Materials and methods

2.1. *In situ* chlordecone exposure and sampling

Post-larvae of *Macrobrachium rosenbergii* were provided by a hatchery farm (OCEAN-SA, Guadeloupe, FWI) in a geographic area free of CLD contamination. Pretests have previously been carried out to evaluate the presence of CLD in tissues of prawns from the hatchery farm and results showed no contamination (concentrations below detection limit) (data not shown). Prawns were transferred into two farming ponds, naturally filled by rivers. The first, called "control site", was located in Pointe-Noire (North Basse-Terre, Guadeloupe: 16°22'49"N, 61°77'69"W), and the second one, called "contaminated site", was located in Saint-Claude (South Basse-Terre, Guadeloupe: 16°01'93"N, 61°68'37"W), which is a pond supplied by the River Rivière Aux Herbes, a CLD-contaminated river (0.33 µg L^{-1} average measured between 2003 and 2008 by the DIREN Guadeloupe). The farm based in Pointe-Noire is still in operation, while the Saint-Claude farm has had to cease operations because CLD concentration in prawns was higher than the French and European maximum residue limit of 20 ng g^{-1} wet weight (Anon., 2008; Ministère de l'Écologie du Développement durable et de l'Énergie, 2015a). Water temperature, pH and dissolved oxygen were measured throughout the experiment, which took place from March 2012 to November 2012 (Table 5.1). These values are in accordance with optimal water temperature, pH and dissolved oxygen commonly used in prawn farms (New, 2002).

Table 5.1 - Water parameters of Pointe-Noire (control site) and Saint-Claude (contaminated site) measured during the 240 days of exposure. LOD = 0.01 µg L^{-1} means limit of detection.

Parameter	Pointe-Noire	Saint-Claude
CLD concentration (µg L^{-1})	< LOD	0.33 ± 0.10
Temperature (°C)	27.6 ± 1.5	27.9 ± 1.6
pH	8.47 ± 0.43	9.00 ± 0.44
Dissolved oxygen (mg L^{-1})	7.18 ± 2.19	6.62 ± 4.59

After 90 and 240 days of exposure, 20 females and 20 males were collected in each site. For each gender, five prawns (corresponding to 5 replicates) were used for CLD concentration assessments, five prawns (corresponding to 5 replicates) were used to measure 20-HE concentration, and ten prawns (corresponding to 10 replicates) were used to measure vitellogenin (Vg) and the vitellogenin receptor (VgR) gene relative expression. Vg gene expression was measured in hepatopancreas since this organ is one of the main synthesis site of Vg in the giant freshwater prawn *M. rosenbergii* (Soroka et al., 2000), while the synthesis site of VgR is the gonadal tissue (Roth and Khalaila, 2012). CLD concentration was also measured in both control and exposed prawns. After sampling, individuals were transferred to laboratory where hepatopancreas and gonadal tissue were immediately dissected, frozen in liquid nitrogen and store at -80°C until analysis.

Before dissections, body length and body weight were measured and no significant difference ($p > 0.05$, two-way ANOVA test) was observed between individuals from the control site and those of the contaminated site, taking into account time exposure (i.e. 90 days Pointe-Noire: 10.20 ± 0.52 cm and 10.21 ± 1.74 g, Saint-Claude: 10.96 ± 0.45 cm, 15.00 ± 3.16 g; 240 days Pointe-Noire: 12.03 ± 0.97 cm and 21.79 ± 4.18 g, Saint-Claude: 12.72 ± 0.74 cm, 23.42 ± 4.74 g). Individual sex was confirmed during dissections, thanks to the observation of gonadal tissues.

2.2. Chlordecone concentration in *Macrobrachium rosenbergii*

CLD concentration was analyzed in *M. rosenbergii* tissue according to a method adapted from Debier et al. (2003), Guldner et al. (2010) and Multigner et al. (2010). Briefly, prawns were freeze-dried with a Benchtop 3 L Sentry Lyophilisator (VirTis, USA). 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) and a hexanic solution of PCB congener 112 (Dr. Ehrenstorfer, Germany) was added to the samples as a surrogate internal standard. After the extraction, samples were cleaned-up with 98 % sulfuric acid (Merck, Germany) in order to remove organic matters (e.g. lipids, lipoproteins, carbohydrates). Then, a volume of 5 µL of nonane were added in the collected phase as a keeper, samples were evaporated under a gentle nitrogen stream and resuspended in n-hexane additionned with a solution of PCB 209 as an injection volume internal standard (Dr. Ehrenstorfer, Germany).

In parallel of the sample extractions, a procedural blank (i.e. ASE extraction without biological matrix allowing to control the extraction and the clean-up procedure), and a Quality Control (QC) were carried out. The QC was performed to control the CLD recovery by using a CLD-free biological matrix (here, freeze-dried *Penaeus monodon*) spiked with a defined concentration of CLD in order to obtain a nominal concentration of 2.5 ng g^{-1} wet weight.

The purified extracts, procedural blank and QC, were analyzed by high-resolution gas chromatography. The analytical parameters were described by Guldner et al. 2010 and Multigner et al. 2010. CLD was identified based on its retention time previously determined with a linear calibration curve (1.5 to 200 pg μL^{-1}, $r > 0.99$) established with CLD certified solutions (Riedel-de Haën, Germany). The quantification of CLD was achieved by means of the internal standard method. The CLD concentrations in each sample and in the QC were corrected with the percentage recovery of the surrogate PCB 112 and the initial sample weight. The recovery efficiency was within the limits recommended by SANCO (SANCO/12571/2013 European commission, 2013).

The limit of detection (LOD) was 0.02 ng g^{-1} wet weight and the limit of quantification (LOQ) was 0.06 ng g^{-1} wet weight. The CLD concentrations in *M. rosenbergii* were measured in five replicates per condition and means were calculated. CLD concentrations were expressed in ng g^{-1} wet weight.

2.3. 20-Hydroxyecdysone concentration

The concentrations of 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). 20-HE concentration assessment was entirely described in Lafontaine et al. (2016) (Chapter 4, p 113).

2.4. Quantification of vitellogenin and vitellogenin receptor gene relative expression

2.4.1. Total RNA extraction and cDNA synthesis

Total RNAs were extracted from 30 mg of tissue using the RNeasy lipid tissue mini kit (Qiagen, Germany), following the manufacturer's instructions. Quality of RNA was verified by electrophoresis on a 1.5% agarose gel in TAE buffer (Tris 40 mM, Acetic acid 1 mM, EDTA 40 mM) and visualisation under UV light, and RNA concentrations were measured using a NanoDrop ND-1000 spectrometer (NanoDrop, USA). Synthesis of cDNA was performed with 150 ng of total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fisher Scientific) and random hexamer primers. The reverse transcription polymerase chain reaction (reverse-PCR) was performed at 42 °C for 60 min with a reaction mixture containing: 10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl2, 1 mM dNTP mix, 2.5 µM of random hexamer primers, 2.5 U/µL of Revert Aid MuLV RT and 1 U/µL of Ribolock RNase Inhibitor.

2.4.2.Quantitative real-time PCR (RT-qPCR)

Evaluation of Vg and VgR gene expression levels was carried out by using RT-qPCR. Specific primers were designed to amplify the corresponding cDNAs according to Roth and Khalaila (2012) and the specific gene sequences of *M. rosenbergii* available in GenBank database (Vg: accession number AB056458.1; VgR: accession number GU454802.1) (Table 5.2). Actin (accession number AY626840.1) (Qiu et al., 2008) and 18S (accession number KM101531.1) (Roth and Khalaila, 2012) fragments were tested as housekeeping genes.

Table 5.2 - Sequences and reaction efficiencies of each primer pair for each of the studied genes

Genes	Species	Primers		Efficiency (mean ± SD)
Vg	***M. rosenbergii***	**Forward 5'-3'**	GCGAAAAGGTAAAGCACGGAGT	1.957 ± 0.083
		Reverse 5'-3'	ACGGCGCAAGAAATGTAATGC	
VgR	***M. rosenbergii***	**Forward 5'-3'**	CTCCCTTGACTACGTCTGCAAC	1.911 ± 0.068
		Reverse 5'-3'	GCATTGCATCTTGAGGTCTCG	
18S	***M. rosenbergii***	**Forward 5'-3'**	TAGCAATTCGCCGTCGTTATTC	2.127 ± 0.141
		Reverse 5'-3'	CTACCCCCGGAACTCAAAGACT	

RT-qPCR were performed in 384-well plates with an ABI Prism 7900HT system (Applied Biosystems) using Mesa Green qPCR MasterMix (Eurogentec). A total of three technical repeats were run for each cDNA gene and primer pair (Table 2). For each reaction, an equal amount of cDNA (4 µl of the cDNAs diluted 50-fold and prepared from 150 ng of total RNAs) were completed with a reaction mix containing 5 µL of Mesa Green qPCR MasterMix and 2.5 pmol of each primers in a final volume of 10 µL. The following standard thermal profile was used: 2 min at 50°C, 10 min at 95°C, 40 repeats of 15 s at 95°C and 60 s at 60°C. At the end of the 40 repeats, dissociation curves of the amplified products were established with the following thermal steps: 15 s at 95°C, 15 s at 60°C, and 15 s at 95°C. The quality of the reactions was checked through the amplification and dissociation curves. In addition, reaction efficiencies were determined for each RT-qPCR using the LinRegPCR software v2013 (Ruijter et al., 2009).

Mean reaction efficiencies were then determined for each primer pair from all reactions (>100 reactions; table 2) and used to calculate relative gene expression levels using 18S as housekeeping gene for normalization with the qBase software (Biogazelle, Hellemans et al. 2007). The 18S was selected to normalize the data, as it displayed the highest stability across the sample set (no significant differences upon a three-way ANOVA-test, with $p > 0.05$, taking into account "CLD exposure", "duration of exposure" and "gender" as factors).

2.5. Statistical analysis

Statistical analyses were carried out with the STATISTICA 10 Software (StatSoft 2012, USA). The data met normality and variance homogeneity which were tested with the Shapiro–Wilk and the Bartlett tests, respectively. First, a MANOVA test was performed in order to test the influence of gender, CLD and exposure duration on the whole measured parameters (Table 5.3). Then, to investigate the CLD bioaccumulation as well as the effects of CLD, gender and duration of exposure on the relative expression of Vg and VgR gene and 20-HE concentration, data were analyzed using a three-way ANOVA test (Table 5.3), followed by Tukey HSD post-hoc tests which were performed to describe significant differences. A probability value of less than 0.05 was regarded as significant. The correlations between measured parameters were analyzed using the Pearson correlation coefficient.

Table 5.3 - Univariate and multivariate analyses of variance (ANOVA/MANOVA) investigating variations in vitellogenin (Vg) and vitellogenin receptor (VgR) gene expressions and 20-HE concentrations according to chlordecone exposure (CLD), gender and duration of exposure.

MANOVA **Sources of variation**	***p*-values**		
Gender	< 0.0001		
CLD	< 0.0001		
Duration of exposure	0.06		
Gender × CLD	0.04		
Gender × Duration of exposure	0.002		
CLD × Duration of exposure	0.01		
Gender × CLD × Duration of exposure	0.03		
ANOVA	***p*-values**		
Sources of variation	**Vg gene**	**VgR gene**	**20-HE level**
Gender	< 0.0001	< 0.0001	0.07
CLD	0.36	0.002	< 0.0001
Duration of exposure	0.70	0.33	0.009
Gender × CLD	0.64	0.003	0.75
Gender × Duration of exposure	0.41	0.51	< 0.0001
CLD × Duration of exposure	0.025	0.06	0.68
Gender × CLD × Duration of exposure	0.011	0.03	0.02

3. Results and discussion

3.1. Chlordecone concentration in *Macrobrachium rosenbergii*

CLD concentrations were measured in post-larvae provided by the hatchery, before the transfer in each farming pond (T0, Table 5.4). CLD concentrations were below the LOD (i.e. 0.02 ng g^{-1}), which confirmed that prawns were free of CLD contamination at the beginning of the experiment. CLD concentrations were also measured in *M. rosenbergii* transferred in the control and the contaminated farms after 90 and 240 days of exposure (Table 5.4).

Table 5.4 - CLD concentrations (mean ± S.D.), on a wet weight basis, measured in *M. rosenbergii* sampled in the control (Pointe-Noire) and the contaminated site (Saint-Claude), as well as in post-larvae, before the transfer in each farming pond (T0). Asterisks indicate significantly different values of CLD concentrations between control and contaminated samples. LOD means limit of detection.

CLD concentration in prawns (ng g^{-1})		Pointe-Noire	Saint-Claude
T0		< LOD	< LOD
90 days	Males	9.09 ± 9.20	858.50 ± 92.09*
	Females	13.01 ± 3.79	646.51 ± 58.40*
240 days	Males	13.57 ± 4.04	767.03 ± 144.64*
	Females	9.04 ± 3.69	1247.39 ± 238.22*

ANOVA results showed a significant influence of the exposure site ($p < 0.05$) on the CLD concentrations in prawns. In control prawns, the very low concentrations of CLD detected could be explained by the bioaccumulation of trace amounts of CLD (lower than the LOQ in water, i.e. 0.01 µg L^{-1}) detected in the river supplying the Pointe-Noire farming pond (Bonan and Prime, 2001; Ministère de l'Écologie du Développement durable et de l'Énergie, 2015b). The results obtained from field studies are generally more environmentally realistic and ecologically meaningful than laboratory tests (Connon et al., 2012; Crane and Babut, 2007), but they are difficult to interpret, because of several confounding factors (e.g. season, others chemicals). However, the CLD bioaccumulation observed here is consistent with the fact that CLD has a high potential of bioaccumulation in aquatic organisms, based on its physicochemical properties, even when concentrations are very low (ATSDR et al., 1995).

As expected, a significant accumulation of CLD was observed in prawns sampled in the contaminated pond (Saint-Claude). This result is in agreement and of the same order than those of Monti (2007) who measured CLD concentrations of 2204 ng g^{-1} in *Macrobrachium carcinus* and 1134 ng g^{-1} in *M. faustinum*, sampled in the River Rivière aux Herbes, which is the river supplying the Saint-Claude pond, used as contaminated site in the present study. On the contrary, the CLD concentrations measured in *M. rosenbergii* in the present study were higher than those measured in the same organism in another *in-situ* study (Gaume et al., 2014).

This difference is probably due to the duration of exposure, since Gaume et al. (2015) studied a 96h-exposure compared to our study where prawns were exposed for 90 and 240 days. Nevertheless, results of these studies show clearly that the CLD is constantly accumulated in *M. rosenbergii*, especially after a long term exposure.

3.2. 20-hydroxyecdysone concentration

Results revealed that 20-HE concentration was significantly influenced by CLD exposure. Indeed, it was observed that 20-HE concentrations were from 1.3-fold to 1.9-fold lower in prawns exposed in the contaminated site compared to the control prawns, whatever the duration of exposure (Figure 5.1). This decrease was significant in females *M. rosenbergii* exposed for 90 days and in males and females exposed for 240 days.

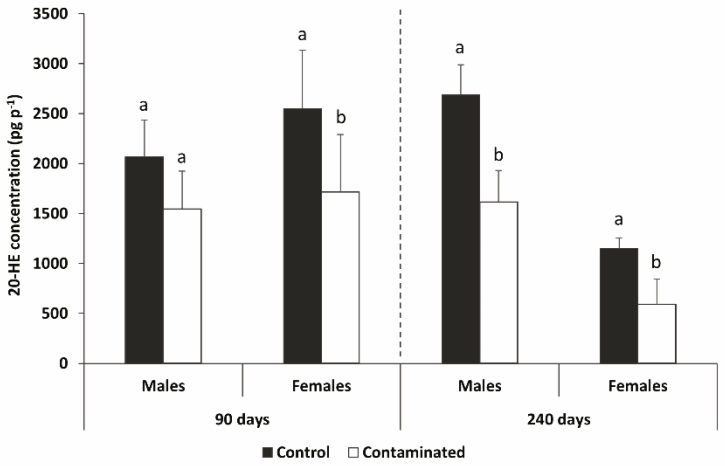

Figure 5.1 - 20-HE concentrations (pg g^{-1}; mean ± S.D.) in males and females *M. rosenbergii*, sampled after 90 and 240 days of exposure in control and chlordecone-contaminated sites. Different letters above the bars indicate significant differences for each duration of exposure (Tukey's HSD test, p-values < 0.05).

Moreover, a significantly negative correlation between 20-HE concentrations and CLD concentrations measured in prawns was obtained (*p*-value = 0.0019, r = - 0.70). A decrease of 20-HE hormone in *M. rosenbergii* has already observed in presence of CLD in our previous study in laboratory conditions (Lafontaine et al., 2016a). The same result obtained here supports the hypothesis that CLD could act as an anti-ecdysteroid compound and be an endocrine disruptor in invertebrates.

Chlordecone can be considered as a steroid receptor ligand (Kavlock, 1996) and various EDCs have been shown to act as ecdysteroid synthesis inhibitors or ecdysteroid receptors (EcR) antagonists in crustaceans (LeBlanc, 2007), leading to a possible decrease of ecdysteroid concentrations (Forget-Leray et al., 2005; Rodriguez et al., 2007). Consequently, CLD could be responsible of the decrease 20-HE concentration by binding directly to EcR, as a consequence of a potential anti-ecdysteroid activity (Zou and Fingerman, 1999c; Zou, 2005). The secretion of 20-HE by the crustacean Y-organ is under negative control of the MIH (molt inhibiting hormone), which is a hormone secreted by the X-organ/sinus gland complex (Rodriguez et al., 2007).

20-HE hormone might have a feedback control on the production and/or release of MIH from X-organ/sinus gland complex located in the eyestalk or on the ecdysteroid synthesis in Y-organs (Dell et al., 1999; Techa and Chung, 2015). The presence of EcRs in eyestalks and Y-organs of decapod species has been demonstrated and supports these suggestions (Chang and Mykles, 2011; Chung et al., 1998; Dell et al., 1999; Techa and Chung, 2013). The binding of CLD on EcR in X- or Y-organ could thus disrupt 20-HE synthesis. However, as other EDCs, CLD may interfere with the endocrine system by several mechanisms, at any step of the transduction pathway of the hormones (Hyne, 2011; Rodriguez et al., 2007). Methyl farnesoate (MF), the secretion of which is inhibited by the mandibular organ inhibiting hormone (MOIH) from the X-organ, stimulates the secretion of 20-HE (Rodriguez et al., 2007). The decrease of 20-HE concentration observed in exposed prawns could also be due to the disruption of the mandibular organ (through MOIH) following an interaction of CLD with X-organ. These effects of CLD on 20-HE concentration could therefore affect the organism growth and development in the long term, but also the reproduction of prawns since the 20-HE is involved in the reproduction process (Hyne, 2011; LeBlanc et al., 2000; Rodriguez et al., 2007; Snyder and Mulder, 2001).

3.3. Expression of vitellogenin gene

Results revealed that expression of Vg gene was significantly influenced by gender of prawns, as well as the interaction between gender, CLD concentration and exposure duration and the interaction between CLD concentration and exposure duration (Table 5.3).

Results showed that expression of Vg gene was not completely silent in males and that strong differences in Vg gene expression were observed between males and females ($p < 0.0001$). These results confirm the baseline level of Vg in male crustaceans as reported in the copepod *Tigriopus japonicus* (Lee et al., 2008) or in the amphipod *Gammarus fossarum* (Jubeaux et al., 2012). Moreover, no significant difference ($p > 0.05$) was detected between the Vg gene expression measured in CLD-exposed and control males after 90 days of exposure, although a trend of increase was observed after 240 days of exposure (Figure 5.2A). Conversely, in females, a trend of decrease was observed after 90 days of exposure, while a significant increase ($p = 0.04$) of Vg gene expression was measured after 240 days of exposure, compared to respective controls (Figure 5.2B).

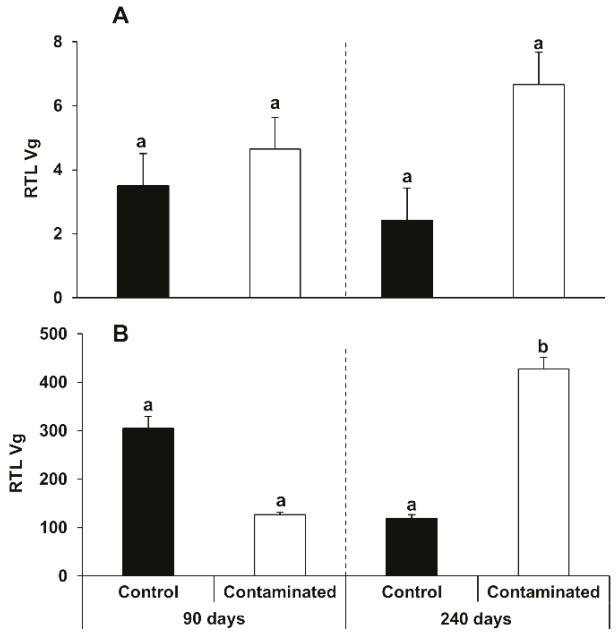

Figure 5.2 - Relative Transcript Levels (RTL) of Vg gene in hepatopancreas of males (A) and females (B) *M. rosenbergii*, sampled after 90 and 240 days of exposure in control and chlordecone-contaminated sites. Expression levels are relative to rRNA 18S and are the average of 10 replicates (means ± SE). Different letters above the bars indicate significant differences for each duration of exposure (Tukey's HSD test, p-values < 0.05).

In addition, a significantly positive correlation (*p*-value = 0.01, r = 0.70) between Vg gene expression and the CLD concentrations measured in prawns was obtained, suggesting a relationship exists between the CLD exposure and the modification of Vg gene expression in *M. rosenbergii*. The increase of Vg gene expression after a CLD exposure has been observed in some vertebrates, through the interaction with the estrogen receptor (Donohoe and Curtis, 1996; Flouriot et al., 1996; Hammond et al., 1979). For example, it was measured an increase of the Vg synthesis in the juvenile trout *Oncorhynchus mykiss* fed during 33 weeks with CLD contaminated food (0.4 mg kg^{-1} day^{-1}) (Donohoe and Curtis, 1996). However, Thornton et al. (2003) assumed the loss of the estrogen receptor in ecdysozoans, which invalidates the hypothesis of an interaction between CLD and an estrogen receptor in *M. rosenbergii*.

Nevertheless, previous investigations showed an increase of Vg gene expression in crustaceans exposed to EDCs (e.g. xenoestrogen compounds) (Billinghurst et al., 2000; Ghekiere et al., 2006; Huang and Chen, 2004; Huang et al., 2006; Oberdörster et al., 2000; Sanders et al., 2005). For example, in female shrimp *Neocaridina denticulata,* Vg gene expression was induced by exposure to chlordane at 0.001 and 0.01 µg L^{-1} (Huang et al., 2006), suggesting that chlordane may cause some reproductive impairments and alterations in endocrine functions.

As 20-HE and Vg synthesis is hormonally-dependent in crustaceans (Hyne, 2011), our results could be explained by an interaction of the CLD with the endocrine system of *M. rosenbergii*. Shrivastava and Princy (2014) indicated that MIH inhibits the 20-HE synthesis in Y-organ on the one hand, and stimulates vitellogenesis in hepatopancreas on the other hand. As all of the chemical insecticides, CLD is not species-selective with regard to targets of toxicity and it has neurotoxic properties since it interferes with the nervous system of the target organisms (Costa, 2015). Therefore, CLD could affect the secretion of neurohormones in crustaceans by binding with key receptors such as steroid receptor (Kavlock, 1996), or EcR, but no information is available regarding the potential interaction between CLD and EcR. However, CLD could interact with X-organ of *M. rosenbergii*, through EcR, and causing an increase of the synthesis and/or release of MIH, which would explain the decrease of 20-HE concentration coupled with an increase of the Vg gene expression, observed here.

CLD exposure could also inhibit the GIH (gonad inhibiting hormone) synthesis, resulting in an increase of the Vg production. Another hypothesis could be that the increase of Vg expression would be due to an increase of the gonad stimulating hormone (GSH) from thoracic ganglion or vitellogenin stimulating ovarian hormone (VSOH) from ovary, which seem to play a similar role as 17β-estradiol in egg laying vertebrates (Hasegawa et al., 1993; Kusk and Wollenberger, 2007). Finally, the last assumption is related to the low levels of 20-HE (as observed here after CLD exposure) coupled with the secretion of GSH by the thoracic ganglion, which could initiate the production of vitellogenin (Hyne, 2011). This assumption is supported by a negative, but not significant correlation between 20-HE concentration and Vg expression. The 20-HE/Vg relationship was previously investigated by Young et al. (1993) in *P. monodon*, who observed low 20-HE concentration when high Vg concentration were measured.

3.4. Expression of vitellogenin receptor gene

Results revealed that expression of VgR gene was significantly influenced by gender, CLD exposure, as well as by the interaction between these two factors (Table 5.3). Moreover, the level of expression of VgR gene was impacted by the interaction between gender, CLD exposure and duration of exposure. In males (Figure 5.3A) and females (Figure 5.3B), the level of VgR expression was significantly increased in exposed prawns compared to respective controls, whatever the duration of exposure. These observations suggested that a relationship exists between the CLD exposure and the modification of VgR gene expression in both *M. rosenbergii* genders.

Moreover, a strong significant positive correlation between VgR gene expression and the CLD concentrations in prawns was also observed (p-value = 0.04, r = 0.64) and support this relationship. In addition, the results obtained in males showed that, in addition to the Vg gene, males *M. rosenbergii* expressed also the gene of vitellogenin receptor.

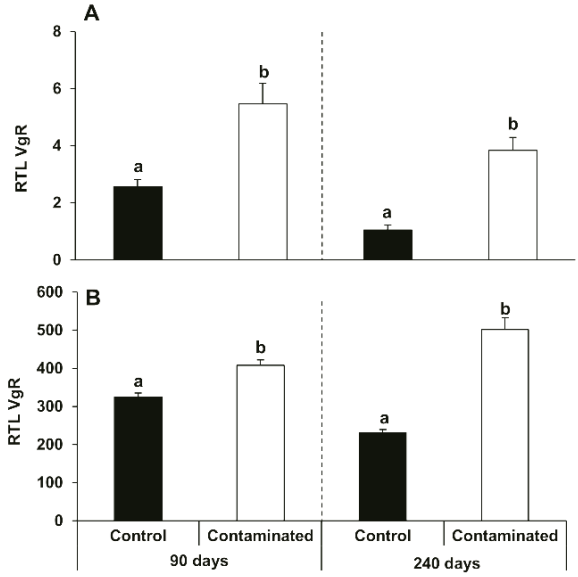

Figure 5.3 - Relative Transcript Levels (RTL) of VgR gene in gonads of males (A) and females (B) *M. rosenbergii* sampled after 90 and 240 days in control and chlordecone-contaminated sites. Expression levels are relative to rRNA 18S and are the average of 10 replicates (means ± SE). Different letters above the bars indicate significant differences for each duration of exposure (Tukey's HSD test, p-values < 0.05).

The VgR is a receptor on oocyte membrane having a high affinity for Vg proteins, and which mediates the endocytic process required for the internalization of Vg in oocytes by a receptor-mediated endocytosis (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998; Tiu et al., 2008). The presence of this Vg-specific receptor on oocyte membrane has been showed in invertebrates such as insect species (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998), or shrimps *Penaeus monodon* (Tiu et al., 2008) and *M. rosenbergii* used in this study (Roth and Khalaila, 2012). However, although the VgR plays an important role in oocyte maturation through the Vg incorporation in the gonadal tissue (Tiu et al., 2008), only a few studies focused on VgR gene expression or VgR levels in crustaceans (Subramoniam, 2011). Moreover, to our knowledge, no study has considered the modification of VgR gene expression in presence of EDCs. In the present study, CLD significantly increased the expression of VgR gene in both genders, whatever the duration of exposure. So far, it is difficult to compare our results to previous studies because none dealt with the effects of EDCs on this receptor.

However, the induction of VgR expression could be explained by the increase of Vg expression as suggested by Tiu et al. (2008) in the shrimp *P. monodon*. In their study, the authors supposed that VgR could be over-expressed in order to provide more receptors for increased amounts of Vg. This assumption is supported by a significant positive correlation (p-value = 0.003, r = 0.88) between Vg and VgR expressions. Otherwise, the induction of VgR expression could be due to an effect of CLD similar to that of the MIH. As suggested above, CLD could be an agonist of MIH, resulting in both a decrease of 20-HE and an increase of Vg. The interaction between CLD and the X-organ could also inhibit the GIH synthesis, resulting in an increase of both Vg and VgR. In the same way, this increase of VgR expression could be the consequence of the synthesis of the GSH by a direct action of CLD on the thoracic ganglion, or indirectly for example through a disturbance of the serotonin. Indeed, in crustaceans, serotonin is identified as a neurotransmitter that stimulates release of some neurohormones such as CHH (crustacean hyperglycemic hormone) or MIH (Keller and Beyer, 1968; Mattson and Spaziani, 1985) and inhibits the release of GIH (Kulkarni and Glade, 1991; Tinikul et al., 2008). Processes regulated by serotonin may become a target of environmentally relevant endocrine disruptors, such as insecticides because of their neurotoxic properties (Brooks et al., 2003; Henry et al., 2004; Mazurová et al., 2008). The assumption on the serotonin involvement in the Vg and VgR gene expressions is supported by the fact that Ruttanakorn et al. (2014) observed an increase of Vg concentration in *M. rosenbergii*, after serotonin injection.

Our results suggest that CLD could act on endocrine pathways involved in the reproduction and molt processes. It could act through the X-organ, which is one of the main neuroendocrine organs in crustaceans, or through Y-organ or thoracic ganglion, or several endocrine structures simultaneously. The incorporation of Vg by VgR into the oocytes is essential to the reproduction process of the organism (e.g. survival and development of eggs) (Roth and Khalaila, 2012). Although it was already observed in vertebrates that changes in Vg expression could cause disruptions of reproduction and population dynamics on the long-term (Bosker et al., 2010; Kidd et al., 2007; Martín-Díaz et al., 2005), in invertebrates, this question is less advanced. However, it was observed in several crustacean species, that a modification of Vg gene expression causes reproduction impairments as well as growth disturbances (Hannas et al., 2011; Tokishita et al., 2006).

Moreover, impairments in reproduction (i.e. infertility of females) have been related to a deficiency of VgR in *Drosophila melanogaster* females (Schonbaum et al., 1995). According to these previous studies and our results, it appears that CLD could disrupt the reproduction and/or development of the decapod crustacean *M. rosenbergii* through the modification of molting hormone (20-HE) and Vg and VgR gene expression. However, due to the fact that this study was carried out in field conditions, confounding factors should be considered in further studies to incriminate the observed effects only to the CLD exposure.

4. Conclusion

The present study is the first to link the CLD effects on 20-HE concentration and both Vg and VgR gene expressions in crustaceans. Results revealed that the expression of Vg and VgR genes in *M. rosenbergii* were modified following a CLD exposure, and that CLD decreased 20-HE concentration as previously observed in the laboratory experiment. Since Vg, VgR and 20-HE are hormonally-controlled, these observations suggest a potential disruption of the endocrine system of crustaceans after a CLD exposure. Consequently, CLD seems to be an EDC for *M. rosenbergii*, and more generally for crustaceans. However, this study only revealed a possible relation of putatively endocrine disrupting effects of CLD in an environmentally relevant context, but did not focus on the underlying mechanisms. Further investigations are thus needed to explain how CLD acts on hormonal control and/or metabolic pathways, especially by focusing on the X/Y-organs, their receptors (e.g. ecdysteroid receptor), as well as their secreted hormones (e.g. MIH, GIH).

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Chapter 6 – Proteomic Approach

Publication 4

Proteomic analysis of the giant freshwater prawn, *Macrobrachium rosenbergii*, exposed to chlordecone

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Abstract

The present work is one of the first study investigating the impacts of xenobiotics on the proteome of a crustacean species using gel-free shotgun proteomic approach. We investigated the effects of chlordecone, an organochlorine insecticide, on the proteome of *Macrobrachium rosenbergii*, a decapod crustacean, by measuring variations of protein expression in the hepatopancreas of organisms exposed to three environmental relevant concentrations of chlordecone (i.e. 0.2, 2 and 20 µg L^{-1}) using a gel-free proteomic analysis. Our results revealed that the proteome of *Macrobrachium rosenbergii* was influenced by chlordecone exposure. Expression of several proteins was significantly up- or down-regulated in exposed prawns compared to controls. Most of these proteins are involved in important physiological processes such as ion transport, defense mechanisms and immune system, cytoskeleton dynamics, or protein synthesis and degradation. Moreover, proteins involved in the hormonal control of reproduction or development processes showed significant changes in their expression, highlighting that chlordecone is potentially an endocrine disruptor compound for crustaceans. However, future investigations are needed to complete understanding of action mechanisms of CLD on proteome and endocrine system of crustaceans. Proteomic approach could elucidate the underlying molecular pathways involved in stress response and leading to adverse impacts of chemicals in invertebrates and in a larger scale in populations.

Keywords: proteomics; chlordecone; *Macrobrachium rosenbergii*; endocrine system

1. Introduction

During the last decades, proteomic approach has been developed progressively in the field of ecotoxicology in order to increase the understanding of adverse impacts of chemicals in exposed organisms (Rodríguez-Ortega et al., 2003; Sanchez et al., 2011; Wright et al., 2012). Proteomic analyses aim to obtain a quantitative description of protein expressions to identify changes in the expression patterns following exposure to environmental stress conditions such as temperature fluctuation, parasitism, and exposure to environmental pollutants (Cao et al., 2009; Giusti et al., 2013b). Moreover, analysis of the effects of pollutants on the proteome of an exposed species could allow to investigate potential biomarkers (Ankley et al., 2009; Rodríguez-Ortega et al., 2003; Sanchez et al., 2011). Indeed, biomarker responses (e.g. antitoxic enzymes, metallothioneins, vitellogenin) are widely employed to investigate the presence of xenobiotics, such as endocrine disrupting compounds (EDCs), and evaluate their consequences on biota (Hiramatsu et al., 2005; Ringwood et al., 2008; Rodríguez-Ortega et al., 2002).

Until recently, most ecotoxicological studies involving the proteomic approach were carried out using the two-dimensional gel electrophoresis (2D) method, which is based on the separation of proteins by two-dimensional gel electrophoresis, first according to the isoelectric point of proteins and secondly based on the molecular weight of proteins, and their subsequent identification by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) (Gomiero et al., 2006; Görg et al., 2004). However, although 2D is commonly used, this approach is labor intensive and has some limitations such as under-selection of some protein categories, limited dynamic range, co-migration of proteins, and the necessity to run many replicates (Görg et al., 2004; Zhou et al., 2012). To overcome some of these limitations, a new proteomic approach called “shotgun” proteomic or gel-free proteomic has been developed over the years (Baggerman et al., 2005). In this approach, proteins are extracted from tissues and then immediately cleaved into peptides using proteolytic enzymes. Next, peptides are separated in liquid chromatography coupled with a tandem mass spectrometric analysis. The identification of these peptides by mass spectrometry allows to determine the protein content of the initial sample (Williams, 1999).

Moreover, this approach allows to study a multiplicity of proteins simultaneously and thus, analyze all proteins present in samples (Cao et al., 2009; Williams, 1999). Until now, among invertebrates, most proteomic studies have focused on bivalve species (Sanchez et al., 2011). Few ecotoxicoproteomic studies have been carried out using crustaceans (Boulangé-Lecomte et al., 2016; Gismondi et al., 2015; Trapp et al., 2014) and no study on protein expression changes in response to a chlordecone exposure through proteomic approach has been reported.

Chlordecone (CLD) is an insecticide commonly used in the French West Indies (FWI) in particular in Guadeloupe to control the banana weevil *Cosmopolites sordidus* from 1972 to 1993. A few years after the introduction and use of CLD, widespread pollution of soils, rivers, wild animals and aquatic organisms was reported (Cavelier, 1980; Snegaroff, 1977). Moreover, because of its high K_{oc} (Soil Organic Carbon Water Partitioning Coefficient), K_{ow} (Octanol-Water Partition Coefficient) and its affinity for lipids, CLD is persistent and accumulates in food webs (Cabidoche and Lesueur-Jannoyer, 2012; Clostre et al., 2013). Since Hammond et al. (1979) revealed that CLD can bind to estrogen receptors in rat, many studies investigated the endocrine effects of CLD in various vertebrates species (Curtis and Beyers, 1978; Donohoe and Curtis, 1996; Eroschenko, 1981; Guzelian, 1982) and in a few invertebrates species (Oberdörster and Cheek, 2001; Schimmel et al., 1979; Zou and Bonvillain, 2004). However, in aquatic ecosystems, endocrine effects of CLD were mainly studied in vertebrates and information about its effects on invertebrates is still limited, since previous studies mainly concerned the observation of morphological characteristics impacted by CLD. However, Legrand et al. (2016) highlighted the alteration of the expression of a panel of genes involved in reproduction, development and growth in the crustacean *Eurytemora affinis* after CLD exposure. Moreover, Gaume et al. (2014) underlined that CLD exposure triggers the induction of genes involved in the antioxidative defense (e.g. catalase and glutathione peroxydase), or in the biotransformation process (i.e. cytochrome P450 enzymes and glutathione-S-transferase) in *Macrobrachium rosenbergii*. Giusti et al. (2014) showed reduction of the oviposition and the fecundity of the gastropod *Lymnaea stagnalis* exposed to CLD at environmental relevant concentrations.

Finally, we observed in a previous study that CLD exposure of the decapod *Macrobrachium rosenbergii* affected 20-hydroxyecdysone concentration (i.e. molting hormone), chitobiase activity (i.e. molting enzyme), vitellogenin (i.e. female-specific protein involved in the reproduction process) gene expression and vitellogenin receptor gene expression (Lafontaine et al., 2016a, 2016b). All these hormones and proteins are controlled by the endocrine system, suggesting that CLD could be an EDC in exposed invertebrates.

The present study aimed to investigate the variations of protein expressions in the detoxification tissue (i.e. the hepatopancreas) of *M. rosenbergii* exposed to environmental concentrations of CLD, using a "gel-free" proteomic approach. This decapod crustacean is one of the most important economic resource in Guadeloupe and can be considered as a good model for the wild *Macrobrachium spp.* living in freshwater ecosystems in these regions. Results could improve our understanding of the toxic action of chlordecone, and allow the identification of proteins useful in the development of biomarkers of endocrine disruption disturbing the reproduction and the population fitness.

2. Materials and methods

2.1. Tested organisms

Post-larvae of *M. rosenbergii* (approximately 2 g, 1.4 cm cephalothorax length, sexually immature) were provided by an aquaculture farm (OCEAN-SA) located at Pointe-Noire (Guadeloupe, FWI) in a geographic area free of CLD contamination. Before the exposure experiment, pretests were carried out to evaluate the CLD concentration in tissues of prawns from Pointe-Noire and results have showed no contamination (concentrations below the limit of detection) (data not shown). Prawns used for the proteomic experiment were transferred to the laboratory (UMR BOREA, University of the French West Indies, Guadeloupe), and acclimated for one week in glass aquaria filled with 28 L of tap water prefiltered through activated carbon. Aquaria were under constant aeration with a 12 h light / dark photoperiod.

During acclimation, prawns were fed once daily with one artificial shrimp pellet per individual (complete food for rearing, Le Gouessant, France). A

constant water temperature of 27.6 ± 0.2°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 about 96 % throughout the experiment, whatever the conditions.

2.2. Experimental design

A total of 16 post-larvae of *M. rosenbergii* were sampled (i.e. 4 prawns per condition) after a 30 days-exposure at three CLD concentrations, i.e. 0.2 µg L^{-1}, 2 µg L^{-1} and 20 µg L^{-1}, which were chosen for their environmental relevance in surface water in Guadeloupe. Indeed, according to the Guadeloupean DIREN (Regional Department for the Environment), Guadeloupean rivers are contaminated by CLD at concentrations that ranged from 0.2 to 4 µg L^{-1} with a maximum of 8.6 µg L^{-1} measured in the River Grande Anse in 2003 (GREPP, 2004; InVS-Inserm, 2009). A "solvent control" consisting of tap water prefiltered through activated carbon spiked with 56 µL of acetone, was run. During the 30 days of exposure, *M. rosenbergii* were fed daily with one artificial shrimp pellet per individual (complete food for rearing, Le Gouessant, France). Exposure media were renewed every 96 hours in order to maintain the CLD concentrations constant. At the end of the 30 days of exposure, prawns were sampled, and immediately frozen in liquid nitrogen and stored at -80 °C until proteomic analysis.

2.3. Proteomic analysis

2.3.1. Extraction of protein fractions

The samples (i.e. hepatopancreas) were solubilized in Tris HCl 10 mM, pH 7.4, SDS 4%, 0.5 µL of Protease Inhibitor Cocktail EDTA-free and DNAse. The samples were sonicated twice for 30 seconds, and homogenized by vortex for 30 minutes at room temperature, before storage overnight at 4°C. The protein concentration of each sample was quantified using a *RC DC*™ Protein Assay Kit (Biorad, USA).

The samples were reduced and alkylated before applying the 2D Clean-Up kit (GE Healthcare Life Sciences, USA) according to the manufacturer's recommendations, in order to eliminate impurities not compatible with mass

spectrometry analysis. After the washing steps, protein pellets were solubilized in 50mM bicarbonate ammonium. Each sample was digested for 16h at 37°C in trypsin solution (ratio trypsin/total proteins (w:w) 1/50) and then, 3h at 37°C in another trypsin solution (ratio trypsin/total proteins (w:w) 1/100 in 80 % acetonitrile). After the digestion step, samples were resuspended in 0.1 % formic acid. For each sample, an aliquot corresponding to 3.5 µg of digested proteins was purified using a Zip-Tip (Billerica, USA) C18 High Capacity according to the manufacturer's recommendations.

Then, samples were evaporated to dryness in a speed vacuum. Peptides were conditioned at 2.86 µg in ammonium formiate 100 mM, with 150 fmoles in Yeast Alcohol Dehydrogenase (ADH, accession number P00330) per volume injected for the MassPREP protein digestion standard mixtures (MPDS, Waters corporation, USA). The internal standards spiked with MPDS mix 1 or MPDS mix 2, allow technical verification of the whole 2D-UPLC separation, MS^E data acquisition with additional ion mobility separation and PLGS identification process. ADH being present at ratio MPDS mix1/MPDS mix 2 = 1.

2.3.2. Samples analyses on a *nano*UPLC-SynaptTM HDMSTM G2 system

Protein samples were analysed using a *nano*Acquity UPLC© (Waters corporation, USA) separation system. This system uses a unique combination of two C18 chromatographic separation performed at different pH (i.e. pH = 10 and then pH = 3). Protein samples were then analysed with the Synapt™ HDMS™ G2 mass spectrometer (Waters corporation, UK) which uses an electrospray ionisation source (ESI) and allows sensitive detection (lower limit around 0.1 to 1 fmol of protein) with high resolution and high accuracy (within 10 ppm) for the analysed peptides. All the peptides are fragmented and their sequence and identity can be obtained after databases searches and correlation to the accurate mass measured for each parent peptide fragmented.

2.3.3. Identification of proteins and PLGS analysis

As the transcriptome of the crustacean decapod *M. rosenbergii* has not been sequenced yet, protein identification was performed by homology using ProteinLynx Global Server Software v2.5 (PLGS, Waters, USA) and the Crustacea database extracted from UNIPROT (www.uniprot.org). This database search also involves the search on a randomized database recomputed from the original database to evaluate the risk of false positive protein identification. For identification, the minimum to consider is at least two different peptides per protein identified and a control of the false-positive rate, which must be as low as possible (the false-positive rate will be of maximum 1 % because of the setting used in PLGS database search). Biological functions were obtained by using the UniProtKB section of the UniProt website (www.uniprot.org) and the AmiGO section of GeneOntology website (http://amigo1.geneontology.org).

2.3.4. Analysis of PLGS results

After samples analyses in LC-ESI-MS/MS^{E}, PLGS Software has been used to compare proteins identified in each condition two by two. Then, the expression levels of proteins have been normalized using the expression level of the standard protein alcohol dehydrogenase (ADH – see section 2.3.1). Results obtained were sorted according to three parameters. First, only proteins identified without ambiguity were selected. Then, only the proteins having a *p*-value lower than 0.05, and higher than 0.95 were retained. Indeed, a *p*-value is calculated by PLGS Expression E (Richardson et al., 2012), and values between 0 and 0.05 represent a 95% likelihood of under-expression, while a values between 0.95 and 1 indicate a 95% likelihood of over-expression. Finally, a 1.5-fold expression change were used as significant level (ratio of the protein amount less than 0.67 and greater than 1.5).

3. Results

3.1. Protein identification

This study was carried out to investigate the difference in protein expression of *M. rosenbergii* between control conditions (i.e. without pollutant stress) and stress conditions, i.e. exposure to CLD at three concentrations. "Shotgun" proteomic analysis of hepatopancreas of *M. rosenbergii*, allowed us to identify 120 proteins without ambiguity (i.e. false-positive rate less than 1 %) in all experimental conditions. Among these proteins, multiple comparisons of protein expressions revealed that the expression of 62 proteins were significantly different in exposed prawns compared to control, according to the 1.5-fold change criterion (Table 6.1). Results revealed that about 60 % of modified proteins (i.e. 38 proteins) were up-regulated ($0.95 \leq p\text{-value} \geq 1$) and about 40 % (i.e. 24 proteins) were down-regulated ($0 \leq p\text{-value} \geq 0.05$).

The Venn diagram shows the number of proteins whose abundance was significantly altered in each CLD exposure condition, compared to control (Figure 6.1). We observed that the higher the CLD exposure concentration, the higher the number of proteins impacted, which is in agreement with a dose-response relationship. Indeed, most proteins were impacted in prawns exposed to 2 µg L^{-1} and 20 µg L^{-1} of CLD. Indeed, most proteins were impacted in prawns exposed to 2 µg L^{-1} and 20 µg L^{-1} of CLD. Approximately 50% of the proteins altered following a CLD exposure were only disrupted by one concentration of CLD, compared to control. Only 1 and 4 proteins were simultaneously altered by two CLD concentrations, and 15 proteins were altered, whatever the CLD concentration of exposure.

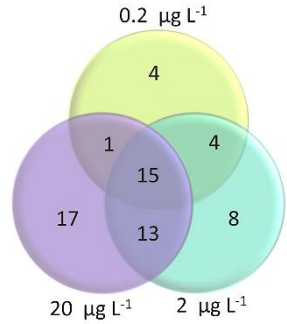

Figure 6.1 - Venn diagram of total proteins with at least 1.5-fold expression change and significantly identified in hepatopancreas of *M. rosenbergii* exposed for 30 days to the three chlordecone concentrations.

The identified proteins were functionally categorized based on the UniProtKB annotation of biological processes. The proteins differently expressed in exposed prawns compared to controls were involved in 9 biological functions, i.e. endocrine system, protein synthesis and degradation, cytoskeleton, ion transport, immunity and defenses, ATP metabolism, signal transduction and cell communication, carbon metabolism, glucose metabolism (Table 6.1, Figure 6.2). Figure 6.2 highlights five biochemical processes that comprised almost 80 % of the total of identified proteins impacted. Results showed that the most disrupted processes were the defense mechanisms and immunity, represented by 32 % of total altered proteins. Figure 6.2 also highlights that 15% of proteins whose abundance varied were involved in protein synthesis and degradation, 11 % in cytoskeleton and muscle movements, 10 % in glucose metabolism or ion transport and 6 % in the endocrine system. All other biological functions were represented by 5 % or less of modified proteins.

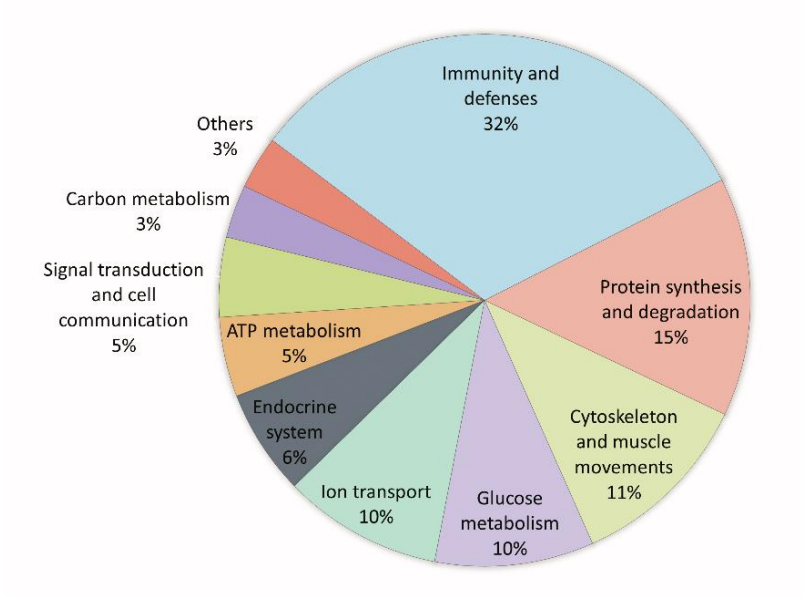

Figure 6.2 - Graphical view representing the percentage of proteins within each functional category as a function of the total protein number with at least 1.5-fold expression change and significantly identified in hepatopancreas of *M. rosenbergii* exposed to chlordecone.

Table 6.1 - Proteins with at least 1.5-fold changes, in at least one exposure condition, in hepatopancreas of *M. rosenbergii* exposed for 30 days at 0.2, 2 and 20 µg L^{-1} of CLD, and significantly identified ($p \leq 0.05$ for downregulated proteins, $p \geq 0.95$ for upregulated proteins) with the Uniprot Crustacea database. PLGS Score = Score of protein identification calculated by PLGS (high score means high confidence of identification).

	Upregulated protein name	**Accession N°**	**Taxonomy**	**PLGS score**	**Biological function**
1	14-3-3 protein epsilon	H9CWV0	*Scylla paramamosain*	25152	Signal transduction
2	70 kDa heat shock protein	D6BP38	*Palaemonetes varians*	3903	Immunity and defenses
4	ADP-ribosylation factor 1	C1BTJ2	*Salmon louse*	1910	Signal transduction
7	Alpha-spectrin	D0UN94	*Libinia emarginata*	1985	Cytoskeleton
8	Arginine kinase	E2JE77	*Macrobrachium rosenbergii*	25441	ATP metabolism
9	ATP synthase	D2CNK5	*Litopenaeus vannamei*	6645	ATP metabolism
10	Beta-1,3-glucan-binding protein	P81182	*Litopenaeus vannamei*	167	Immunity and defenses
11	Bip	J7K1E9	*Litopenaeus vannamei*	3456	Immunity and defenses
12	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	P35316	*Artemia franciscana*	2542	Ion transport
13	Calmodulin	B6DYD6	*Procambarus clarkii*	32493	Immunity and defenses
14	Calreticulin	E2DRF0	*Penaeus monodon*	7418	Protein synthesis and degradation
15	Calsequestrin	F8QXM4	*Scylla paramamosain*	2415	Ion transport
16	Catalase	H8XYP6	*Macrobrachium rosenbergii*	3373	Immunity and defenses
17	Cathepsin L	D7F2M6	*Palaemonetes varians*	23285	Immunity and defenses
18	Chitinase 3	H8YI19	*Pandalopsis japonica*	28637	Immunity and defenses
22	Cytochrome P450	H6UXP1	*Macrobrachium nipponense*	9886	Immunity and defenses
23	Elongation factor 2	I6VB26	*Scylla paramamosain*	9432	Protein synthesis and degradation
26	Enolase	I6P4W6	*Macrobrachium rosenbergii*	35490	Glucose metabolism
27	Farnesoic acid O-methyltransferase	A0PGI8	*Litopenaeus vannamei*	3174	Endocrine system
28	Ferritin	I1VWN8	*Macrobrachium rosenbergii*	11888	Immunity and defenses
30	Glucose-regulated protein 78	B8LF10	*Fenneropenaeus chinensis*	5292	Immunity and defenses
32	Glyceraldehyde 3-phosphate dehydrogenase	G3C6U6	*Hippa adactyla*	13148	Glucose metabolism

33	Heat shock protein 70	Q194W6	*Callinectes sapidus*	5394	Immunity and defenses
37	Lectin 1	I6W7T5	*Macrobrachium rosenbergii*	1569	Immunity and defenses
38	Lectin 2	I6V2P8	*Macrobrachium rosenbergii*	38288	Immunity and defenses
39	Lectin 3	I6W5B6	*Macrobrachium rosenbergii*	7194	Immunity and defenses
40	Lipopolysaccharide and beta-1,3-glucan binding protein	C7DZ96	*Macrobrachium rosenbergii*	29040	Immunity and defenses
45	Phosphoenolpyruvate-carboxykinase	Q86R97	*Neohelice granulata*	1187	Glucose metabolism
46	Prophenoloxidase	Q58HZ8	*Macrobrachium rosenbergii*	258	Immunity and defenses
47	Proteasome subunit alpha type	E9GIX9	*Daphnia pulex*	1174	Protein synthesis and degradation
48	Protein disulfide-isomerase	C0JBY4	*Litopenaeus vannamei*	7676	Immunity and defenses
50	Ribosomal protein	D7F2L2	*Palaemonetes varians*	4553	Protein synthesis and degradation
51	Sarcoplasmic calcium-binding protein 1	P05946	*Astacus leptodactylus*	21632	Ion transport
52	Sarcoplasmic/endoplasmic reticulum calcium ATPase	P86911	*Chionoecetes opilio*	3092	Ion transport
55	Superoxide dismutase [Cu-Zn]	Q45Q33	*Macrobrachium rosenbergii*	39780	Immunity and defenses
56	Transglutaminase	F1JZV5	*Macrobrachium rosenbergii*	345	Immunity and defenses
61	Vitellogenin	Q8ISB2	*Penaeus semisulcatus*	165	Endocrine system
62	V-type proton ATPase	D3PHZ2	*Lepeophtheirus salmonis*	352	Ion transport

	Downregulated protein name	**Accession N°**	**Taxonomy**	**PLGS score**	**Biological function**
3	Actin 1	O96657	*Penaeus monodon*	49838	Cytoskeleton
5	Alpha-1,4 glucan phosphorylase	E9G2G6	*Daphnia pulex*	4181	Glucose metabolism
6	Alpha-amylase	E9GXM0	*Daphnia pulex*	930	Carbon metabolism
19	Clathrin	D0UQ16	*Neogonodactylus oerstedii*	1524	Ion transport
20	Crustacyanin-like lipocalin	A4Z4V4	*Macrobrachium rosenbergii*	5561	Pigmentation
21	Crustin	B8LG64	*Macrobrachium rosenbergii*	2423	Immunity and defenses
24	Endo-1,4-beta-glucanase	Q1A366	*Macrobrachium lar*	6409	Carbon metabolism
25	Enhancer of split mbeta protein	C1BU45	*Lepeophtheirus salmonis*	1200	Protein synthesis and degradation
29	Gelsolin cytoplasmic	Q27319	*Homarus americanus*	899	Cytoskeleton
31	Glutamate dehydrogenase	Q0KHB4	*Litopenaeus vannamei*	5701	Protein synthesis and degradation

34	Hemocyanin	F5CEX2	*Macrobrachium nipponense*	17775	Oxygen transport
35	Histone H2A	D2DSH4	*Scylla paramamosain*	10552	Protein synthesis and degradation
36	Histone H3	I6P4G7	*Macrobrachium rosenbergii*	14244	Protein synthesis and degradation
41	Male reproductive tract specific Kazal type proteinase inhibitor	Q1EF71	*Macrobrachium rosenbergii*	49666	Endocrine system
42	Myosin heavy chain isoform 1	E9FZS9	*Daphnia pulex*	30703	Cytoskeleton
43	Na+/Ca2+-exchanger	Q8WPE2	*Porcellio scaber*	1109	Signal transduction
44	Pen a 1 allergen	Q3Y8M6	*Farfantepenaeus aztecus*	10021	Immunity and defenses
49	Pyruvate kinase	B1N690	*Litopenaeus vannamei*	3444	Glucose metabolism
53	Sodium/potassium-transporting ATPase subunit alpha	Q95PC2	*Pachygrapsus marmoratus*	8141	Ion transport
54	Sperm gelatinase	I6R3T3	*Macrobrachium nipponense*	5911	Endocrine system
57	Triosephosphate isomerase	K0E682	*Litopenaeus vannamei*	2895	Glucose metabolism
58	Tropomyosin	D3XNR9	*Macrobrachium rosenbergii*	16125	Cytoskeleton
59	Troponin	P05547	*Astacus leptodactylus*	22629	Cytoskeleton
60	ubiquitin	D7RF65	*Eriocheir sinensis*	12638	Protein synthesis and degradation

3.2. Comparative analysis of proteins identified and involved in hormonal process

As CLD is suspected to be an endocrine disruptor in invertebrates, and especially in *M. rosenbergii* (Lafontaine et al., 2016a, 2016b), we focused the analysis on altered proteins involved in the endocrine system. Four modified proteins involved in the signaling pathways of hormones were highlighted (Figure 6.3). Among these proteins, 3 were involved in the reproduction process, and 1 in the development process. The vitellogenin (Vg) protein was significantly up-regulated in prawns exposed to 20 µg L^{-1} of CLD. The male reproductive tract specific kazal type proteinase inhibitor (MRPINK) and the sperm gelatinase (MSG) were significantly down-regulated in prawns exposed to 2 and 20 µg L^{-1} of CLD. The farnesoic acid o-methyltransferase (FAMeT) was up-regulated in all the exposed prawns. However the up-regulation was only significant in prawns exposed to 20 µg L^{-1} of CLD.

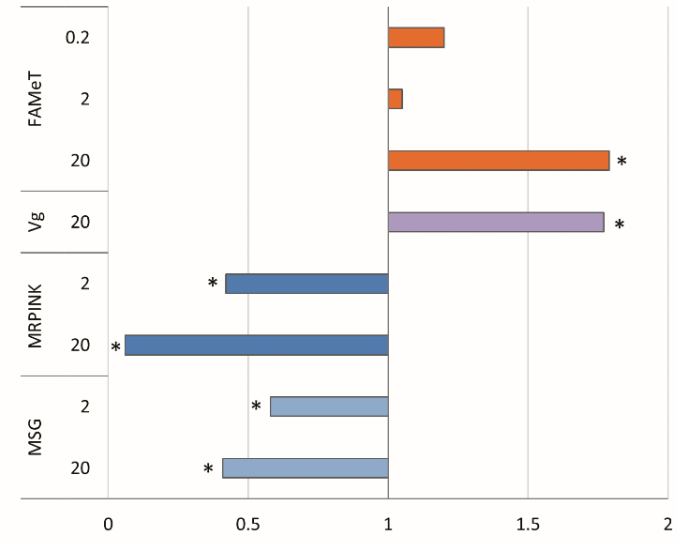

Figure 6.3 - The four proteins involved in hormonal process and whose abundance was modified in *M. rosenbergii* exposed to CLD as compared to controls. The three conditions of exposure were represented by the concentrations (i.e. 0.2 means 0.2 µg L^{-1}, 2 means 2 µg L^{-1}, 20 means 20 µg L^{-1}). Vg = Vitellogenin; MRPINK = Male reproductive tract specific kazal type proteinase inhibitor; MSG = Sperm gelatinase; FAMeT = Farnesoic acid o-methyltransferase. Asterisks represent a statistically significant difference compared to controls.

4. Discussion

As aquatic vertebrates, aquatic invertebrates are exposed to several EDCs (Kloas et al., 2009; Meyer-Reil and Köster, 2000; Sanchez et al., 2011). However, few studies have been conducted on these species to evaluate the impacts of EDCs (Sanchez et al., 2011) by using a proteomic analysis.

4.1. Global overview

The Venn diagram indicated a dose-response relationship since the number of modified proteins increased with CLD concentration of exposure. Results also showed that 10 biological processes were impacted by the three CLD exposures such as the stress response, cytoskeleton, protein synthesis and degradation of endocrine system. Most altered proteins were involved in immunity and defense processes (Table 6.1), including detoxification process, biotransformation process (cytochrome P450), stress (oxidative) response (Hsp70, Bip, calmodulin, transglutaminase), antioxidant mechanism (superoxide dismutase, catalase, ferritin) and immune system (lectins, chitinase, crustin, prophenoloxidase, protein disulfide-isomerase). Most of these proteins were up-regulated in response to presence of CLD and its toxicity in tissues of exposed prawns.

Xenobiotics are known to induce biotransformation process in exposed organisms (James and Boyle, 1998). Biotransformation is a complex process that involves phase I and phase II enzymes which transform endogenous compounds or xenobiotics in order to increase their water solubility, and thus facilitate their excretion by organisms (Koenig et al., 2012; Snyder, 1998). In crustaceans, this mechanism mainly occurs in the detoxifying organ, the hepatopancreas (James and Boyle, 1998; Snyder and Mulder, 2001). The increase of CYP450 enzyme expression observed in this study (n°22, Table 6.1), could be an induced response due to the CLD exposure as observed by Gaume et al. (2014) in *M.rosenbergii*. Indeed, cytochrome P450 monooxygenases (CYP450) are the main detoxification enzymes involved in the phase I of the biotransformation process, and one of the more widely used biomarkers for biochemical response pathways (Snyder and Mulder, 2001).

Moreover, exposure to xenobiotics and CYP450 activity may also lead to increased oxidative stress which induces an up-regulation of antioxidant enzymes to remove reactive oxygen species (ROS) produced during this oxidative stress (Leung et al., 2011). The ROS may cause damages to cell structure, proteins and nucleic acids. Among antioxidant enzymes, superoxide dismutase (SOD) and catalase play a key role in cell protection from ROS (Mahaffey et al., 1982; Rodríguez-Ortega et al., 2002). Therefore, the increase of SOD and catalase (n°55 and 16, Table 6.1) activity observed in the present work, could allow the elimination of ROS produced during a CLD exposure. This hypothesis is supported by the increase of catalase gene expression observed by Gaume et al. (2014) in *M. rosenbergii* exposed to CLD. Moreover, the increase of antioxidant enzyme activity in presence of CLD reflects the activation of defense mechanisms in exposed organisms to thwart ROS toxicity. The enzymes involved in the detoxification of xenobiotics, including biotransformation and antioxidant enzymes, were used as biomarkers of pesticide exposure in various aquatic species (Fercha et al., 2013; Gaume et al., 2014; Gismondi et al., 2012; Snyder and Mulder, 2001).

Among the proteins involved in immunity, the expression of cathepsin L was also up-regulated in exposed prawns, as compared to control. Cathepsin L is a proteolytic enzyme involved in protein degradation during immunity and digestion, but may also play a role in the uptake of vitellogenin during ovarian maturation and ecdysis in crustaceans, since this enzyme appears to be under the ecdysteroid control (Matsumoto et al., 1997; Qian et al., 2014; Zhao et al., 2013). The modification of its expression, due to CLD exposure, could impact the molting and the reproduction processes of exposed prawns. The CLD effect on these hormonally-dependent processes is developed below (see section 4.2).

Chlordecone is an organochlorine insecticide that acts by altering sodium channels, essential for the transmission of nerve impulses in organisms (Guzelian, 1982; Newhouse et al., 2009). Indeed, CLD was reported to produce neurotoxicity by inhibiting the Na^+/K^+-ATPase activity in fish (Desaiah and Koch, 1975) and rat (Bansal and Desaiah, 1985; Guzelian, 1982; Mishra et al., 1980). Our results showed the down-regulations of the Na^+/K^+-ATPase activity (n°53, Table 6.1) and the Na^+/Ca^{2+}-exchanger (n°43) in *M. rosenbergii* exposed to CLD.

The Na^+/K^+-ATPase play a key role in osmoregulation and regulation of membrane functioning. Its inhibition induces the increase of intracellular concentration of Na^+ by diffusion through the plasma membrane, following the concentration gradient (Guzelian, 1982). This increase of Na^+ inhibits the Na^+/Ca^{2+}-exchanger and the Ca^{2+} release from the cell, leading to an increase of Ca^{2+}. The increase of intracellular concentration of calcium activates the contractile proteins and causes convulsions which cause death of the target organism. Moreover, the increase of Ca^{2+} concentration in the cell, could activate the Ca^{2+} transfer into the lumen of the sarcoplasmic reticulum (Silvestre et al., 2010). This suggestion is supported by our results which showed up-regulation of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (n°12, 51 and 52, Table 6.1), calreticulin and calsequestrin (n°14 and 15, Table 6.1), which are proteins that sequester calcium in sarcoplasmic reticulum, rendering it inactive. As Ca^{2+} regulation is crucial for exoskeleton development of crustacean, its deregulation by CLD exposure could thus induce impairments in the molting process and development.

Results also showed an up-regulation of the enzyme ATP synthase and arginine kinase (n°8 and 9, Table 6.1) which could be the consequence of the activation of proton pumps, which require energy. Indeed, the up-regulation of ATP synthase and arginine kinase suggests that there is a significant energy mobilization in hepatopancreas of *M. rosenbergii* exposed to CLD. Previous investigations of the EDC effects on invertebrate proteome have underlined an increase of arginine kinase, e.g. in *Porcellio scaber* exposed to bisphenol A and vinclozolin (Lemos et al., 2010) or in *Gammarus pulex* exposed to polybromodiphenyl ethers (Gismondi et al., 2015). Environmental stress may cause impairments in protein expressions and, in proteomic analysis, some proteins appear to be systematically deregulated after exposure to chemicals such as elongation factors and cytoskeleton constitutive proteins (Leroy et al., 2010). Indeed, CLD had an impact on the expression of the elongation factor 2, alpha spectrin, actin, myosin, tropomyosin or troponin which could affect the protein synthesis and cellular structure (Bourchookarn et al., 2008; Leroy et al., 2010). Moreover, glucose metabolism was also found to be significantly modified in *M. rosenbergii*, following a CLD exposure.

Indeed, essential proteins and enzymes (enolase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, triosephosphate isomerase…) involved in glycolysis and glucogenesis were deregulated in prawns exposed to CLD. This impact of CLD on glucose levels has already been observed in mouse (Fujimori et al., 1983).

4.2. Altered proteins involved in reproduction and development processes

Chlordecone has been identified to have endocrine disruption effects on vertebrates (Donohoe and Curtis, 1996; Flouriot et al., 1996; Hammond et al., 1979), and invertebrates (Lafontaine et al., 2016a, 2016b). Our proteomic analysis underlined some deregulations of proteins hormonally-controlled and involved in reproduction or development processes.

4.2.1. Vitellogenin

The expression of vitellogenin (Vg) was significantly up-regulated in *M. rosenbergii* exposed to 20 µg L^{-1} of CLD compared to control. The presence of Vg in sexually immature prawns, as observed here, has already been reproted in the oriental river prawn, *Macrobrachium nipponense* and the glass prawn, *Palaemon elegans* (Bai et al., 2016; Sanders et al., 2005). Synthesized during the vitellogenesis in females, Vg is the extraovarian precursor of yolk proteins, called vitellins (Vn) (Jasmani et al., 2004; Matozzo et al., 2008; Sankhon et al., 1999). Vn, which is an intraovarian essential compound for embryonic and larval development, is formed from Vg during its sequestration by the oocytes (Jasmani et al., 2004; Tseng et al., 2001). In vertebrates, Vg protein is generally synthesized in response to endogenous estrogens and is under the control of the estrogen receptor pathway (Sumpter and Jobling, 1995). However, Thornton et al. (2003) suggested that ecdysozoans have lost the steroid receptor family, and Thomson et al. (2009) underlined the absence of receptors of the 3A group (estrogen receptor) and the 3C group (androgen, progestogen receptors) in *Daphnia pulex*.

In crustaceans, the Vg synthesis is regulated by several hormones: the VIH (vitellogenesis inhibiting hormone) secreted by the X-organ/sinus gland complex, the VSH (vitellogenesis stimulating hormone) secreted by the thoracic ganglion or the VSOH (vitellogenesis stimulating ovarian hormone) secreted by ovaries (Hyne, 2011; Kusk and Wollenberger, 2007; Rodriguez et al., 2007). Moreover, the molting hormone, 20-hydroxyecdyone (20-HE), which is secreted by the Y-organ under negative control of the MIH (molt inhibiting hormone) from the X-organ, is also involved in vitellogenesis (Rodriguez et al., 2007).

The increase of Vg in prawns exposed to CLD could be explained by the interaction of CLD with the signalling pathways of these hormones through the X-organ of *M. rosenbergii*, which is one of the main neuroendocrine organs in crustaceans. This hypothesis is in line with our previous study which demonstrated that Vg gene expression increased in *M. rosenbergii* exposed to environmental CLD concentration (Lafontaine et al., 2016b). Moreover, other investigations showed an increase of Vg gene expression in crustaceans exposed to EDCs (e.g. xenoestrogen compounds) (Billinghurst et al., 2000; Ghekiere et al., 2006; Huang and Chen, 2004; Huang et al., 2006; Oberdörster et al., 2000; Sanders et al., 2005). For example, in females of the shrimp *Neocaridina denticulata*, Vg gene expression was induced by exposure to chlordane at 0.001 and 0.01 µg L^{-1} (Huang et al., 2006). *In the same way,* Yano and Hoshino (2006) showed that Vg gene expression was significantly increased in ovaries of *Marsupenaeus japonicus* exposed to four concentrations of the estrogenic 17β-estradiol (i.e. 3.6, 36.7, 367 and 3671 nM) compared to controls.

The increase of Vg levels could cause some reproductive impairments such as gonad damages, oocyte production impairments, abnormal structure of oocytes, decrease of the reproductive capacity and alterations in endocrine functions (Huang et al., 2006; Jubeaux et al., 2012; Oetken et al., 2004).

4.2.2.Male reproductive tract specific kazal type protease inhibitor

Results revealed that the male reproductive tract specific kazal type protease inhibitor (MRPINK) and the sperm gelatinase (MSG) were significantly decreased in *M. rosenbergii* exposed to 2 and 20 µg L^{-1} of CLD compared to control. The MRPINK and MSG have been identified from adult *M. rosenbergii* (Cao et al., 2007; Li et al., 2008; Qian et al., 2012; Yang et al., 2013), and our results revealed that they are also present in sexually immature prawns. MRPINK is linked to the male prawn reproduction and plays an important role during the fertilization process, mainly in sperm-oocyte interactions (Cao et al., 2007; Li et al., 2008). Indeed, MRPINK was identified and characterized as having an inhibitory effect on both the gelatinolytic and proteolytic activities of prawn sperm (Li et al., 2009, 2008). These enzymes are involved in the degradation of the vitelline coat by sperm proteases, during the fertilization process, and are therefore necessary for sperm penetration of the egg envelope (Li et al., 2008; Vacquier, 1998).

Our results revealed a significant decrease of MRPINK in prawns exposed to 2 and 20 µg L^{-1} which may result in an increase of gelatinolytic and proteolytic activities of sperm. The disruption of the activity of sperm protease during spermatozoid penetration of the egg envelope could have an impact on reproductive capability. Moreover, MRPINK specifically inhibits the activity of *M. rosenbergii* sperm gelatinase (MSG) (Cao et al., 2007; Li et al., 2009, 2008; Qian et al., 2012). MSG is linked to the male reproductive tract and is also involved in the fertilization process, even if, its specific role is still unknown. The decrease of MRPINK and MSG in CLD-exposed prawns could prevent the normal progress of the reproduction and could have an impact on the population dynamics on the long-term.

4.2.3. Farnesoic acid o-methyltransferase

Finally, proteomic analysis highlighted an up-regulation of the farnesoic acid o-methyltransferase (FAMeT) which was increased in *M. rosenbergii* exposed to CLD, as compared to control. The FAMeT is a key enzyme involved in the conversion of farnesoic acid (FA) to methyl farnesoate (MF) (Duan et al., 2014; Li et al., 2013). MF, which is an analogue of the juvenile hormone of insects, is a crustacean hormone synthesized by the mandibulary organ and involved in regulation of several physiological processes such as growth, reproductive development, ovarian development or metamorphosis (Abdu et al., 1998; Chan et al., 2005; Chang, 1995; Duan et al., 2014; Li et al., 2013; Makkapan et al., 2011; Toyota et al., 2015). Therefore, FAMeT may play key roles in regulation of the reproduction and growth of crustaceans (Duan et al., 2014; Silva Gunawardene et al., 2002). The increase of the FAMeT protein, observed here, could lead to an increase of MF by higher FA conversion.

Previous studies showed that some insecticides have a methyl farnesoate agonist activity (Oda et al., 2005; Olmstead and Leblanc, 2002; Tatarazako et al., 2003; Wang et al., 2005). Moreover, several studies showed that changes in the MF levels appeared to stimulate or inhibit larval development and metamorphosis in exposed crustaceans resulting in the production of organisms with mixed larval and juvenile physical traits (Abdu et al., 1998; LeBlanc, 2007; Yamamoto et al., 1997). Furthermore, it was shown that high MF levels in ovigerous daphnids resulted in high male/female ration in offspring (Olmstead and Leblanc, 2002; Rider et al., 2005; Tatarazako et al., 2003), these studies allowed to determine the role of MF as the endogenous signal induced by environmental cues that triggers sexuality in daphnids. It has been demonstrated that MF may stimulate the synthesis and secretion of ecdysteroids from the Y-organ and regulate the Vg production and uptake, which is in accordance with the Vg up-regulation observed here (Chan et al., 2005; Chang et al., 1993; Mak et al., 2005; Nagaraju, 2007).

These proteins involved in hormonal control all play a key role in the regulation of the endocrine system. The modification of their expression could induce adverse effects on reproduction and/or development and thus impact the crustacean populations on the long term.

Indeed, our results suggest that CLD could induce a decrease of the efficiency of the sperm/oocyte interaction leading to impaired fertilization success and thus to lower egg production; CLD also induced an increase of FAMeT that could lead to an acceleration of growth. The identification of proteins disrupted by chemicals is essential to understand the mechanisms of action of xenobiotics and could also lead to the development of exposure biomarkers.

5. Conclusion

This study revealed that the proteome of *Macrobrachium rosenbergii* was influenced by CLD exposure. Several proteins were significantly up- or down-regulated in exposed prawns compared to controls. Most of these proteins are involved in the ion transport, defense mechanisms and immune system, cytoskeleton, or protein synthesis and degradation. Our results provide evidence that CLD does not target one single process in cells but that it may induce several toxic effects, including immunotoxicity, neurotoxicity, developmental toxicity and reproductive toxicity. Indeed, proteins involved in reproduction and development processes, also showed significant deregulation. As these proteins are closely linked with the hormonal system of crustaceans, our results indicate that CLD potentially disrupts the hormonal system of *M. rosenbergii* like endocrine disruptor compounds.

Although this proteomic analysis revealed the impact of CLD on the protein expression of *M. rosenbergii* , and notably on the regulation of proteins involved in endocrine functions of crustaceans, further investigations are needed to complete our understanding of toxic mechanisms of CLD on the proteome and the endocrine system of crustaceans, especially by focusing on hormonal pathways, such as the X/Y-organs, their receptors (e.g. ecdysteroid receptor), as well as the production of associated hormones (e.g. MIH, VIH).

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