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EFFECT OF HEAVY METAL TOXICITY ON CALANOID COPEPODS: EXPERIMENTAL APPROACH

Defended **by Esther Uzoma KADIENE on 8th July 2019, in Villeneuve d'Ascq (Lille, France)**

Committee members:

Pr. Hans-Uwe DAHMS, Kaohsiung Medical University, Taiwan	Reviewer
Dr. Isabella BUTTINO, ISPRA, Italy	Reviewer
Pr. Baghdad OUDDANE, Université de Lille, France	President
Pr. Hong-Yi GONG, National Taiwan Ocean University, Taiwan	Examiner
Pr. Michèle TACKX, Université Paul Sabatier - Toulouse 3, France	Examiner
Pr. Jiang-Shiou HWANG, National Taiwan Ocean University, Taiwan	Co-director
Pr. Sami SOUISSI, Université de Lille, France	Co-director

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ECOLE DOCTORALE-Sciences de la Matière, du Rayonnement et de l'Environnement

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EFFET DE LA TOXICITE DES METAUX TRACES SUR LES COPEPODES CALANOÏDES : APPROCHE EXPERIMENTALE

Soutenue par **Esther Uzoma KADIENE le 8 Juillet 2019, à Villeneuve d'Ascq (Lille, France)**

Devant le **jury composé de**:

Pr. Hans-Uwe DAHMS, Kaohsiung Medical University, Taiwan	Rapporteur
Dr. Isabella BUTTINO, ISPRA, Italy	Rapporteur
Pr. Baghdad OUDDANE, Université de Lille, France	Président
Pr. Hong-Yi GONG, National Taiwan Ocean University, Taiwan	Examinateur
Pr. Michèle TACKX, Université Paul Sabatier - Toulouse 3, France	Examinateur
Pr. Jiang-Shiou HWANG, National Taiwan Ocean University, Taiwan	Co-directeur
Pr. Sami SOUISSI, Université de Lille, France	Co-directeur

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Abstract

As a result of growing concern in the face of increasing environmental pollutants, several studies were carried out on copepod ecology. This thesis focused on biotic factors that affect metal toxicity of calanoid copepods.

Firstly, cadmium (Cd) toxicity between two copepods in the order; Calanoida; *Eurytemora affinis* (Poppe 1880) from a temperate region (Seine Estuary, France) and *Pseudodiaptomus annandalei* (Sewell 1919) from a subtropical region (Danshuei Estuary, Taiwan), were determined based on their sex and reproductive states. Results from this investigation revealed that both copepods have different levels of sensitivity to cadmium toxicity and also, their sensitivity to cadmium was significantly dependent on their sex and reproductive states. In addition, an investigation of cadmium toxicity on copepod life history traits was made using *P. annandalei* as a model species. Cd showed negative effects on growth, reproduction and lifespan of this copepod species. The results revealed further that Cd toxicity was also dependent on the developmental stages of the copepods.

To understand the reason for sex-specific sensitivity to cadmium, an investigation on the bioaccumulation of Cd was carried out. In the natural environments, copepods could bio accumulate metals either directly from the water or indirectly from consumed diets. Before, both routes of metal uptake were tested, some preliminary test was carried out on the rate at which copepod diet (microalgae) were accumulating Cd. Microalgae accumulation of Cd was tested by exposing *Diacronema lutheri* to Cd under different conditions (salinity and temperature). At the end, it was observed that increased temperature and low salinity influenced the uptake of Cd in the algae. *P. annandalei* was then exposed to Cd in water and through their diets, using information from above results. This investigation revealed that *P. annandalei* uptake of Cd was significantly more from water than from the diets.

A hypothesis was developed based on why copepod uptake of Cd from water was higher than from dietary Cd exposure. It was assumed that metal uptake from water is a more important route in the bioaccumulation of metals than through diet route because of

oral intake. This hypothesis was tested by exposing copepods to a dyed medium. By the aid of a microscope, the dyed medium was observed to have entered inside the gut of the copepod through the mouth opening. Oral intake of water by copepods was confirmed by a bioaccumulation test.

A molecular study on the transcriptomic assay and sex-specific differential expression of *P. annandalei* exposed to Cd was carried out. The results showed that *P. annandalei* responded to Cd toxicity in a sex-specific manner and that females were less sensitive to Cd than male copepods. Moreover, multigenerational exposure of *P. annandalei* to Cd showed a possible development of adaptation, particularly in female copepods. Although, copepods could develop adaptive mechanisms to tolerate toxic chemicals, an increasing concentration of metals in the aquatic environment in addition to maternal transfers of metals over several generations could increase metal concentrations in copepods. A long term exposure could reduce their fitness, thereby compromising copepod population structure.

This study showed that mortality, life history traits and molecular responses of model copepod species can provide important bio-indicators for environmental risk assessments.

Keywords: Cadmium, Toxicity, Calanoid copepod, Sex, Life history traits, Bio-accumulation, Oral intake of water, Transcriptome, Multigeneration.

Résumé

En raison des préoccupations croissantes concernant le devenir de l'écologie des copépodes face à l'augmentation des polluants environnementaux, plusieurs études sont en cours. Cette thèse cible les facteurs biotiques qui influent sur la toxicité des métaux chez les copépodes calanoïdes. Tout d'abord, la toxicité du cadmium (Cd) entre deux copépodes de l'ordre ; calanoida *Eurytemora affinis* (Poppe 1880) d'une région tempérée (estuaire de la Seine, France) et *Pseudodiaptomus annandalei* (Sewell 1919) d'une région subtropicale (estuaire de Danshuei, Taiwan), ont été déterminés sur la base de leur sexe et de leur état de reproduction. Les résultats de cette étude ont révélé que les deux copépodes avaient des niveaux de sensibilité différents à la toxicité du cadmium et que leur sensibilité au cadmium était également fortement dépendante de leur sexe et de leur état de reproduction. En outre, une étude de la toxicité du cadmium dans les traits de vie du copépode a été testée en utilisant *P. annandalei* comme espèce modèle. Les résultats ont révélé que la toxicité du cadmium était également dépendante du stade de développement des copépodes. De plus, le Cd a eu un effet négatif sur la croissance, la reproduction et la durée de vie du copépode. Afin de comprendre la raison de la sensibilité au cadmium spécifique au sexe, la bioaccumulation du cadmium chez les deux sexes a été réalisée. De plus, dans les environnements naturels, les copépodes peuvent bioaccumuler les métaux via une voie directe dans l'eau ou via les aliments consommés. Dans un premier temps, des essais préliminaires ont été effectués pour identifier la cinétique de bioaccumulation des méatux chez les micro-algues utlisées pour nourrir les copepods. L'accumulation du Cd dans les microalgues a été testée en exposant *Diacronema lutheri* dans différentes conditions de salinité et température. À la fin, il a été observé que l'augmentation de la température et la faible salinité favorisent l'absorption de Cd par les micro-algues. *P. annandalei* a ensuite été exposé au cadmium dans l'eau et par le biais de son régime alimentaire, en utilisant les informations fournies par les résultats ci-dessus. Cette expérience a révélé que l'absorption de Cd par *P. annandalei* provenait nettement plus d'eau que de la nourriture. Une hypothèse a été développée sur les raisons pour lesquelles l'absorption de Cd par l'eau par les copépodes était plus élevée que par l'exposition par Cd via l'aliment. En d'autres termes,

l’absorption de métaux par l’eau est une voie plus importante dans la bioaccumulation des métaux que par la voie alimentaire en raison de l’absorption orale. L'hypothèse a été démontrée en exposant les copépodes à un colorant alimentaire et, à l'aide d'un microscope, il a été constaté que le colorant était entré dans l'intestin du copépode par l'ouverture de la bouche. L'absorption orale d'eau par le copépode a été confirmée par un test de bioaccumulation. Une étude moléculaire sur le test transcriptomique et l'expression différentielle spécifique au sexe du copépode de *P. annandalei* exposé au Cd a été réalisée. Les résultats ont montré que les copépodes de *P. annandalei* répondaient à la toxicité du cadmium de manière spécifique au sexe, et expliquaient pourquoi la femelle était moins sensible au cadmium que les copépodes mâles. De plus, l'exposition multigénérationnelle de *P. annandalei* au cadmium a montré un développement possible de l'adaptation, en particulier chez les copépodes femelles. Bien que les copépodes puissent développer des mécanismes adaptatifs pour tolérer les produits chimiques toxiques, une concentration croissante de métaux dans le milieu aquatique ainsi que des transferts de métaux par la mère sur plusieurs générations pourraient augmenter la concentration de copépodes. Une exposition à long terme pourrait réduire leur forme, compromettant ainsi la structure de la population de copépodes. Cette étude a montré que la mortalité, les traits d'histoire de vie et les réponses moléculaires des espèces modèles peuvent fournir d'importants bioindicateurs pour l'évaluation des risques environnementaux.

Mots clés : Cadmium, Toxicité, Copépode calanoïde, Sexe, Traits d'histoire de vie, Bioaccumulation, Absorption orale d'eau, Transcriptome, Multigénération.

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Chapter 1. General introduction

1.1. Research background

Trace metals such as copper, cadmium, lead, or zinc are increasingly contaminating marine, brackish, and freshwater environments. The flow of contaminants into estuaries mostly comes from intra-estuarine tributaries, remobilization of stored contaminants in the environments, including direct discharges from industries (Fisson, 2015; Moshenberg, 2013). The Danshui estuary in North-Eastern Taiwan experiences high influx of untreated domestic discharge and both treated and untreated industrial discharges from its tributaries and it is heavily polluted with trace metals and their concentrations vary with location and seasons (Fang and Lin, 2002; Fang et al., 2006; 2014; Jeng and Han, 1994; Jiann et al., 2014). Metals, such as copper and zinc, can be functionally essential at low concentrations. However, these metals can become toxic when they exceed a particular threshold concentration in the water. The rate of metal concentrations in the environment and the level of toxicity to organisms among other factors are affected by the geochemical behaviour of metals, chemical speciation, the presence of other toxicants or environmental conditions and the physiology and condition of the organism (Ansari et al., 2004). Metals cannot be broken down in the environment, but their transfer, bioavailability, or toxicity are affected by conditions such as low pH, low hardness, low suspended matter level, high redox potential, low salinity (Cole et al., 1999), and temperature (Boeckman and Bidwell, 2006; Khan et al., 2006; 2007).

Cadmium is widely distributed in the Earth's crust at an average concentration of approximately 0.1 mg/kg, and it enters the aquatic environment through geological or anthropogenic activities (FAO/BOBP, 1999; Tchounwou et al., 2012). Increasing influx of this metal from geological or anthropogenic activities increases its concentration in aquatic environment (Neff, 2002). Although, cadmium has no vital or biological benefits on organisms (Chang et al., 1996; Singh et al., 2011), however, for certain organisms or under certain conditions, it can be beneficial (Lane et al., 2005). Concentrations of toxic chemicals in the tissue of marine organisms are often in equilibrium with the natural concentrations in seawater. Which means the tissue of marine organisms contains natural

background concentrations of many natural occurring chemicals such as most metals which may not be toxic.

Phytoplankton and zooplankton are important primary and secondary producers in the aquatic food web, the threats they face from metal toxicity shown by numerous studies is of ecological concern. Phytoplankton production can be affected by pollution from heavy metals resulting in a decrease of biomass yield (Napan et al., 2015) and they can also transfer metals along the different trophic levels.

1.2. Copepod species of interest

Copepods are essential trophic links among higher aquatic organisms; thus, they mediate in the bio-accumulation and bio-magnification processes of toxic pollutants in the aquatic food webs (Fisher et al., 2000; Watras et al., 1998). The productivity and abundance of copepods face threats from environmental pollutants. However, toxicity of metals (which is the degree to which a metal can induce harm) to copepods varies because the rates of uptake, excretion, and detoxification are inter- and intra-species as well as metal specific (Fang et al., 2014; Hsiao et al., 2010; Luoma and Rainbow, 2005; Ritterhoff and Zauke, 1997) and can depend on their life stage and/or sex.

One of the prevalent estuarine zooplanktons is the calanoid copepod *Eurytemora affinis* Poppe 1880, which is a dominant species in the zooplankton community of temperate regions, Northern European and North American estuaries (David et al., 2005; Quintin, 2013; Winkler et al., 2011). Another calanoid copepod of interest is *Pseudodiaptomus annandalei* Sewell 1919, a dominant euryhaline species found perennially in coastal and estuarine ecosystems in the tropical and subtropical Indo-Pacific region. *P. annandalei* is a relatively abundant and dominant brackish water species found in the Danshui estuary in Northern Taiwan; this species is also abundantly present in aquaculture ponds, where they are essential live feed organisms for fish larvae (Chen et al., 2006; Doi et al., 1997; Golez et al., 2004; Hwang et al., 2010a; Liao et al., 2001; Sarkar et al., 1985).

E. affinis often exhibits different traits from distinct populations or environments. These differences are believed to be genetic, resulting from an evolutional history, to

phenotypic plasticity or from acclimation to culture conditions (Souissi et al., 2016), and this is reflected in their different levels of responses or sensitivity to toxic pollutants. *E. affinis* and *P. annandalei* share a number of similarities (Fig. 1.1.) such as their ability to be cultured in laboratory conditions; they both exhibit sexual dimorphism and optimum culture conditions including culture medium of salinity 15 to 20 for both species and a temperature range of 10 to 18°C and 25-30°C for *E. affinis* and *P. annandalei* respectively. Both species have similar mating behaviour, where the male seizes a female by her posterior abdomen using his geniculate right antennule (Beyrend-Dur et al., 2011; Katona, 1975). Their females are both egg carriers; *E. affinis* female have a single large egg sac, the number of egg production varies but can reach more than 30 eggs/female (in optimum conditions) (Devreker et al., 2009). Whereas, *P. annandalei* have a pair of oval-shaped egg sacs situated on each side of the female urosome. Each sac can contain about 4-14 eggs (Golez et al., 2004). Both copepod species can produce a second pair of viable clutches with high hatching success after a single mating, and even a second clutch (Beyrend-Dur et al. 2011; Devreker et al 2009). Clutch size varies widely with density, temperature and age of female (Devreker et al., 2009; Su et al., 2005). For example, maximum density of all stages of *P. annandalei* culture is around 5 individuals/mL (Su et al., 2005). Both species have three developmental phases, the naupliar stages (N1-N6), the copepodid stages (C1-C5) and the adult stage (C6). Development time varies in both species and usually depends on environmental conditions (Beyrend-Dur et al., 2011; Devreker et al., 2007). *P. annandalei* have higher production rate than *E. affinis* because of their shorter embryonic development and latency time (Beyrend-Dur et al., 2011; Su et al., 2005). The male and female morphology of both copepod species are structurally similar, the female body size in both species is larger than their males but both species have very similar body size.

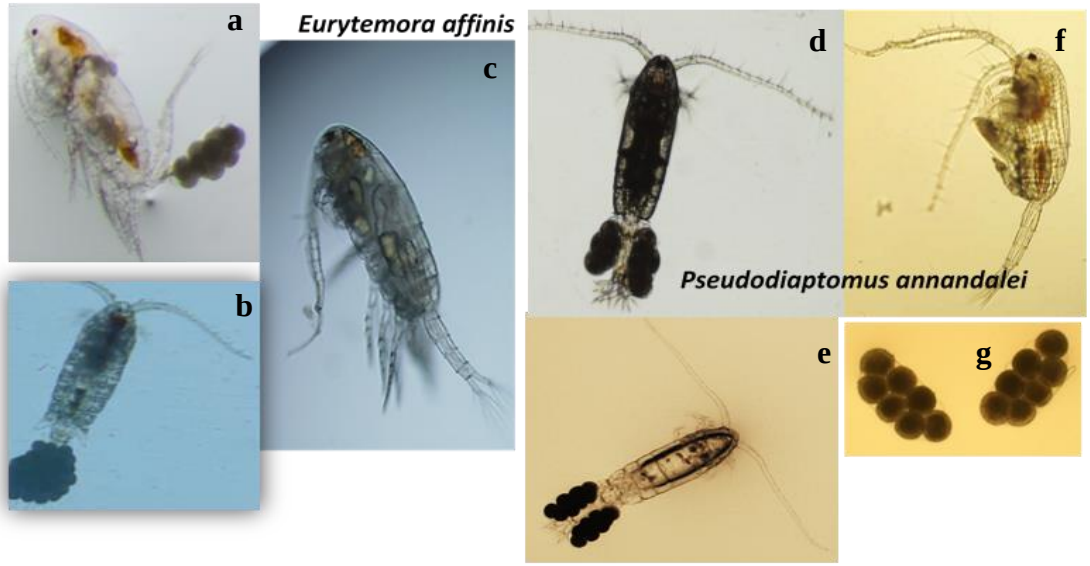

Fig. 1.1. *Eurytemora affinis* ovigerous female (a and b) and male (c). *Pseudodiaptomus annandalei* ovigerous females (d and e), male (f) and a pair of egg sacs (g).

1.3. Metal toxicity in copepods

The biological features of copepod such as availability, relevance, ease of handling, cultivability, sensitivity, abundance, size (Cutts, 2003; Devreker et al., 2004; Souissi et al., 1997; 2010; Wang et al., 2014; Zhang and Uhlig, 1993) makes them suitable candidates as reference species in aquatic toxicology. *P. annandalei* and *E. affinis* exhibit sexual dimorphism (Beyrend-Dur et al., 2011; Katona, 1975), this make it possible for investigation on sex-specific toxicity to chemicals to be carried out. Their fast growth and short life cycle makes them suitable for toxicity testing of their life history traits and multigenerational studies of chemical toxicity.

The deleterious effect of heavy metals in the aquatic environment has been well documented. Metal contaminations in estuaries can negatively affect the population densities of copepods (Van Damme et al., 1984). Bioaccumulation and bioavailability of metals can be controlled by factors such as the organism's physiology and behaviour (Simpson and Batley, 2007). Bioaccumulation can be influenced by physio-chemical changes. For example, although metal accumulation is barely influenced by pH. However, acidic pH favours cadmium accumulation by algae (Jennett et al., 1983). Cadmium exist

in natural waters as Cd^{2+}, CdOH, and $Cd(OH)_2$ depending on pH and Eh (Weber and Posselt, 1974). Bioaccumulation of metals by marine organisms involves interactions between aqueous speciation of the metal and the biochemistry of the organism (Simkiss and Taylor, 1989).

The bioaccumulation of chemicals from food is called trophic transfer. A process whereby chemicals are passed through a food chain or food web by trophic transfer, reaches increasingly higher amount in the tissue of animals at each trophic level is refer to as bio-magnification (Neff, 2002).

Dietary toxicity is dependent upon the organism and life stage that are exposed, as well as the diet type, form of the metal, and the daily dose received. Metals in the lumen of the gut have the potential to alter digestive processes and nutrient uptake without direct interaction with the gut epithelia. These effects can include disruption in the activity of digestive enzymes and intestinal microorganisms, changes in mucus secretion rate, interference with neuroendocrine functions that impact gut motility and hormone secretion, and direct effects on hormones and nutrient absorption processes (McGeer et al., 2004). The mechanism involved in the uptake of contaminants by microalgae generally involves rapid physico-chemical adsorption and ion exchange processes that occur at the cell surface, and is metabolism independent and a slow active absorption, accumulation and degradation. (Arias et al., 2017).

In the dissolved phase, metals can exist as free ions as well as in a variety of complexed forms. For many metals in aquatic systems it is the free ionic form which is believed to be responsible for toxicity. Dissolved cations such as Na^+, K^+, Ca^{2+}, and Mg^{2+} can competitively inhibit metal uptake (Paquin et al., 2002). Direct uptake of metals from water occurs by either adsorption onto cell or organism surfaces, or absorption across cell walls or body surfaces such as the gill and/or gut. Assimilation of cadmium from water is about 0.18 to 0.35 percent (Wang et al., 1996; Wang and fisher, 1997). *Acartia tonsa* assimilate 40 percent of the cadmium through their phytoplankton food and more than 50 in their tissue is from the dissolved phase in the ambient water (Fisher et al., 2000). Other studies have shown similar results of metal accumulation from water to be higher than from food

(Borchardt, 1983; Riisgard et al., 1987). Cadmium accumulated from water is retained primarily in the gills and kidney, whereas cadmium from food is retained primarily in the kidney, gut and liver in fish (Neff, J. M., 2002). The rate of uptake of cadmium across the gill of marine invertebrates is inversely proportional to the concentration of calcium in the ambient water, suggesting that calcium and cadmium are competing for an active uptake site through the inhibition of Ca^{2+} influx (Reid and McDonald, 1988; Verbost et al., 1989). However, most cadmium uptake appears to be passive, through diffusion across biological membranes, or by adsorption of cadmium to the surface of external epithelia (Carpene and George, 1981; Clausen et al., 1993; Sidoumou et al., 1997). The uptake rate from water generally decreases until a steady state is reached between the metal in the water and in organism tissues (McGeer et al., 2004).

Metal becomes toxic when the bioaccumulated amount exceeds that which the organism can tolerate, however, some studies show that the change in toxicity is not consistently related to bioaccumulation. In order for metal to cause toxicity, an interaction at a site of toxic action occurs following a disruption in normal processes (McGeer et al., 2004). A chemical must be in a bioavailable form that can move through or bind to surface coatings (skin, gill or gut epithelium, cell membrane, cuticle) of an organism (Neff, 2002). The effect of cadmium exposure to copepods can be acute or chronic, depending on concentrations and duration of exposure. Acute exposure to 300 to 400ug/L dissolved cadmium as $CdCl_2$ or chronic exposure to 50 to 100µg/L has been reported to cause very high mortalities in some species. Under chronic exposure, some marine species have been shown to exhibit growth reduction, depressed respiration, molt inhibition, shortened life span, altered enzyme activity and abnormal muscular contraction in the range of 0.5 to 10 µg/L (Eisler, 1985).

Copepods accumulate metals by assimilating them from their food or by absorbing them from water (Wang and Fisher 1998) and the uptake pathway can determine its internal distribution (Hare et al 1991; Wang and fisher 1998). Hook and Fisher (2001), showed that copepod exposed to dissolved metal resulted in metal deposition in the exoskeleton and exposure to dietary metal resulted in metal deposition in internal tissues. Indicating that

enrichment of metal concentration in internal tissues, by dietary metal affects vitellogenesis (the process of yolk accumulation). Tissue burdens tend to vary widely on a seasonal basis due to differences in growth rate, body composition, sexual condition, nutrition, salinity and temperature (Amiard et al., 1986; Coleman et al., 1986; Coimbra and Carraca, 1990; Swaileh and Adelung, 1994). Growth may partially or completely dilute the net accumulation of metals over time, so that metal concentration in tissues increase gradually, remain the same, or even decrease gradually during long-term exposure to metals under natural environmental conditions (Bryan, 1979; Fischer, 1988; Lytle and Lytle, 1990; Jorgensen and Pedersen, 1994).

Promising biomarkers are being developed in the area of genomics, gene expression, and proteomics. These techniques are used to measure real-time changes in specific messenger RNA. The underlying concept is that exposure to a particular chemical (e.g., Cd) will result in the transcription of messenger RNA sequences specific to proteins involved in the metabolism and/or detoxification of Cd. This may provide a chemical specific "fingerprint" for the type and quantity of chemical involved in the exposure (McGeer et al., 2004), and further physiological changes as a result of continuous exposures and the potential alterations in copepod community structure as a consequence.

1.4. General objectives

The aim of this study was to investigate the toxic effects of acute and chronic exposure of metal in individual and cohort copepods. To investigate the level of toxicity of metal based on factors such as developmental stage, sex and reproductive stages, in order to evaluate the impact on the growth, survival, reproduction and ultimately, population structure of copepod in a long-term.

1.5. Large scale copepod cultures

E affinis (Poppe, 1880) used in this study was cultured at the LOG-Marine Station of Wimereux, France. The strain of *E. affinis* was first collected and isolated from the Seine estuary at Tancarville Bridge, France, and then acclimated in the laboratory following the

protocol described by Souissi et al. (2016). *P. annandalei* Sewell 1919 used in this study was cultured in the laboratory of the Institute of Marine Biology, National Taiwan Ocean University, Taiwan. The strain was initially collected from the coastal brackish ponds in Tungkang, Southern Taiwan, and maintained in the laboratory. The *E. affinis* and *P. annandalei* strains were maintained in 2-L beakers in a laboratory incubator at 18 and 26 °C, respectively, under a light regime of 12-h light and 12-h dark. We used the protocol described by Souissi et al. (2010, 2016) and (Beyrend-Dur et al., 2013; Devreker et al., 2009) to maintain the copepods throughout generations. The copepods used in the experiments were provided from the large-scale culture systems. The seawater used for the *E. affinis* culture was pumped from the English Channel near the Wimereux marine station and filtered several times up to 1-µm. The seawater used for the *P. annandalei* culture was pumped from the ocean near the National Taiwan Ocean University (Northern Taiwan) and filtered up to 1-µm. *E. affinis* copepods were fed a microalgae mixture of *Isochrysis galbana* and *Rhodomonas baltica* or *Rhodomonas salina* cultivated in the Conway medium, and *P. annandalei* copepods were fed a mixture of *I. galbana* and *Nannochloropsis oculata* or *Diacronema lutheri* cultivated in the Conway medium, following the method described by Sadovskaya et al. (2014). The water used for both the large-stock copepod species culture and the experiment was diluted with distilled water to obtain salinity 15.

1.6. Algae culture

The algal strains used for experiments includes *Isochrysis galbana*, *Rhodomonas salina* and *Diacronema lutheri*. They were maintained in the laboratory in 250 mL Erlenmeyer flasks. Large volume needed for each study were cultured in 2 L flasks with autoclaved seawater of salinity 35 and enriched with Conway medium. Cultures were aerated with sterile air. The cultures were kept in a laboratory incubator at 18 °C (in France) and 25 °C (in Taiwan) and a photoperiod of 12L:12D cycle under a fluorescent light with an intensity of about 2500 lux. Conway medium composed of: 100 mg $NaNO_3$, 20 mg NaH_2PO_4, 45 mg Na_2EDTA, 33.6 mg H_3BO_3, 0.36 mg $MnCl_2$, 1.3 mg $FeCl_3$, 0.021 mg $ZnCl_2$, 0.02 mg $CoCl_2 \cdot 6H_2O$, 0.02 mg $CuSO_4 \cdot 5H_2O$, 0.09 mg $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.2 mg thiamine HCl

(vit. B1) and 0.01 mg cyanocobalamin (vit. B12) per litre of autoclaved seawater. The algae cultures used in the experiments were harvested at an advanced exponential growth phase with cell densities > 12 x 10^6 cells mL^{-1}

Chapter 2.

Differences in lethal response between male and female calanoid copepods and life cycle traits to cadmium toxicity

Kadiene, E. U., Bialais, C., Ouddane, B., Hwang, J. S., & Souissi, S. (2017). Differences in lethal response between male and female calanoid copepods and life cycle traits to cadmium toxicity. Ecotoxicology, 26(9), 1227-1239.

2.1. Introduction

The median lethal concentration is the concentration of cadmium that will kill 50% of a population exposed to it. The study of median lethal concentration of a heavy metal pollutant like cadmium involves a 96-hour acute exposure of the species to different concentration of the metals. Several studies have shown that LC50 of cadmium varies between species and their life stages. Forget et al. (1998), assessed the toxicity of cadmium using short-term 96-h semistatic bioassays to establish the LC50 values for all life stages, and their results showed cadmium to be very toxic to the copepod *Tigriopus brevicornis* and the LC50 value for the nauplius stage was 17.4 µg liter-1, copepodid stage was 29.7 µg liter-1 and that of adult stage (ovigerous female) was 47.9 µg liter-1. This showed that the nauplius stage were more sensitive to cadmium than the adult stage, perhaps this could be as a result of the adult developed ability to detoxify or excrete the contaminant or the sensitivity level might be related to the size of the adult stage compared to the nauplius stage. Moreover, Sullivan et al. (1983) showed that the 96-h LC50 of cadmium was >120 µg liter-1 for nauplii of the estuarine copepod *Eurytemora affinis*. This difference in LC50 of naupliar to cadmium toxicity shows that different copepod species possess different tolerance level and ability to detoxify of excrete cadmium.

Laboratory analyses of the lethal concentrations (LC) of heavy metals in aquatic organisms have revealed their quantitative responses and sensitivity levels, providing guidelines for the standardization and regulation of heavy metal influx into the aquatic environment. Several studies have highlighted the critical ecological effect of lethal and sublethal concentrations of toxic metals. However, very few studies have attempted to elucidate the responses of copepods to toxic metals based on their life stage or reproductive state. Sex can be a factor in how copepods respond to stressors (Dipinto et al., 1993; Sornom et al., 2010; Sroda et al., 2011). Michalec et al. (2013), examined the effect of sublethal concentrations of three water pollutants, including cadmium, on the swimming behaviour of *E. affinis* and reported an increase in the swimming speed and activities of adult copepods, as an escape mechanism from the pollutants, and this hyperactivity was higher in males than in females. Male and female copepods may respond differently when

exposed to unfavourable conditions. Therefore, investigating sex differences in copepods' responses to environmental pollutants is crucial to understand changes in their population. The sensitivity of copepods to heavy metals, particularly cadmium, varies with species (Marcus, 2004) and their reproductive life stage (Hsiao and Fang, 2013).

The literature regarding the sex-specific sensitivity to heavy metals in both copepods, *P. annandalei* (Chen, 2011; Jiang et al., 2012; 2013) and *E. affinis* (Cripe and Cripe, 1990; Hall et al., 1995; Sullivan et al., 1983) is scant. Moreover, these studies have focused on the late developmental stages of copepods with little or no consideration of the sex-based sensitivity to heavy metal toxicity. Therefore, the current study investigated the sex-specific and female reproductive stage and life cycle trait responses to cadmium toxicity of *E. affinis* and *P. annandalei*.

2.2. Materials and Methods

2.2.2. Differences in the responses of male and female copepod species to cadmium

The 50% LC (LC50) of cadmium was determined for the males and females of *E. affinis* and *P. annandalei*. Various concentrations of cadmium (40, 80, 150, 220, and 360 µg/L) were prepared in 100-mL beakers for a total of six treatments including the control (0 µg/L Cd). All treatments were prepared in triplicate. After preparing the media, 25 males and 25 females were identified under a stereomicroscope and separated into their respective pre-labelled beakers. The beakers were covered with an aluminium foil and kept in the incubator at 18 °C (*E. affinis*) and 26 °C (*P. annandalei*) under a light regime of 12 h light and 12 h dark with no feeding and no aeration during the 96 h exposure. Dead copepods were identified under a stereomicroscope every 24 hours; they were identified as those that were not moving for few seconds and by further touching them gently with a very fine and tiny glass tip to stimulate movement. If there was still no movement, the copepods were considered dead, recorded, and discarded.

2.2.3. Toxicity difference between the reproductive stages of female copepod species to cadmium

Ovigerous female (OVF) and nonovigerous female (NOF) *E. affinis* and *P. annandalei* copepods were separately tested with or without exposure to the sublethal cadmium concentration (40 µg/L). Similar environmental conditions were provided, and mortality observations were made as described in Experiment 1.

2.2.4. Toxicity effect of cadmium on the life cycle traits of *E. affinis* and *P. annandalei* copepods

Both species were exposed to cadmium concentrations lower than their respective female 96 h LC50 values from Table 1. The treatment culture medium included 40µg/L (for *E. affinis*) and 160µg/L (for *P. annandalei*) of cadmium and the control without cadmium in triplicates. Ovigerous (n=20) females of both species were randomly sorted from a batch culture and transferred to a 200µm mesh false bottom suspended in 2-liter beakers containing the aerated culture medium. *E. affinis* and *P. annandalei* were kept in their temperature-controlled environments. We used the same protocol as described in Souissi et al. (2010, 2016). Females were incubated until nauplii were hatching. Later the females were removed and their nauplii were allowed to develop to adults. They were fed every 2 days with 10 ml of red microalgae *Rhodomonas sp.* and *Isochysis galbana* (Tiso) (~ 5000 cells mL^{-1}) at its exponential growth phase. The culture water was changed once the nauplii reached copepodid stage. Algae (10 mL) were centrifuged and the supernatant was discarded and re-suspended with the respective culture medium. When individuals reached the adult stage and ovigerous females were observed, the whole population was collected and preserved in alcohol.

2.2.5. Life cycle traits and morphological measurements

Copepod population density and female morphology

Samples were counted under a stereo microscope according to their developmental stages, as: copepodids, males, females (non-ovigerous) and ovigerous females. At least 20 ovigerous females from each treatment were sorted randomly from the fixed samples. Photos of the females were taken with an inverted microscope (OLYMPUS IX71, Tokyo, Japan), then image analysis software package Image J 1.41 (Rasband 1997–2014) was used

to measure the prosome length as described in Souissi et al. (2010). Theoretical production (ThP) was calculated using the following equation:

$$\text{ThP} = \overline{CS}_{F0} \times NFemOv_{F0}$$

where CS_{F0} is the average clutch size in the stock culture (F0) and $NFemOv_{F0}$ is the number of females incubated in each beaker (n=20).

Sex ratio and percentage (%) of ovigerous females

Sex ratio (males/females) and the percentages of ovigerous females (%OVF) (100 × ovigerous females/ 'non-ovigerous females + ovigerous females') were calculated from each treatment

Fecundity

The fecundity of females was estimated by counting the eggs in each female's ovisac (s) (clutch size) of the same prosome size measured females from the fixed sample as in Souissi et al. (2016).

Survival rate

The survival of individuals in a generation cycle *F1* (S_1) was calculated using the following equation (as in Souissi et al. 2016):

$$S_1 = 100 \times \frac{Ntot_{F1}}{\overline{CS}_{F0} \times NFemOv_{F0}}$$

Where $Ntot_{F1}$ is the total number of individuals produced from generation *F1*, $\overline{CS}_{F0}$ and $NFemOv_{F0}$ are the mean clutch size and initial number of ovigerous females incubated to start generation *F1* (fixed at 20 ovigerous females).

Statistical analyses

Dead copepods were recorded as percent mortality = (no. of dead copepods/25) × 100. Probit analysis was performed using Microsoft Excel 2013, and the LC50 was calculated as described by Tlili et al. (2016). Mortality was corrected for probit analysis by using Abbot's formula. Analysis of covariance (ANCOVA) was used to compare the coefficients

of male and female regression lines of both species and the sensitivity of both sexes was compared by estimating the common slope. Data are expressed as the mean ± standard deviation (SD). Significant differences were analysed using one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered significant. SPSS, v.18.0 (SPSS Inc., Chicago, IL, USA), was used for the statistical analysis.

In the life cycle experiment, a two-sample F and T-test was used systematically to evaluate the statistical significance ($P< 0.05$) of the mean difference between all experimental treatments and species. The objective of the study was to compare the effect of Cd on the life cycle traits within both species (*E. affinis* control and Cd; *P. annandalei* control and Cd) and between both species (*E. affinis* control and *P. annandalei* control; *E. affinis* Cd and *P. annandalei* Cd). Theoretical production (ThP) was used to compare the total production (TP) (control and Cd) within and between both species

2.3. Results

2.3.1. Differences in the male and female copepod responses to cadmium

Fig. 2.1 shows the 96 h concentration–mortality regression lines for both sexes of *E. affinis* and *P. annandalei*. Mortality increased with an increase in the cadmium concentration. Mortality after 24 h Cd exposure was less than 30% for *E. affinis*, and *P. annandalei* had even lower mortality (<5% in males; none in females). Furthermore, 100% mortality was not observed at any Cd concentration tested (40–360 µg/L) after 96 h for either species.

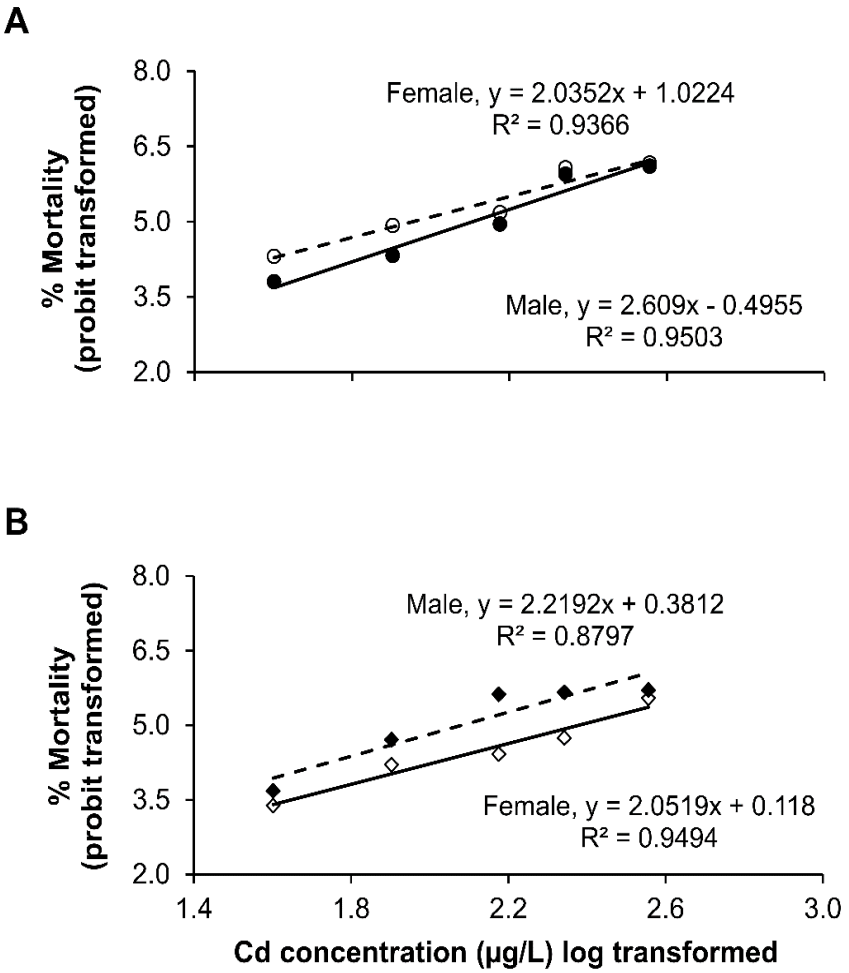

Fig. 2. 1. 96-h concentration–mortality curves for male and female copepods exposed to cadmium. (A) *Eurytemora affinis* (B) *Pseudodiaptomus annandalei*. Symbols (closed circles (●) and diamonds (♦) for males, open circles (○) and diamonds (◊) for females) are experimental regression lines.

2.3.2. Lethal concentrations

Mortality was probit transformed, and lethal concentration (LC) values extrapolated from regression lines are listed in Table 2.1. LC10, LC20, and LC50 values and their confidence intervals for males and females of both copepod species are shown in Table 2.1. Because of the slow response of the copepod species to Cd, the LCs and a reasonable confidence interval (CI) could not be calculated after 24–48 h for *P. annandalei* and *E. affinis*, except for the LC50 of female *E. affinis* after 48 h (Table 2.1), because there was no significant mortality response to Cd exposure.

The sensitivity of both species' sexes increased with exposure time and was significantly different ($P < 0.05$; Fig 2.2). After 96 h, *P. annandalei* males (LC50 = 120.6 µg/L Cd, 95% CI = 119.3–121.9) were about twice as sensitive as *P. annandalei* females (LC50 = 239.5 µg/L Cd, CI = 238.1–240.8), and *E. affinis* females (LC50 = 90.0 µg/L Cd, 95% CI = 88.7–91.4) were approximately 1.4 times more sensitive than *E. affinis* males (LC50 = 127.75 µg/L Cd, 95% CI = 126.5–129). Although *E. affinis* males (LC50 = 127.7 µg/L Cd) and *P. annandalei* males (LC50 = 120.6 µg/L Cd) had similar sensitivity, *E. affinis* females (LC50 = 90.0 µg/L Cd) were 2.7 times more sensitive than *P. annandalei* females (LC50 = 239.5 µg/L Cd; Table 2.1).

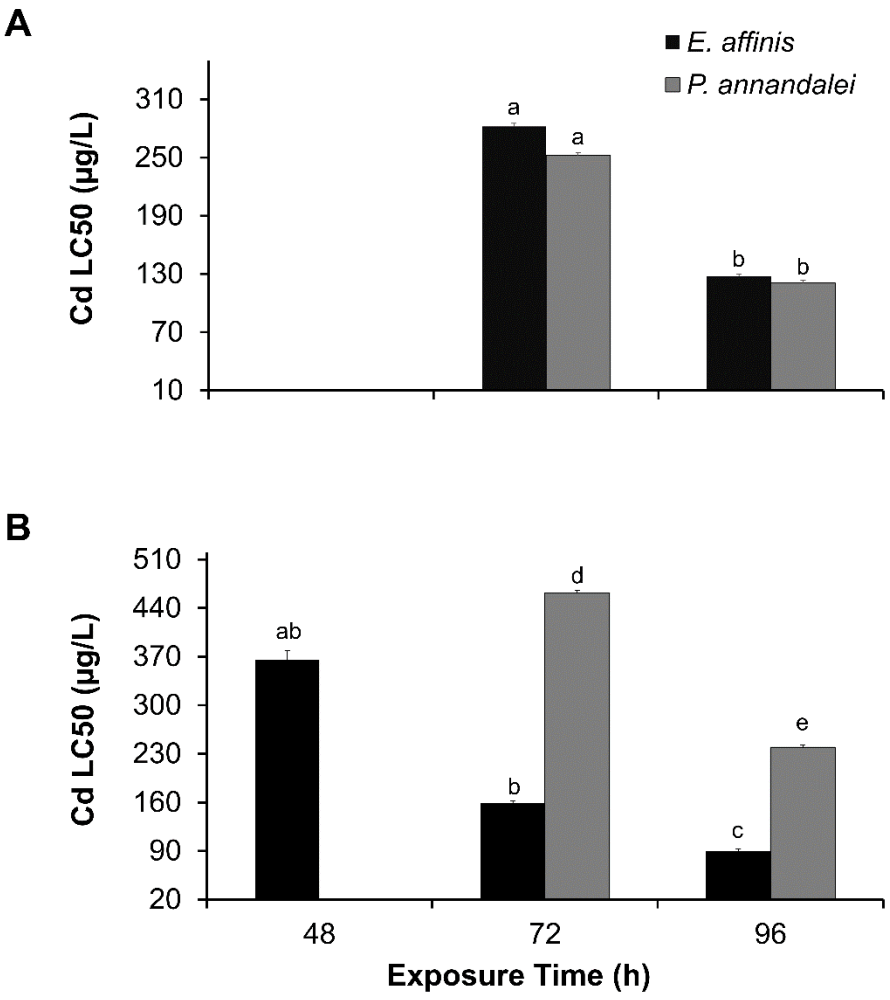

Fig. 2. 2. 50% lethal concentration (LC50) of cadmium for temperate (*Eurytemora affinis*) and subtropical (*Pseudodiaptomus annandalei*) copepods after 96 h of exposure; (**A**) Male, (**B**) Female. Values are LC50 ± SD (n = 3)

2.3.3. Sex-specific responses within copepod species

Eurytemora affinis

The females showed significantly different responses to Cd after 48 and 72 h, whereas the males only showed a significantly different response after 72 h ($P < 0.05$). The sensitivity between the males and females was also significantly different ($P < 0.05$). After 96 h, both sexes separately showed significant ($P < 0.05$) responses to Cd; however, the sensitivity between males and females was not significantly different ($P > 0.05$).

Pseudodiaptomus annandalei

After 48 h, the response of the individual sex to Cd and the sensitivity difference between these sexes were not statistically significant ($P > 0.05$). After 72 and 96 h, the responses of the individual sex to Cd were significantly different ($P < 0.05$); however, the difference between their sensitivity was not significant ($P > 0.05$) after 72 h but significant ($P < 0.05$) after 96 h.

2.3.4. Sex-specific responses between copepod species

E. affinis male and female responses were compared with *P. annandalei* male and female responses to Cd exposure by comparing their coefficient of regression lines after estimating the common slopes. The difference between the sensitivity of *E. affinis* and *P. annandalei* males to Cd was not significant ($P > 0.05$) after 48–96 h. However, *E. affinis* females were significantly ($P < 0.05$) more sensitive to Cd than *P. annandalei* females, as shown by the LC50 values in Table 2.1. Overall, *E. affinis* copepods appeared to be more sensitive to Cd than *P. annandalei*; however, although the males of both species did not significantly differ ($P > 0.05$) in sensitivity after 96 h, the females differed significantly ($P < 0.05$) in sensitivity after 72 and 96 h.

Table 2. 1. Acute lethal concentration (LC) of cadmium (µg/L) for subtropical (*Pseudodiaptomus annandalei*) and temperate (*Eurytemora affinis*) species of copepod after 48 h, 72 h, and 96 h exposure, 95% confidence interval (CI), ($P < 0.05$).

	Male			**Female**		
	LC10 (95% CI)	LC20 (95% CI)	LC50 (95% CI)	LC10 (95% CI)	LC20 (95% CI)	LC50 (95% CI)
P. annandalei						
72 h	62.30	100.71	252.2	90.68	158.61	461.86
	(60.29 - 64.31)	(99.11 - 102.30)	(250.82 - 253.59)	(89.06 - 92.30)	(157.18 - 160.03)	(460.03 - 463.7)
96 h	31.90	50.36	120.59	56.83	93.14	239.46
	(30.09 - 33.70)	(48.80 - 51.93)	(119.30 - 121.89)	(55.18 - 58.49)	(91.72 - 94.56)	(238.08 - 240.85)
E. affinis						
48 h	-	-	-	-	-	365.54
						(361.24 - 369.84)
72 h	61.76	104.04	281.94	29.15	52.19	158.88
	(60.09 - 63.44)	(102.62 - 105.47)	(280.48 - 283.4)	(27.01 - 31.28)	(50.47 - 53.9)	(157.51 - 160.25)
96 h	41.22	60.79	127.75	21.12	34.75	90.04
	(39.68 - 42.76)	(59.39 - 62.18)	(126.5 - 129)	(19.1 - 23.14)	(33.04 - 36.46)	(88.7 - 91.38)

2.3.5. Toxicity differences to cadmium between female copepods at different reproductive stages

After 96 h, we observed higher mortality in the NOFs than in the OFs for both species in both the control and 40 µg/L Cd treatment group. The OF mortality of *E. affinis* was significantly higher ($P < 0.05$) than that of *P. annandalei* both in the control and 40 µg/L Cd treatment group. Similarly, the NOF mortality of *E. affinis* was significantly higher ($P < 0.05$) than that of *P. annandalei* both in the control and in 40 µg/L Cd treatment group (Fig. 2.3A and B). These results showed higher survival in *P. annandalei* females than in *E. affinis* females.

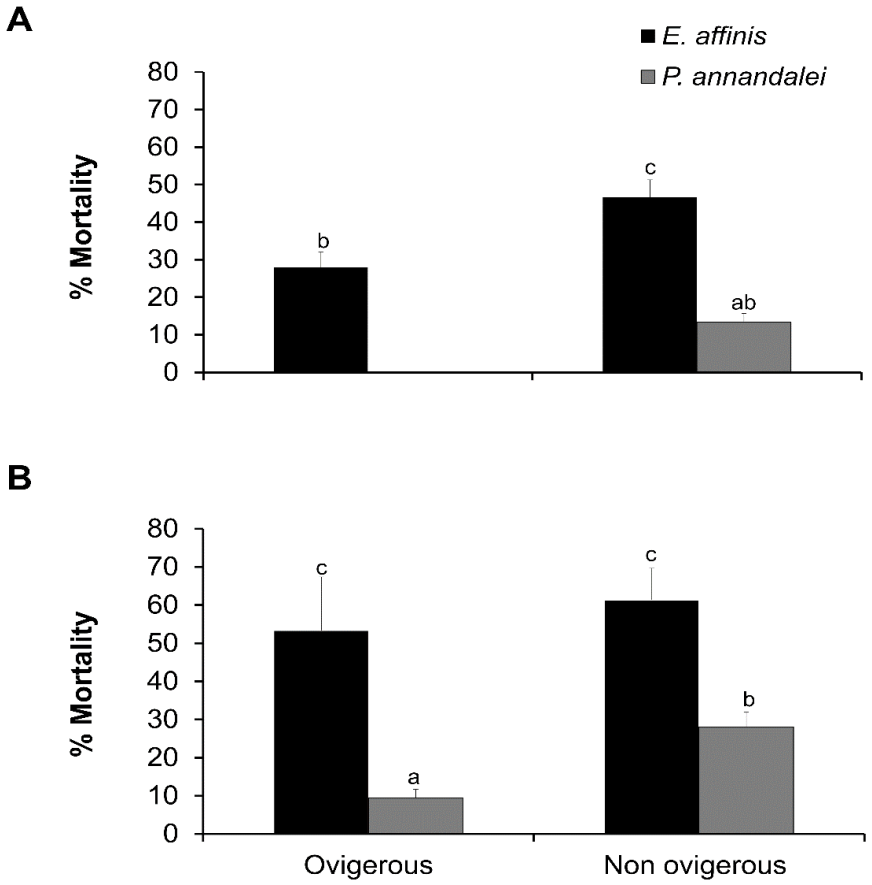

Fig. 2. 3. 96-h % mortality of ovigerous and nonovigerous female copepods of two species, *Eurytemora affinis* (black) (18 °C) and *Pseudodiaptomus annandalei* (grey), (26 °C), with or without exposure to 40 µg/L cadmium; (**A**) Control, (**B**) 40µg/L Cd. Values are mean ± SD (n = 3)

2.3.6. Toxicity effects of cadmium on the life cycle traits of *E. affinis* and *P. annandalei* copepods

Copepod population density, total production and female size

Figure 2.4 shows a decreasing trend in the number of individual copepods produced and the total production (TP) in both species. Copepodids and males of both species and females (non-ovigerous) of *P. annandalei* exposed to Cd were significantly lower ($P < 0.05$) than those in the control group. Ovigerous females of both species exposed to Cd and those in the control group were not significantly different ($P > 0.05$). However, the TP of both species exposed to Cd decreased significantly ($P < 0.05$) than those in the control group. Prosome length of both species was not significantly different between those in the control and Cd exposed group. The theoretical production of *E. affinis* was significantly higher ($P < 0.05$) than the TP of *E. affinis* exposed to Cd (Fig. 2.5) and those in the control group. The TP of those exposed to Cd was significantly lower ($P < 0.05$) than those in the control group. In addition, the theoretical production of *P. annandalei* was not significantly ($P > 0.05$) different from the TP of *P. annandalei* in the control group, but the TP of *P. annandalei* exposed to Cd was significantly lower ($P < 0.05$) than those in the control group.

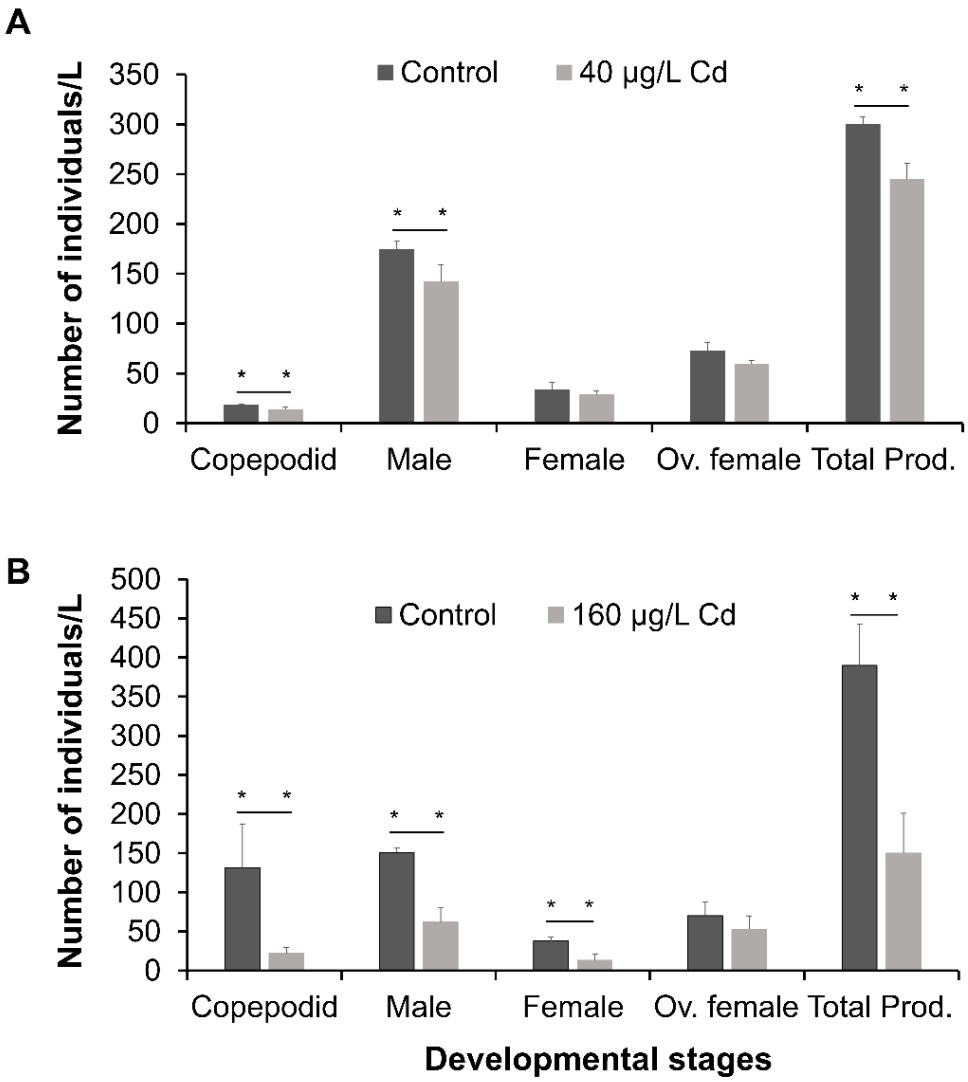

Fig. 2. 4. Effect of cadmium on the number of individual population; Copepodid, Male, Female (non-ovigerous), Ovigerous female and Total production of *Eurytemora affinis* (A) (18 °C) and *Pseudodiaptomus annandalei* (B), (26 °C). Values are mean ± SD, asterisk (*) indicates significant difference, *P< 0.05*

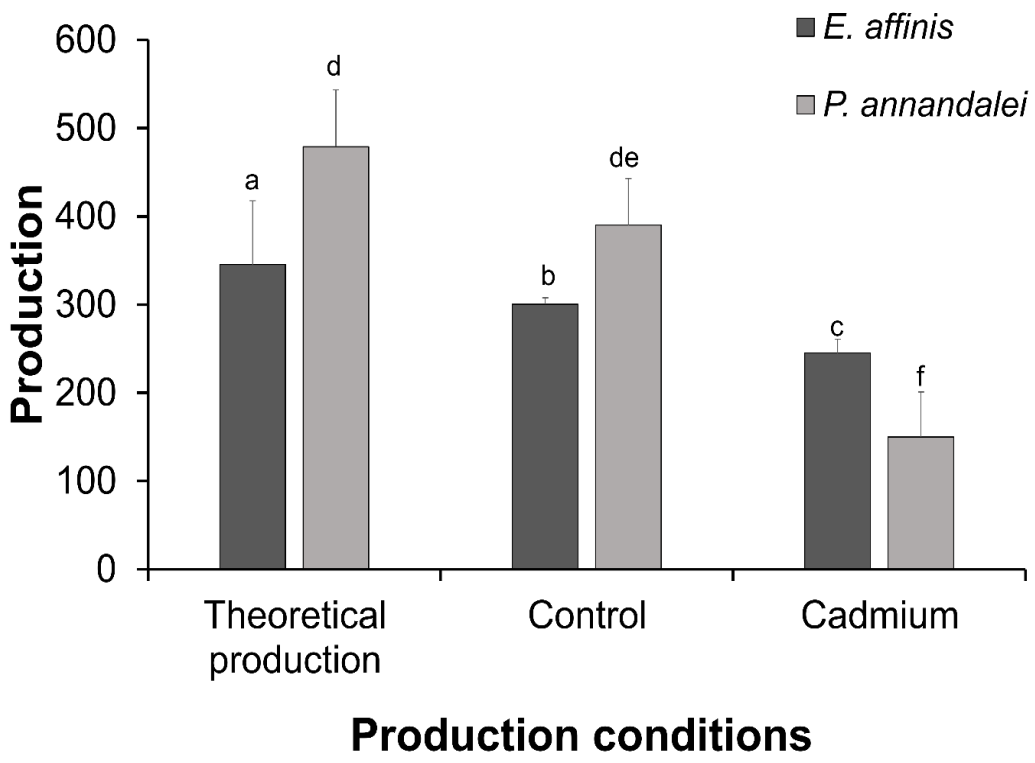

Fig. 2. 5. Effect of cadmium on the production (F1 generation) of *Eurytemora affinis* (18 °C) and *Pseudodiaptomus annandalei* (26 °C) at different conditions. Values are mean ± SD

Sex ratio and percentage of ovigerous females

Sex ratio (male: female) and percentage of ovigerous females (% OVF) of *E. affinis* showed a slight decrease when exposed to Cd compared to those in the control group, although not significantly different ($P > 0.05$). Sex ratio of *P. annandalei* (male: female) exposed to Cd similarly decreased but not significantly different ($P > 0.05$) from those in the control group. In addition, *P. annandalei* exposed to Cd showed a significant increase ($P < 0.05$) in % OVF than those in the control group. However, sex ratio (male: female) and % OVF of *E. affinis* in the control and those exposed to Cd were significantly different ($P < 0.05$) from sex ratio (male: female) and % OVF of *P. annandalei* exposed to Cd (Table 2.3).

Fecundity and survival

Clutch size of both species exposed to Cd decreased significantly ($P < 0.05$) compared to the control groups. However, clutch size of *P. annandalei* in the control and Cd exposed groups was significantly ($P < 0.05$) higher than that of *E. affinis* (Table 2.3).

The percent survival of *E. affinis* and *P. annandalei* in the control groups were 87% and 81%, compared to the Cd-exposed groups with 71% and 31%, respectively.

Table 2. 2. Cadmium toxicity of different copepods in different environmental conditions

Copepod species	Environment	Life stage	Time (h)	Cd LC50 (µg/L)	Reference
Pseudodiaptomus annandalei	Estuarine (Sub-tropical)	Adult Male	96	120.6 (26^{o}C)	Present study
P. annandalei	Estuarine (Sub-tropical)	Adult Female	96	239.5 (26^{o}C)	Present study
P, annandalei	Estuarine (Sub-tropical)		96	169	Chen, 2011
Eurytemora affinis	Estuarine (Temperate)	Adult Male	96	127.75 (18^{o}C)	Present study
E. affinis	Estuarine (Temperate)	Adult Female	96	90.04 (18^{o}C)	Present study
E. affinis	Estuarine	Naupliar	96	>120	Sullivan et al., 1983
E. affinis	Estuarine	-	96	147.7	Cripe and Cripe, 1990
E. affinis	Estuarine	-	96	60	Robert et al., 1982
E. affinis	Estuarine	Naupliar	96	51.6(5ppt)	Hall et al., 1995
E. affinis	Estuarine	Naupliar	96	213.2(15ppt)	Hall et al., 1995
E. affinis	Estuarine	Naupliar	96	82.9(25ppt)	Hall et al., 1995
Acartia tonsa	Estuarine	-	96	90	Cripe and Cripe, 1990
Oithona similis	Estuarine	-	96	20.53	Gnanamoorthy et al., 2012
A. tonsa	Marine	-	96	380	Robert et al., 1982

Tigriopus brevicornis	Marine (Temperate)	Adult	96	48	Barka et al., 2001
T. brevicornis	Marine	ovigerous female	96	47.9	Forget et al., 1998
T. brevicornis	Marine	Naupliar	96	17.4	Forget et al., 1998
T. brevicornis	Marine	Copepodids	96	29.7	Forget et al., 1998
T. breuicornis	Marine	larvae	240	78	Le Dean and Devineau, 1985
Tigriopus japonicus	-	adult	96	25.2	Lee et al., 2007
A. tonsa	Marine	-	96	151 (13^{o}C)	Toudal and Riisgard, 1987
A. tonsa	Marine	-	96	29 (21^{o}C)	Toudal and Riisgard, 1987
Tisbe holothuriae	Marine (Temperate)	-	48	906	Moraïtou and Verriopoulos, 1982
T. fulvus	-	naupliar	24	4390	Pane et al., 2008
T. fulvus	-	naupliar	48	2240	Pane et al., 2008
T. fulvus	-	naupliar	72	960	Pane et al., 2008
T. fulvus	-	Female	48	12360	Pane et al., 2008
T. fulvus	-	Female	72	6540	Pane et al., 2008
T. fulvus	-	Female	96	3320	Pane et al., 2008
Tisbe battaglia	Temperate	Adult	96	340	Hutchinson et al., 1994
T. battaglia	Temperate	Naupliar	96	460	Hutchinson et al., 1994

Paralabidocera antarctica	Marine (Artarctic)	Adult	168	237 (-1oC)	Zamora et al., 2015
Oncaea curvata	Marine (Artarctic)	Adult	168	901(-1oC)	Zamora et al., 2015
Stephos longipes	Marine (Artarctic)	Adult	168	1250 (-1oC)	Zamora et al., 2015

LC50 = Lethal concentration resulting in 50% mortality

Table 2. 3. Effect of cadmium (µg/L) on male: female Sex ratio, % Ovigerous females, Prosome length and Clutch size of subtropical (*Pseudodiaptomus annandalei*) and temperate (*Eurytemora affinis*) species of copepod.

		Sex ratio (male: female)	% Ovigerous females	Prosome length (µm)	Clutch size
E. affinis	Control	1.65 ± 0.12^{a}	68.04 ± 7.02^{a}	$775.20\pm2.01.53^{a}$	15 ± 1^{a}
	40µg/L Cd	1.60 ± 0.19^{a}	67.42 ± 3.67^{a}	784.12 ± 15.41^{a}	13 ± 2^{b}
P. annandalei	Control	1.43 ± 0.23^{ab}	63.95 ± 9.35^{a}	889.75 ± 8.56^{b}	24 ± 3^{c}
	160µg/L Cd	0.98 ± 0.10^{b}	82.36 ± 5.44^{b}	889.98 ± 21.47^{b}	20 ± 3^{d}

Different superscript from the same column are significantly different ($P < 0.05$). Values are mean ± SD

2.4. Discussion

2.4.1. Differences between male and female responses to cadmium

The response of copepod population to different heavy metal concentrations has been shown to be species and gender specific (Bao et al., 2008; Lotufo and Fleeger, 1997); moreover, the results of this study showed an increasing trend in mortality with time from 24 to 96 h with an increase in Cd concentration. This result suggests that the concentration and particularly the length of exposure of copepods to Cd played an important role in the increased mortality (Fig. 1A and 1B). Mortality was higher in *E. affinis* females exposed to 40–360 µg/L Cd than in *E. affinis* males; however, the opposite pattern was observed in *P. annandalei*. Furthermore, the 96 h LC50 of *E. affinis* females (90 µg/L Cd) was significantly lower than that of *E. affinis* males (127.8 µg/L Cd). In addition, the 96-h LC50 of *P. annandalei* males (120.6 µg/L Cd) was significantly lower than that of *P. annandalei* females (239.5 µg/L Cd).

Multiple studies investigating Cd toxicity in copepods have reported that LC50 values vary with species and life stages (Table 2.2). A lower concentration results in higher female than male mortality, suggesting that *E. affinis* females are more sensitive to Cd toxicity compared with *E. affinis* males. In accordance with the findings of the current study, McCahon and Pascoe (1988) reported that the LC50 value at 48 h indicated that freshwater amphipod *Gammarus pulex* (L.) females are more sensitive to Cd compared with *G. pulex* (L.) males. Similarly, Sroda and Cossu-Leguille (2011) found that the females of two gammarid species, *G. roeseli* and *Dikerogammarus villosus*, are more sensitivity to Cu compared with the males of these two species.

Many studies carried out on the exposure of copepods to organic pollutants reported that males are more sensitive than females (Bao et al., 2008; Lotufo and Fleeger, 1997; Medina et al., 2002). Similar gender response was observed for *P. annandalei* when exposed to cadmium in this study, where the males were more sensitive to Cd than females. Differences in sex-specific responses to stress are not general but are specific to both the species and the contaminant. When sexes are separated, variations in individual responses

to chemical pollutants can be identified. Different chemicals and exposure times have different pathways and modes of action for different species and their sexes (Boulangé-Lecomte et al., 2014; Hinck et al., 2008; Ko et al., 2014; Stringer et al., 2012; Volz and Chandler, 2004; Yu et al., 2013). Because the physiology of males and females differs, studies on individual responses to chemical pollutants can provide more insights into their tolerance level.

Size differences due to sex or species is believed to account for variations in sensitivity to contaminants; that is, the larger surface area to volume ratio of a smaller animal made them more susceptible to toxic pollutants (Chandler and Green, 1996; Stringer et al., 2012; Wang and Zauke, 2004). According to Hagopian-Schlekat et al. (2001), *Amphiascus tenuiremis* males are smaller than females; thus, they assumed that males could accumulate higher amounts of metals than females after observing that the survival of males following the exposure to Cu, Pb, and Ni was significantly lower than that of females. Furthermore, Stringer et al. (2012) observed that *Quinquelaophonte sp*. males were significantly more sensitive to Zn and atrazine compared with females, which was speculated to be because of different body sizes. However, in *R. propinqua*, despite the size difference between the sexes, no sex-specific differences in sensitivity to Zn, phenanthrene, or atrazine were observed, suggesting that factors other than size differences can affect sex-specific sensitivity. In addition, Medina et al. (2002) claimed that differences between sexes could not account for observed differences in tolerance because their results showed that sex-related differences in sensitivity to the pollutant pyrethroid cypermethrin changed with time.

In this experiment, the males of both copepod species were smaller than the females, but the females of *E. affinis* were more sensitive to Cd than the males; by contrast, the males of *P. annandalei* were more sensitive than the females. The differences in sex-specific sensitivity may not be size related but perhaps related to their physiology; that is, their individual ability to affect uptake, effectively metabolize, and eliminate contaminants can be contaminant specific (Escher and Hermens, 2002). The mode of metal uptake differs and could be from ingested food or from the dissolved phase and more than 50% of

cadmium accumulates from dissolved phase (Wang and Fisher, 1998). In addition, mode of metal elimination differs, which could include elimination through molted exoskeleton (Dittman & Buchwalter 2010; Mirenda, 1986), through deposition in eggs (Dipinto et al., 1993; Oberdörster et al., 2000) or faeces (Benayoun et al., 1974). Possible elimination of cadmium by the test species in this experiment through molting or faeces were not considered since the final stage of development was used and they were not fed during the experimental period.

2.4.2. Differences in responses to Cd toxicity between female reproductive stages of two copepods

In the first experiment, the sample of female copepods contained both OFs and NOFs. The second experiment was conducted to evaluate the reasons underlying the differences in responses to Cd toxicity between males and females. We compared the effect of Cd in OFs and NOFs with and without exposure to a sublethal concentration of Cd. The mortality of NOFs was significantly higher than that of OFs with and without exposure to Cd in both copepod species. In addition, the mortality was higher in *E. affinis* females than in *P. annandalei* females. On the cellular level, heat shock proteins (HSPs) have been shown to be expressed by aquatic organisms under stress conditions. Boulangé-Lecomte et al. (2014) found a weaker expression of HSPs in *E. affinis* males than in females on a basal level (e.g., reproduction cycle), suggesting a sex-specific stress tolerance. Therefore, it is possible that the reproductive state of female *E. affinis* can be a factor affecting the sensitivity to Cd in the present study. McCahon and Pascoe (1988) observed the LC50 of freshwater amphipod *G. pulex* exposed to Cd and found that compared with males, females with eggs were twice as sensitive and females without eggs 13 times more sensitive to Cd.

The low sensitivity of females with eggs to Cd toxicity indicates that they have a more effective mode of toxic elimination. The difference found between OFs and NOFs points to the possibility of OFs eliminating Cd through the eggs they carried. The process of detoxification by depositing toxic waste in female eggs was referred to as ovodeposition by Dipinto et al. (1993), which could account for their higher tolerance to environmental contaminants (Oberdörster et al., 2000). Roberts and Leggett (1980) reported an example

of ovodeposition in which eggs produced by the blue crab *Callinectes sapidus* contained more toxic contaminants (Kepone) compared with muscles. Egg production was concluded to be a major route for eliminating Kepone from female blue crabs (Roberts and Leggett, 1980). The theoretical explanation for these results is that lipophilic compounds such as Kepone have an affinity for lipid-rich eggs. Therefore, whether sensitivity to Cd toxicity is higher or lower in females than in males can have an ecological impact. For example, if female copepods are more sensitive than males, and if the concentration and bioavailability of metal increase in aquatic environments influenced by changes in physiochemical parameters, the rate of female mortality could increase consequently impeding the production of new recruits. Moreover, even when the females of a copepod species seem to be less sensitive than the males (as with *P. annandalei* in the second experiment), continuous exposure to metal pollution can result in bioaccumulation.

2.4.3. Toxicity effect of cadmium on the life cycle traits of *E. affinis* and *P. annandalei*

When assessing the impact of contaminant in the environment, mortality is usually the first endpoint to be considered. Other bioindicators such as reproduction and development of model test species have recently become an important endpoint in the risk assessment of aquatic pollutants (Kwok et al., 2015). Cadmium in the aquatic environment can cause a reduction in recruitment potential of copepod nauplii either through decreasing egg production, reduced hatching success or high mortality at the nauplii or copepodid stages and the degree of effect varies with the level of concentration, exposure duration and species (Jiang et al., 2007; Mohammed et al., 2011; present study). In our complete life-cycle experiment, a decrease in the number of individual copepod developmental stages and an overall decrease in the total population of both copepod species exposed to Cd were observed. Results from this experiment showed that chronic exposure of Cd negatively affected the population and total production of both copepod species. These were significantly lower than their theoretical production. Survival of both species in the control groups were less than 20% of the theoretical production. However, survival of *P. annandalei* exposed to Cd was 50% lower than those of the control groups, whereas *E. affinis* was 20% lower. LC50 values of Cd in the first experiment showed *E. affinis* to be

more sensitive than *P. annandalei*. However, we observed a more significant decrease in the population and survival of *P. annandalei* when exposed to Cd. This is due to mortality occurring through the life cycle, which means that nauplii and/or copepodids of *P. annandalei* are more sensitive than the adult stage. Lira et al. (2011) observed a similar sensitivity change with a decrease in the population density of marine nematode *Rhabditis (Pellioditis) marina* when exposed to Cd.

Constantly changing environmental conditions are commonly unfavorable to inhabiting species. The sex ratio is skewed towards the gender with which shows a better tolerance or adaptive capabilities (Krupa, 2005). The Seine (France) and Danshuei (Taiwan) estuaries have particularly high pollution levels of heavy metals (Dauvin, 2008; Fang and Lin, 2002; Fang et al., 2006; 2014; Hwang et al. 2010a; Jeng and Han, 1994). In both estuaries, the sex ratio of *E affinis* and *P. annandalei* is skewed in favor of males and varies during the year (Beyrend-Dur et al., 2013; Devreker et al., 2010). In this study, there were more males than females in the control and Cd exposed groups of both copepod species except those of *P. annandalei* exposed to Cd. In addition, the male proportion was higher in *E. affinis* than in *P. annandalei*. *P. annandalei* male: female sex ratio was skewed in favour of females, which could be a response to the toxicity of Cd they were exposed to.

An increase in clutch size usually correlates positively with an increase in prosome length (Souissi. et al., 2016). This experiment shows a significant decrease in clutch size. However, a slight increase in prosome length was observed, although not significant. This could be due to the fact that only one generation was observed in our study compared to the multigenerational study by Souissi et al. (2016).

The higher ratio of OVF: NOF and the higher % OVF observed in *P. annandalei* show that even though the population density was lower in the Cd treatment, the surviving copepods were in favour of the female population and their reproductive activities. A high percentage of Cd was reported to be associated with the capsule membrane of the eggs of cuttlefish *Sepia officinalis* (Bustamante et al., 2002) and accumulates in the chorion of *Oncorhynchus mykiss* (Beattie and Pascoe, 1978) and *Oryzias latipes* (Michibata, 1981). If toxic waste is indeed eliminated by the deposition in eggs (De Loof, 2015), this may affect

the eggs' hatching success or naupliar viability. However, this hypothesis has to be tested in the future. Jiang et al. (2007) observed a significant reduction in the number of hatched nauplii of *Acartia pacifica* copepod resting eggs exposed to increasing Cd concentrations. Therefore, the significant reduction observed in *P. annandalei* TP when exposed to Cd (Figure 2.5) could be a result of low hatching success or increased mortality from chronic exposure. The experiment was conducted for one generation and the total population was collected after ovigerous females were observed in high number. Ovigerous females were majorly carrying their first egg sacs. Moreover, female copepods can produce a second clutch even after a single mating. This means that more eggs could be produced as a means of reducing the contamination load. Gismondi et al. (2013), suggested that one of the possible reasons for higher survival observed in *Gammarus roeseli* females than in males could be a result of ovodeposition. The trade-off between reducing the fitness of one clutch and increasing female survival could become an added ecological advantage for female copepods. A study on Cd effects on several generations could, therefore, shed more light on the ecological significance and adaptive potentials to Cd contamination.

In conclusion, the ecological implication of Cd toxicity on copepod ecology is more related to a skewed sex ratio, low egg production, reduced hatchability, and reduced survival that affects the recruitment potential of copepod nauplii resulting in a decreasing copepod population. Korsman et al. (2014), modeling on environmental stress factors in *E. affinis* suggest that exposure to zinc and copper was largely responsible for reduced population densities in a contaminated estuary. As a major link in the aquatic food web, copepod decline could result in major disruptions of ecosystem structure and functioning. To conclude, mortality, reproduction and population growth of model species may provide important bio-indicators for environmental risk assessment.

Chapter 3.

Salinity, temperature and cadmium stress on the growth, biochemical composition of algae and their cadmium bioaccumulation

Esther Kadiene U., Pei-Jie Meng, Irina Sadovskaya, Jiang-Shiou Hwang, Sami Souissi. (In Prep.). Salinity, temperature and cadmium stress on the growth, biochemical composition of algae and cadmium bioaccumulation

3.1. Introduction

Microalgae are primary producers in the aquatic food web. Microalgal production can be affected by pollution from heavy metals and they can also mediate the transfer of bioaccumulated metals to primary consumers like copepods. Environmental factors like salinity, temperature, type and concentration of metals can influence the rate at which microalgae bioaccumulate metals. Particularly for estuarine organisms, salinity is one of the major factors that should be studied (Devreker et al., 2009). Salinity variations in the ocean are caused by various factors including increasing temperature. Temperature increase in the ocean also results from climate change.

Salinity is a major factor in the determination of Cd toxicity (McGeer et al., 2004). In sea water cadmium is mainly in the form of $CdCl^{+}$. A study showed that extent and uptake of cadmium in the freshwater alga *Nitella* sp. depends upon the amount of calcium and magnesium present. Uptake occurs faster and to a greater extent in hard water (Kinkade and Erdman 1975). The relative amounts of dissolved cadmium in the different cadmium complexes varies with salinity. The affinity of cadmium for adsorption to particles decreases with increasing salinity, due to chloride complexation (Li et al., 1984). River water often contains higher concentrations of cadmium than seawater, producing an enrichment of cadmium concentration in the brackish waters of estuaries (DeLeon et al., 1986).

One aspect in the study of metal bioaccumulation in algae, is its application for bioremediation in a polluted environment. Another aspect is to understand its role in the transfer of metals across trophic levels. In order to understand the bioaccumulation or bio-amplification of metal contaminants in aquatic ecosystems, laboratory studies were performed using microalgae. In order to understand the rate of metal uptake by microalgae, and the factors that control or influence the uptake rate, microalgae were exposed to different concentrations of metals under different culture conditions.

3.2. Materials and Methods

3.2.1. Salinity and cadmium stress on growth and Cd uptake of *Diacronema lutheri*

Diacronema lutheri (Droop) Bendif & Véron 2011, was grown at salinities of15 and 35 (control) in 2 liter balloons with and without 60µg/L of cadmium in duplicates (Fig. 3.1). The light regime was maintained as 12:12 Dark: Light cycle. 1 mL of algae was sampled daily in Eppendorf tubes and preserved with 100 uL of lugol solution and cell density was estimated using image analysis on Malassez haemocytometer under an inverted microscope (Olympus, IX71). An aliquot of algae was sampled daily for 7 days and was filtered through pre-weighed membrane filter paper and dried to constant weight following cadmium bioaccumulation analysis. Samples for biochemical analysis (Glucan and fatty acids) were collected at different growth stages.

3.2.2. Effect of salinity, temperature and cadmium concentration on cadmium uptake in *D. lutheri*

D. lutheri was grown in salinity 15, 25 and 35 (control) in 100 mL beakers and spiked with 36, 120, 600 and 1200 µg/L of cadmium and were kept at 3 temperatures: 18, 25 and 35 ºC for 72 hours following sampling for cadmium bioaccumulation analysis.

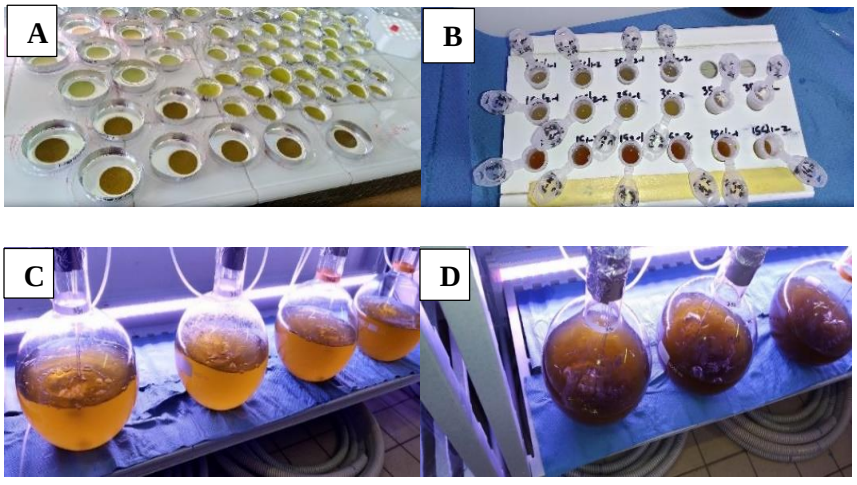

Fig. 3. 1. *Diacronema lutheri* samples in filter papers for cadmium analysis (A), cells in Eppendorf tubes for cell counts (B), experimental cultures at day 1 (C) and day 5 (D).

3.3. Results and Discussion

Figure 3.2 shows the effect of different salinities in addition to cadmium exposure on cell densities of *Diacronema lutheri* culture. Cell density of *D. lutheri* cultured at salinity 15 was lower compared to the control at salinity 35. *D. lutheri* cell density cultured at both salinities and under cadmium (Cd) treatment (35 PSU Cd and 15 PSU Cd), decreased significantly compared to those without exposure to Cd. A multivariate ANOVA test showed significant differences ($P< 0.05$) in cell densities from day 9 between *D. lutheri* cultured at low salinity (15 PSU) and with Cd (15 PSU Cd) and between 15 PSU Cd and 35 PSU Cd, also from day 12 to 15 between *D. lutheri* cultures at 15 and 35 PSU; 15 PSU and 15 PSU Cd; 35 PSU and 35 PSU Cd and between 15 PSU Cd and 35 PSU Cd. The period with significant difference in cell densities (Day 12 to 15) represent the stationary growth phase.

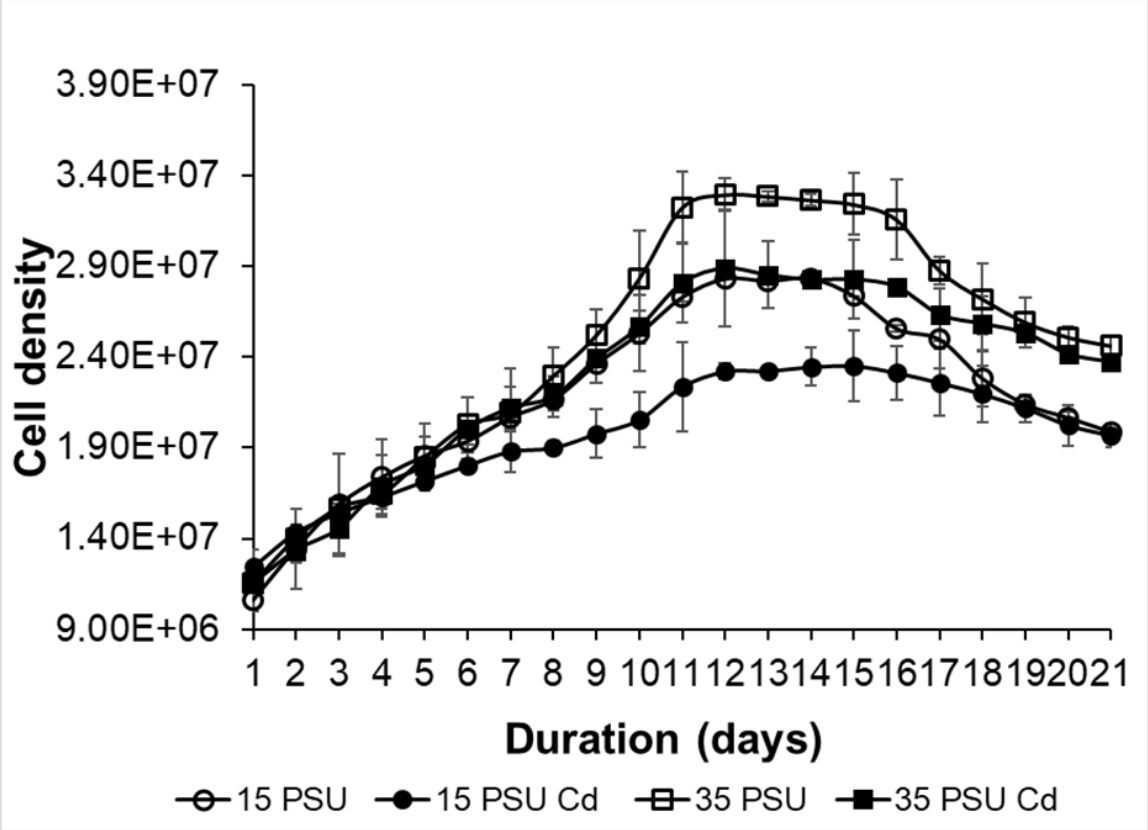

Fig. 3. 2. Growth curve of *Diacronema lutheri* cultured at different treatment conditions. n= 4; P= 0.004.

Figure 3.3 shows the daily uptake of Cd by *D. lutheri* for 7 days cultured at salinity 15 to be significantly ($P< 0.05$) higher than the cultures at salinity 35.

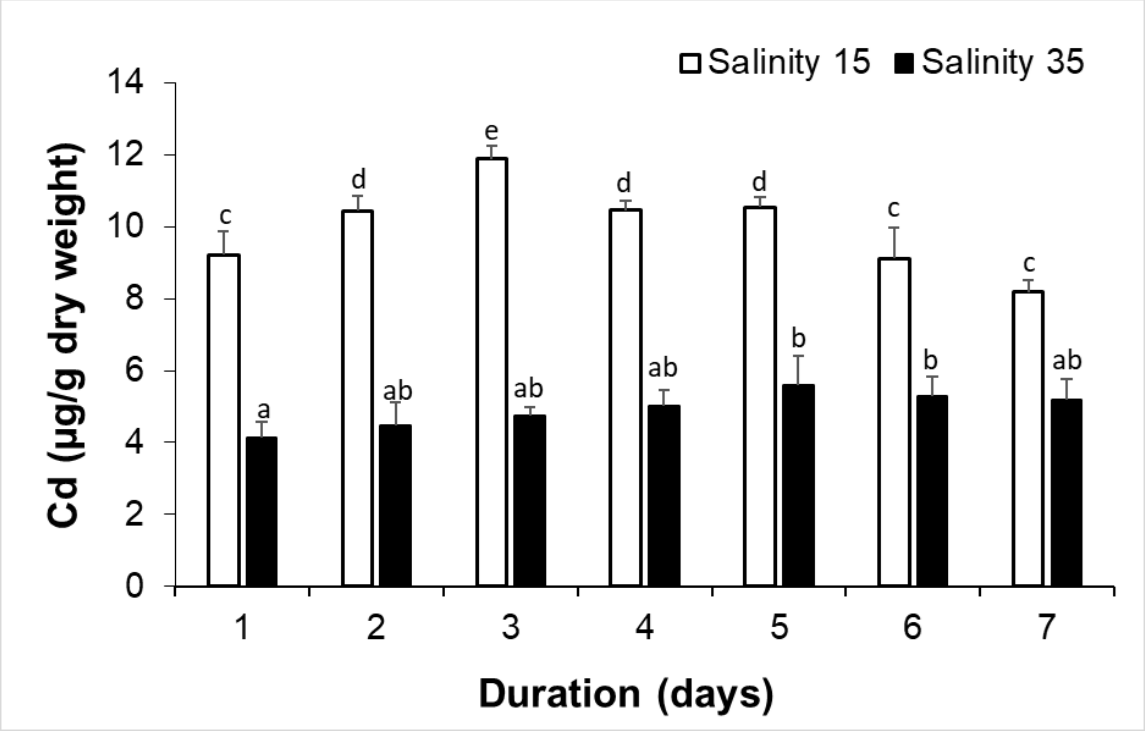

Fig. 3. 3. The uptake of cadmium in *Diacronema lutheri* culture at salinities 15 and 35 with 60µg/L cadmium.

Figure 3.4 shows that lower salinity, higher temperature and high cadmium concentrations increases cadmium uptake in *D. lutheri*. Generally, *D. lutheri* does not efficiently bioaccumulate much cadmium when compared to the concentrations they were exposed to.

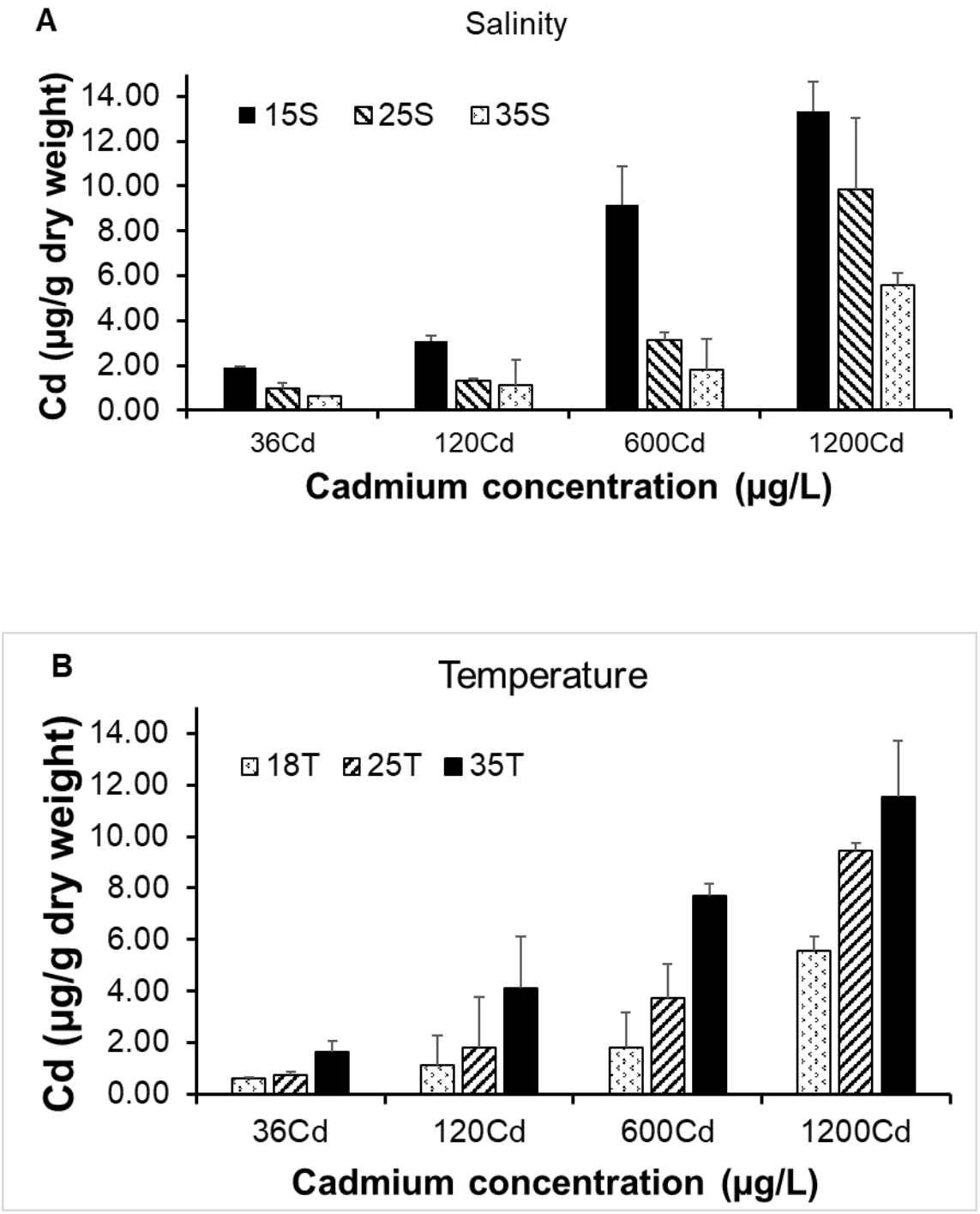

Fig. 3. 4. 72 h cadmium uptake in *Diacronema lutheri* cultured at salinities 15, 25 and 35 (A) spiked with different concentrations of cadmium at different temperatures, 18, 25 and 35 °C (B).

We observed that cadmium exposure negatively affected the growth of *D. lutheri* at both salinity 15 and salinity 35. Furthermore, it was observed that low salinity and high temperature resulted in higher bioaccumulation of cadmium. This implies that the issue of global warming which is also characterized by increasing temperature resulted in an increase of seawater level (seawater dilution; low salinity) and could aggravate metal toxicity in aquatic organisms through increasing metal uptake and it bioavailability.

Chapter 4.

Acute and chronic toxicity of cadmium on the copepod *Pseudodiaptomus annandalei*: A life history traits approach

Kadiene, E.U., Meng, P.J., Hwang, J.S. and Souissi, S., 2019. Acute and chronic toxicity of cadmium on the copepod *Pseudodiaptomus annandalei*: A life history traits approach. Chemosphere, 233, pp.396-404.

4.1. Introduction

The productivity and abundance of copepods face threats from environmental toxic pollutants. However, toxicity of metals (which is the degree to which a metal can induce harm) to copepods varies because the rates of uptake, excretion, and detoxification are inter- and intra-species as well as metal specific (Fang et al., 2014; Hsiao et al., 2010; Luoma and Rainbow, 2005; Ritterhoff and Zauke, 1997).

Copepods accumulate metals by assimilating them from their food or by absorbing them from water (Wang and Fisher, 1998) and the uptake pathway can determine its internal distribution (Hare et al., 1991; Hook and Fisher, 2001; Wang and Fisher, 1998). Dietary toxicity is dependent upon the organism and the life stage that is exposed, as well as the diet type, form of metal, and the daily dose received (Carpene and George, 1981; Clausen et al., 1993; Sidoumou et al., 1997). Studies have shown that cadmium uptake is higher from dissolved phase than through dietary route. For example, *Acartia tonsa* was reported to assimilate 40 percent of the cadmium through their phytoplankton food and more than 50 percent in their tissue is from the dissolved phase in ambient waters (Fisher et al., 2000). Other studies have shown similar results of cadmium accumulation from water to be higher than from food (Borchardt, 1983; Riisgard et al., 1987). Biological factors like age, size, and sex modify uptake and elimination of metals (Wang and Fisher, 1997). Several studies have highlighted the critical ecological effect of lethal and sublethal concentrations of toxic metals. However, very few studies have attempted to elucidate the responses of copepods to toxic metals based on their life stage or reproductive state. Age and sex are commonly important factors for copepod stress response (Dipinto et al., 1993; Forget et al., 1998; Hsiao and Fang, 2013; Sornom et al., 2010; Sroda et al., 2011; Kadiene et al., 2017; Zidour et al., 2019). Sex-specific sensitivity to metals in *P. annandalei* were reported in a previous study (Kadiene et al., 2017).

In the present study, we further showed toxic effects of acute exposure of the developmental stages of *P. annandalei* to cadmium (Cd) as well as the effects of chronic exposure to Cd on growth, development and reproductive performance of the copepod. In addition, we investigated the sex-specific bio-accumulation of cadmium both from water

and from diet in *P. annandalei*. We followed the hypothesis that life history traits, provides critical indications for sublethal effects of toxicants as a comprehensive approach in ecotoxicology.

4.2. Materials and Methods

4.2.1. Experiment 1: Acute exposure of *P. annandalei* to cadmium

Lethal concentrations (LC_{50}) of cadmium in nauplii and copepodids

The evaluation of LC_{50} of Cd in *P. annandalei* nauplii and copepodids was carried out following the protocol of Kadiene et al. (2017). Nauplii and copepodids (n=25) were placed in 100 ml beakers containing 90 ml media of different concentrations of cadmium (0, 20, 40, 80, 150 and 300 µg/L) in triplicate. The beakers were covered with aluminum foil, without aeration, unfed and kept in the incubator at 26 °C, for a duration of 96 hours and mortality was estimated daily.

Effect of cadmium on developmental duration, female lifespan, and total number of nauplii produced per female lifespan

A selected number of ovigerous females were incubated. Nauplii hatched within 24 hours were placed individually in 12-well plates containing 5 mL of medium spiked with 0, 5 (corresponding to No Observed Effect Concentration (NOEC) (refer to results of nauplii LC_{50}), and 40 µg/L Cd (corresponding to 50% lethal concentration for nauplii (refer to results of nauplii LC_{50})). They were monitored every 12 to 24 hours and fed with an uncontaminated diet once in 2 days sufficiently then transferred to a new medium of their respective treatments to permit exposure only from the contaminated water. Presence of molted exoskeletons and the time until they reached the adult stage were recorded.

C5 female and C6 male copepods were selected from the respective treatments in developmental duration tests. A single C5 female was paired with 2 male partners in a new medium with the same treatments (control, 5, and 40 µg/L Cd) in 15mL beakers and 10 replicates. They were fed as described in Ch.1 and frequently observed (every 12 or 24 hours). After spawning (formation of egg sacs) and hatching, the adults were transferred to

a new medium with the same treatment and the number of produced nauplii were counted. Dead males were replaced and the reproduction process was continued until the females died. The total number of nauplii produced by individual females from first clutch to death was enumerated, and the lifespan of the female was recorded.

4.2.2. Experiment 2: Chronic exposure of *P. annandalei* to cadmium

Effect of Cd on cohort populations and sex-specific bio-accumulation of Cd

The experiment consisted of dissolved metal exposure from water only (represented as WCd), a dietary metal exposure (represented as DCd) and control, following Hook and Fisher (2001) and Arias et al. (2016) with modifications. Copepod nauplii were collected by filtering the copepod stock cultures through a 120µm mesh filter and were set to acclimate at the experimental salinity and temperature conditions. 5 L culture containers were spiked with 40 µg/L of Cd (WCd), corresponding to a LC50 of *P. annandalei* nauplii for 12 hours before copepod stocking to allow a sufficient time for Cd to react with ligands which are naturally present in the water (Hook and Fisher, 2001). On the other hand, other sets of 5 L culture containers were prepared without Cd contamination (DCd and control). The copepods were transferred randomly into each of the experimental beakers. The algae (*Diacronema lutheri*) used to feed the copepods in the control and WCd were not contaminated with Cd. *D. lutheri* was inoculated and cultured in 15-20ppt and spiked with 40µg/L of Cd for 4 days and used for feeding copepods in DCd. The algae were centrifuged to remove the culture medium, re-suspended using the medium from the respective treatments, then fed to the copepods. This was done particularly in WCd, to prevent dilution of Cd concentration in the medium or the addition or Cd in the medium of DCd. The copepods were fed every 2 days, according to a standard feeding protocol of the laboratory (Beyrend-Dur et al., 2009; Souissi et al., 2016b). Moreover, algae were still present in observable amounts in their gut when observed under the microscope the next day after adequately feeding the previous day. Salinity 15 was used to culture *D. lutheri* because a preliminary test showed that low salinity could increase Cd uptake in *D. lutheri*. The amount of Cd in *D. lutheri* could bio-accumulate under 40µg/L of Cd exposure for 4 days, ranging from 8 µg/g to 11 µg/g dry weight (DW) throughout the experiment. During

inoculation, Conway medium was used and prepared without the addition of EDTA known to be a ligand that complexes metal ions, and can decrease the concentration of free Cd^{+2} in the solution (Grčman et al., 2001). Copepods were grown to adults at the above conditions, with periodic sampling at nauplius stage (day 3), copepodid stage (day 6), and adult stage. The culture medium was not changed throughout the experiment. When large numbers of ovigerous females were observed, males and females were sorted separately in triplicates for bio-accumulation analysis. The remaining copepods were counted to calculate population densities. The copepods were filtered through acid washed polycarbonate filter paper, rinsed slightly with milliQ water to remove salt and dried at 60^{o}C for 72 hrs and kept in a desiccator until further analysis.

Cadmium analysis

Dried filter papers containing experimental samples were digested for 3h at 120°C using 1 mL of nitric acid (Merck Suprapur, 65%) and 3 mL of hydrochloric acid (Merck Suprapur, 33%) in Teflon tubes. The digested solutions were diluted to a final volume of 10 mL with ultrapure water and stored at 4°C until analysis. Cadmium concentrations were measured in triplicate using GAAS (graphite atomic absorption spectrophotometry, Hitachi Z-5000, Japan). The data quality was assessed and validated using certified reference material samples (NASS-3) including bovine liver (NBS-SRM-1577), orchard leaf (NBS-SRM-1571).

Kinetic uptake model

The kinetic uptake model, expressing the concentration of metal uptake (µg/g/d), was calculated following Tlili et al. (2016) and Zidour et al. (2019):

$$C_{cp} = K_u C_w t \text{ and } C_{cp} = K_u C_D t$$

where C_{cp} is the cadmium concentration in copepod (µg/g dry weight), k_u is the uptake rate constant or the slope of the graph for water exposure ($L.g^{-1}$ (dry weight).day^{-1}) and (g (dry weight).g^{-1} (dry weight).day^{-1}; i.e. day^{-1}) for dietary exposure, C_w is Cd concentration in water (µg/L), C_D is Cd concentration in diet (µg/g dry weight), and t is exposure time (days).

Statistical analysis

Probit analysis was performed using Microsoft Excel 2016, and LC50 was calculated as described by Tlili et al. (2016). Mortality was corrected for probit analysis by using Abbot's formula. Data are expressed as the mean ± standard deviation (SD). Comparisons between means were made by one-way ANOVA, followed by a Tukey's test for identification of the statistically distinct groups. Significant differences were accepted for $p<0.05$. Statistical analyses were performed using SPSS, v.18.0 (SPSS Inc., Chicago, IL, USA).

4.3. Results

Figure 4.1A and 4.1B shows the 96 h concentration–mortality regression lines for *P. annandalei* naupliar and copepodid stages. Mortality increased with an increase in cadmium concentration. Mortality was probit transformed, and lethal concentration (LC) values extrapolated from regression lines. The lethal concentration that resulted in 50% of naupliar mortality was 40.3µg/L Cd and for copepodids it was 120.4µg/L Cd.

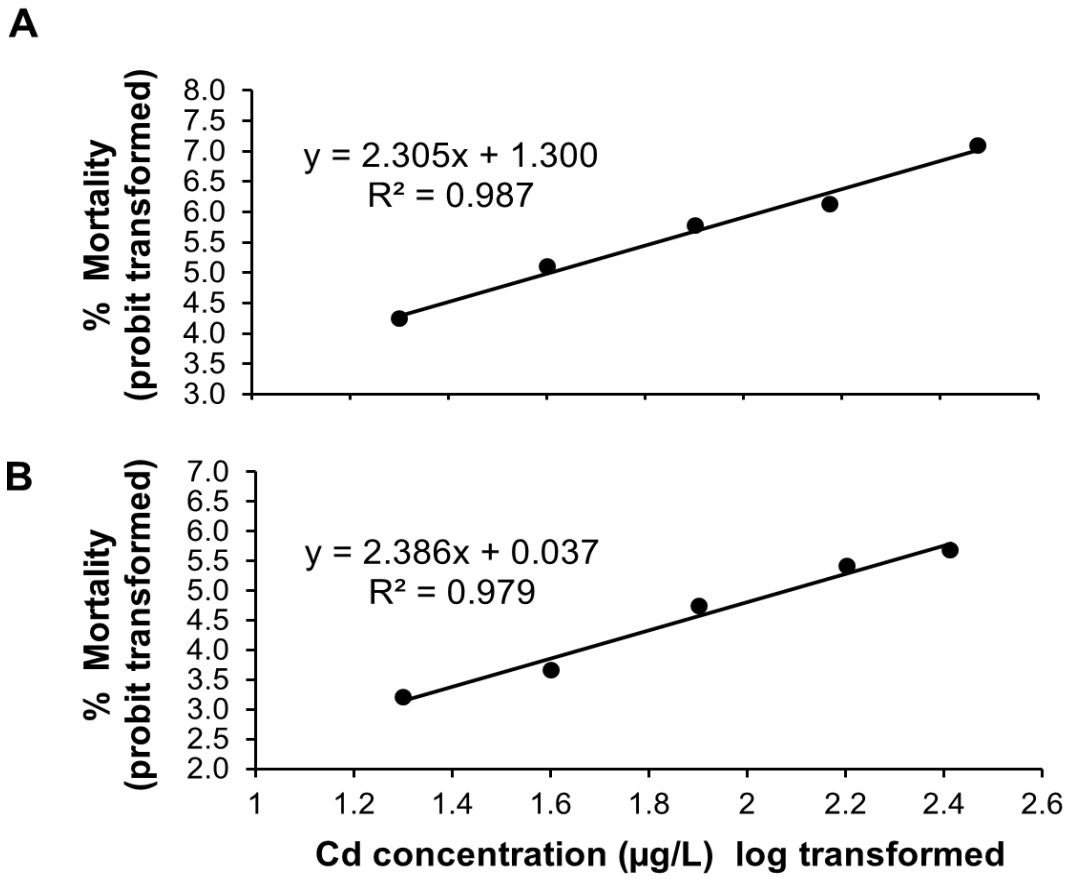

Fig. 4. 1. Ninety-six-hour concentration–mortality curves for *P. annandalei* nauplii (A) and copepodid (B) copepods exposed to cadmium.

Delayed development was observed in individual *P. annandalei* exposed to low (5µg/L) and high (40µg/L) Cd concentrations. Significant difference ($p<0.05$) between the treatments were observed from the early copepodid stages. Naupliar development from N1 to N3 was fast, however, under Cd treatments at least 1 or 2 days' delay was observed between each molt from N3 onward (Fig. 4.2). The duration of copepod development from N1 to C5 was 3.5 days and 5.5 days longer than in the control groups when exposed to 5µg/L and 40µg/L of Cd respectively. In addition, the difference in developmental delay (N1-C5) between high (40µg/L) and low (5µg/L) Cd concentration was 2 days. Mortality in both control and 5µg/L Cd groups were approximately 16% and in those exposed to 40µg/L Cd was 33%.

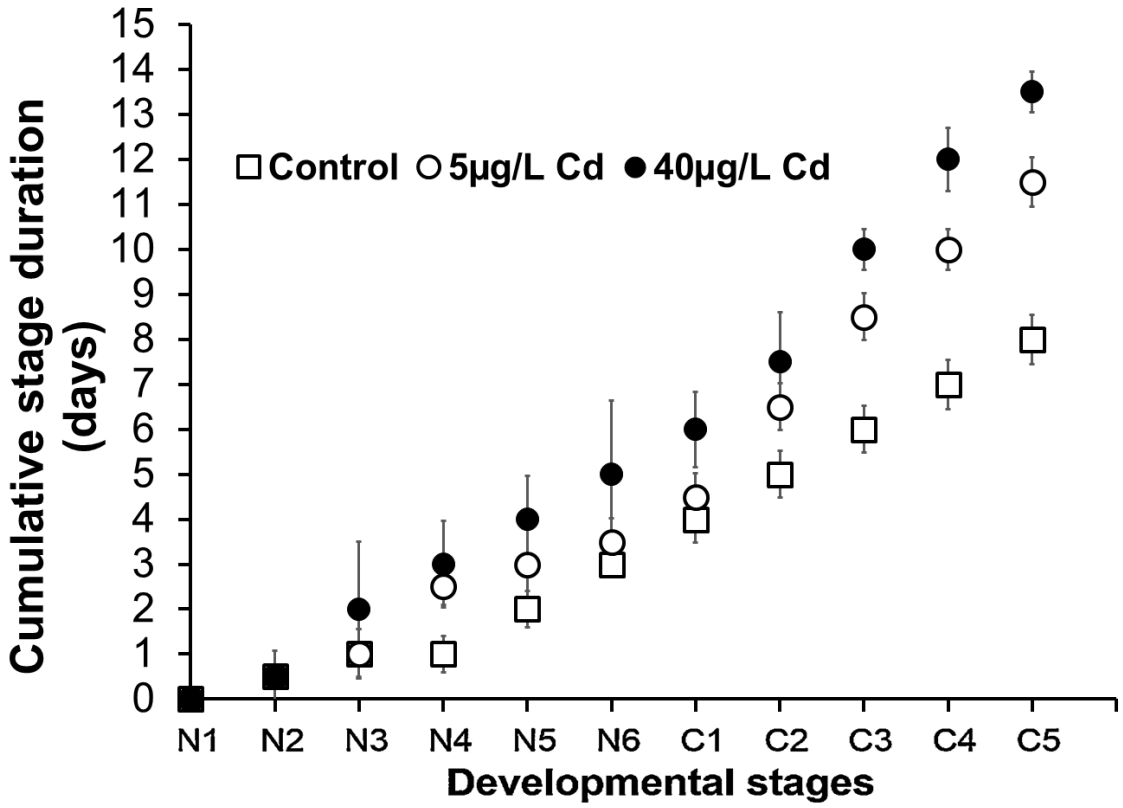

Fig. 4. 2. Developmental duration of *P. annandalei* exposed to 0 (control), 5 and 40 µg/L of cadmium.

Lifespan of female copepods under Cd treatment were lower than in the control, although not statistically significant ($p>0.05$). Female lifespan in the control treatment was

61.0±22.1 days, and was 52.4±17.6 and 53±19.9 days for those under 5 µg/L and 40 µg/L of Cd treatments respectively. The total number of nauplii produced by individual females per lifespan were 440.3±127.2, 450.7±65.2 and 365.7±73.4 in the control, 5 µg/L and 40 µg/L Cd treatments respectively, and were not statistically ($p>0.05$) different.

The life cycle bio-accumulation of Cd in *P. annandalei* copepods exposed to dissolved Cd and dietary Cd are presented in Figure 4.3A and B. After 2 days of exposure to dissolved Cd, the concentration of Cd in *P. annandalei* (late naupliar stages) was 25.81±2.86 µg/g DW, after 6 days (copepodid stages) it was 20.70±0.83 µg/g DW and at the adult stage, it was 20.17±2.02 µg/g DW (Fig. 4.3A). After 2 days of exposure to dietary Cd, the concentration of Cd in *P. annandalei* (late naupliar stages) was 2.15±0.80 µg/g DW; after 6 days (copepodid stages) it was 3.27±0.50 µg/g DW; and at the adult stage, it was 9.00±1.41 µg/g DW (Fig. 4.3B). Experimental calculation of the uptake rate constant (K_u) from aqueous solution and from diet are presented in Table 3.1.

Table 3.1. Uptake rate constant (K_u) calculated from the accumulation kinetics of Cd in *P. annandalei* exposed to dissolved Cd (WCd) and dietary Cd (DCd).

	WCd	DCd
Uptake rate constant K_u	-0.0836 ($L.g^{-1}.day^{-1}$)	0.3147(day^{-1})
r^2	0.7636	0.8575

Sex-specific uptake of Cd from dissolved Cd (WCd) and from dietary Cd (DCd) are shown in Figures 4.3C and D. Cd taken up from water resulted in a concentration of 22.53±2.05 µg/g DW in males and 18.99±2.01 µg/g DW in females. The differences between male and female in WCd were not statistically significant ($p>0.05$) (Fig. 4.3C). Dietary uptake of Cd resulted in a concentration of 11.82±4.35 µg/g DW in males and 7.59±1.41 µg/g DW in females. The differences between male and female in DCd were statistically significant ($p<0.05$) (Fig. 4.3D).

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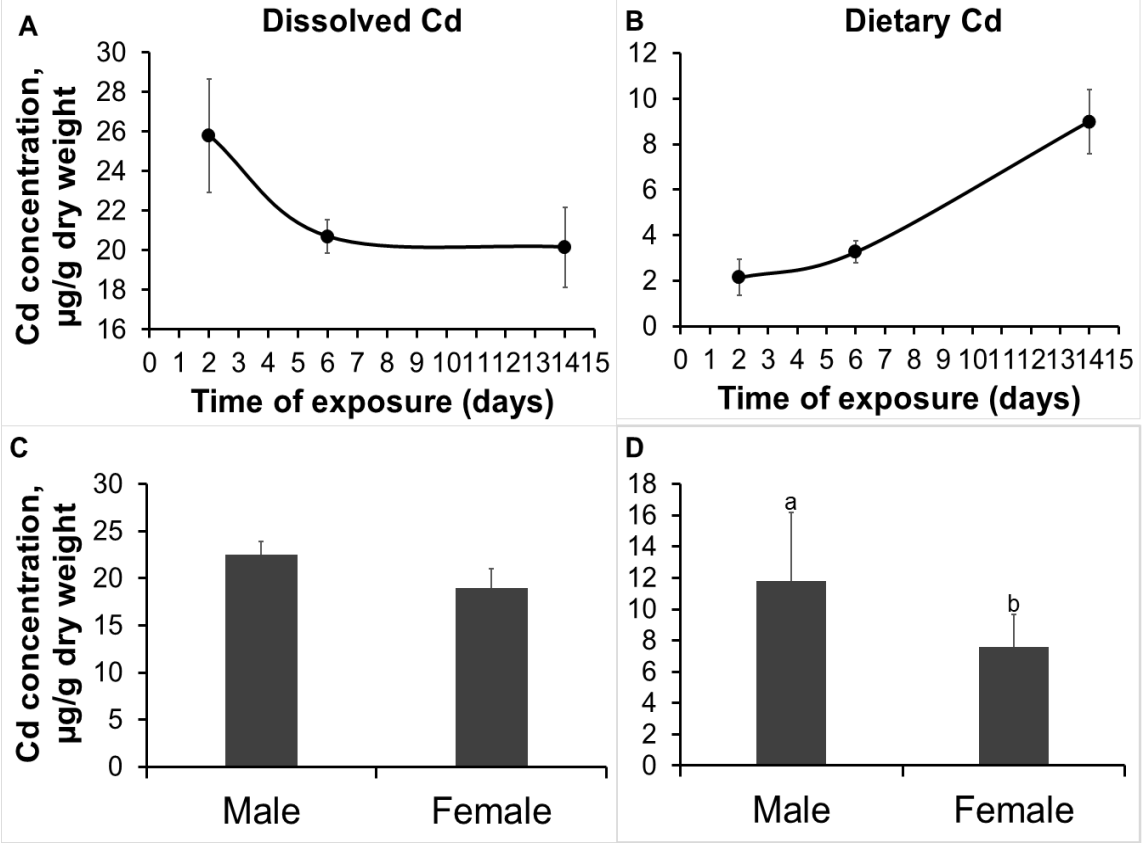

Fig. 4. 3. Cadmium accumulation kinetics in *P. annandalei* exposed to dissolved Cd (WCd) (A) and dietary Cd (DCd) (B) measured in copepod populations at different growth stage (A). Cd concentration in male and female copepods exposed to dissolved Cd (WCd) (C) and dietary Cd (DCd) (D).

Individual and total population of *P. annandalei* copepods exposed to Cd contaminated diet and dissolved Cd (water) are shown in Figure 4.4. The number of copepodids were significantly ($p<0.05$) lower in the control than in WCd. However, the number of male, non ovigerous and ovigerous female copepods were significantly ($p<0.05$) higher in the control, lower in DCd than in control but lowest in WCd. The total population of copepods followed a similar trend, where total population in WCd was significantly ($p<0.05$) lower than in the control group. In the control, the total number of males were significantly ($p<0.05$) higher than the copepodids and the females. In WCd, copepodids were significantly more abundant ($p<0.05$) than males and females.

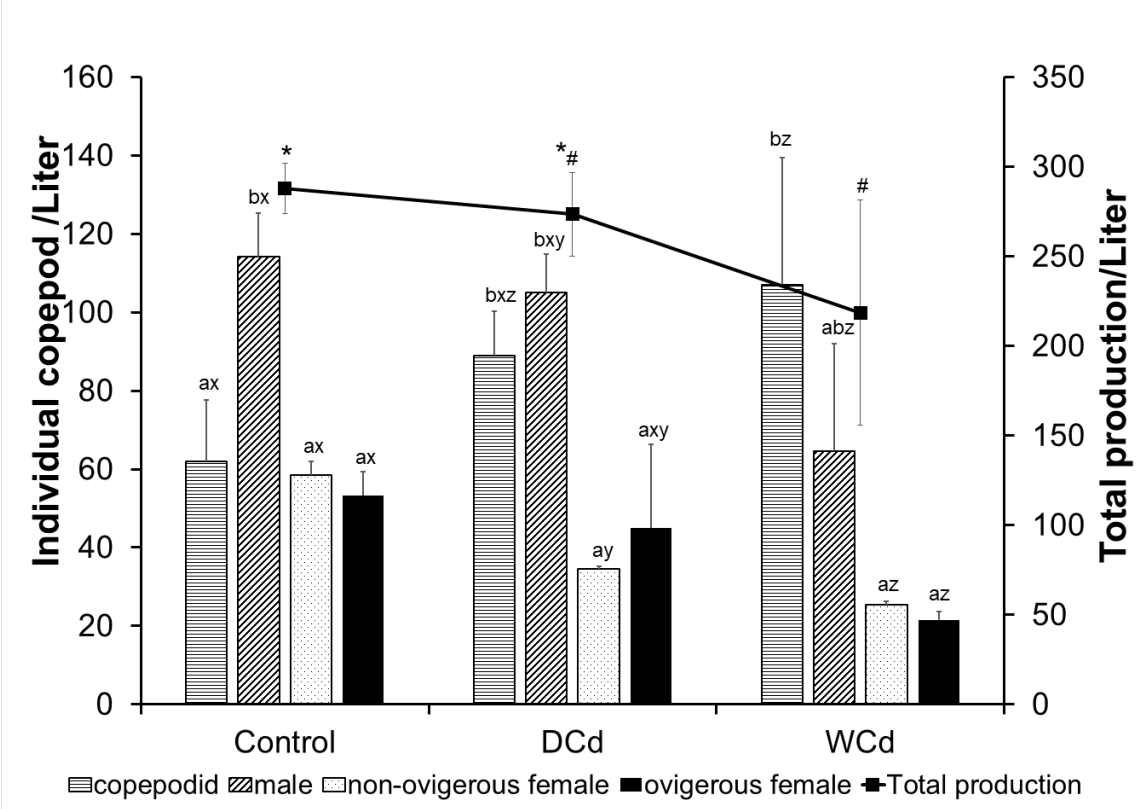

Fig. 4. 4. Individual and total population of *P. annandalei* copepod cohort exposed to a diet containing about 8.4 µg/L of Cd (DCd) and water containing 40 µg/L of Cd (WCd). x, y, z represents significant differences ($p<0.05$) between treatments (control, DCd, and WCd). a, b represents significant differences ($p<0.05$) between individual stages within a treatment (copepodid, male, non-ovigerous female, and ovigerous females). Asterisks (*) and hash (#) represents significant differences ($p<0.05$) between the particular production between treatments.

4.4. Discussion

Studies have shown that the response of copepods to different metal toxicity can be specific to sex (Bao et al., 2008; Lotufo and Fleeger, 1997; Stringer et al., 2012). We supported these findings in a previous study, where males of *P. annandalei* were shown to be more sensitive to cadmium toxicity (LC_{50} of 121 µg/L) than female copepods (LC_{50} of 240 µg/L) (Kadiene et al., 2017). In the present study, we showed that naupliar stages were more sensitive to cadmium toxicity (LC_{50} of 40 µg/L) than copepodid stages (LC_{50} of 120 µg/L). This confirms that toxicity of cadmium (Cd) is also specific to the stage of development. Similar findings were also reported, where nauplii were more sensitive (LC_{50}

of 17 µg/L) to cadmium toxicity than copepodids (LC_{50} of 20 µg/L) for the copepod *Tigriopus brevicornis* (Forget et al., 1998). Dahms et al. (2014) also showed that the early developmental stages of *Tisbe* sp. are more sensitive than advanced stages when exposed to effluents from hydrothermal vents, containing high concentrations of metals. Before this, Verriopoulos and Moraitou-Apostolopoulou (1982), reported that sensitivity of the copepod *Tisbe holothuriae* to cadmium toxicity depends on the stage of development. Furthermore, we showed that cadmium both at low and high concentrations delays the development of individual copepods. A similar delay in development was observed by Gnanamoorthy et al. (2012), in copepods, in sea urchin (*Paracentrotus lividus*) (Filosto et al., 2008) and in shrimp (Munshi et al., 1998). Exposure of *P. annandalei* nauplii to Cd during their developmental stages and further exposure of the female copepods till death negatively affected total number of nauplii produced and reduced female lifespan, which could be a consequence of increasing bio-accumulation of Cd over the exposure period. Similar findings were reported by Moraitou-Apostolopoulou et al. (1979), where an increasing concentration of cadmium affects the longevity of the copepod *Acartia clausi*.

In the present study, *P. annandalei* bio-accumulation of Cd from the dissolved phase was high at the earlier developmental stages of the copepod (nauplii). However, as they grew from nauplii to copepodids and to adults in a static system, Cd concentration in the copepod decreased. In contrast, dietary accumulation of Cd was low at the earlier stages of the copepod (nauplii). However, as they grew from nauplii to copepodids and to adults under periodic feeding of similar concentration of Cd contaminated algae, the Cd concentration in the copepod increased. The increasing Cd concentration with time/ developmental stages could be a result of increased feeding or ingestion rate (Almeda et al., 2010). Bio-accumulation of metals from the dissolved phase alone has been reported to be higher than from the consumption of metal-contaminated diets in mussels and copepods (Borchardt, 1983; Riisgård, 1987; Wang and Fisher, 1996; Wang et al., 1996; Wang and Fisher, 1998). The amount or rate of metal uptake from water can increase over time (Zidour et al., 2019), depending on the copepod stage, metabolism and rate of excretion. In this study, the decrease of Cd concentration in copepod exposed to dissolved Cd over time was probably a result of growth dilution (Borgå, 2013), that is, the decrease

in concentration of Cd with an increase in body size, hence the negative slope or uptake rate constant (K_u). However, the concentration of Cd in all developmental stages of copepod exposed to dissolved Cd were higher than those exposed to dietary Cd. This difference in accumulation could be due to different exposure routes and times, the concentration they are exposed to, the rate of uptake from each route, metabolic or excretion rate (Arunakumara et al., 2008; Berrojalbiz et al., 2009; Gomez et al., 2010; Jennett et al., 1983; Peake et al., 2015). The rate of Cd excretion is slower when copepods are exposed to Cd in water than to a Cd contaminated diet (Wang and Fisher, 1998; Williams et al., 2010; Xu and Wang, 2002). It has been demonstrated that assimilation efficiency of trace elements increases with longer gut passage time (Chong and Wang, 2000; Wang and Fisher, 1996; Xu, 2001). Moreover, the efflux rate of metals is higher following uptake from food than uptake from the dissolved phase in the copepod *Temora longicornis* (Wang and Fisher, 1998). In an egestion study with the branchiopoda *Daphnia magna* and the insecta *Chironomus riparius*, Scherer et al. (2017) found that an exposure to food led to a shorter gut evacuation period of polystyrene spheres. This could imply that with a shorter gut evacuation time, the possibility of reducing or removing toxic pollutants is high. Therefore, the longer the organisms are exposed to contaminants especially from the dissolved phase, the more the contaminants are accumulated (Wallenstein et al., 2009), consequently increasing their toxic effects (Ensibi and Yahia, 2017; Javanshir, 2012; Kadiene et al., 2017). Furthermore, Cervetto et al. (1995) reported that the gut evacuation rate in males was slower than in female copepods. This could explain why the concentration of Cd in male copepods was higher than in female copepods under both exposure routes in the present study. Although, in this study, the Cd concentration in males and females of *P. annandalei* exposed to dissolved Cd were not statistically different, the males were reported to be more sensitive to Cd toxicity than the females in an earlier account (Kadiene et al., 2017). The expression of metallothioneins, an heme-binding protein, is induced when an organism is exposed to metal, their expression is involved in metal homeostasis and detoxification (Amiard et al., 2006; Liberge and Barthélémy, 2007; Wang and Wang, 2010) and could also be expressed in a sex-specific manner. This would mean that the proteins responsible for metal detoxification and elimination of metals maybe

more effectively expressed in females than in male copepods. However, this hypothesis needs to be verified in future investigations by using adequate molecular tools.

The high accumulation observed in copepods exposed to dissolved Cd, more significantly affected individuals and the total population of copepods than when fed a Cd contaminated diet (DCd). The number of copepodids in WCd was higher than in DCd and both were higher than the number of copepodids in the control. This means a large number of copepods stayed longer in the copepodid stage, which corresponds to the delay observed in the individual copepod development in experiment 1 (see Fig. 4.2). In addition, the total copepod population at Cd exposure was lower, probably as a result of high mortality in all stages prior to the adult stage, particularly at the naupliar stage, since the concentration of Cd in water was the LC_{50} of *P. annandalei* nauplii.

In conclusion, bio-accumulation of Cd is dependent on the uptake route, and its toxic effects are dependent developmental stages and can be sex-specific. The long-term implication of a persistent pollutant like Cd in the aquatic environment is of significant concern to copepod population structure. A better understanding of the effect of metals to the population structure of copepods can be demonstrated through long-term or multigenerational approach. Such attempts will not only provide risk assessment of the largest mesozooplankton group in terms of biodiversity and abundance, being a critical mediator, if not keystone taxon between primary producers and secondary consumers. The life history study of copepods as an example can also provide new insights in the use of population issues in the use of model species for ecotoxicology. Such data are particularly useful when they are modelled to provide generalized predictions about single species population effects that in turn will affect community structures in the future.

Chapter 5.

Bioaccumulation of metals in calanoid copepods by oral intake

Kadiene, E.U., Ouddane, B., Hwang, J.S. and Souissi, S., 2019. Bioaccumulation of metals in calanoid copepods by oral intake. Scientific reports, 9(1), p.9492.

5.1. Introduction

The properties of toxic chemicals with respect to their water-solubility (hydrophobic or soluble) play a major role on how they interact with aquatic organisms. For example, when aquatic organisms are exposed to hydrophobic chemicals present in diet and water, diet-borne uptake becomes important since these chemicals are difficult to dissolve in water. However, even for a hydrophobic chemical like 4-nonylphenol, it was suggested that major uptake may also occur through water in the amphipod study (Gross-Sorokin et al., 2003). This makes water an important uptake source for toxicants. Processes involved in the water-borne uptake of toxic chemicals include filtration, passive or facilitated diffusion, active transport or phago/pinocytosis (Timbrell, 2002).

Copepods accumulate metals by assimilating them from their food or by absorbing them from water. Furthermore, the uptake pathway can determine its internal distribution and toxic action (Wang and Fisher 1998). Several studies proposed that direct uptake of metals from water occurs by either adsorption to cell, tissue, organ, or organism surfaces, or via the absorption across cell membranes or organ epithelia such as the gill and/or gut (Carpene and George, 1981; Causen et al., 1993; Sidoumou et al., 1997). Other studies showed that the accumulation of metals such as cadmium from water is higher than from food (Borchardt, 1983; Riisgård et al., 1987; Wang and Fisher, 1996; Wang et al., 1996; Wang and Fisher 1998). Cailleaud et al. (2007), suggested that pollutant uptake by planktonic species is governed by particular mechanisms and not only by adsorption and equilibrium partitioning between water and organisms. Gomes et al. (2004), indicated that uptake of toxic chemicals such as Estrone in *Daphnia magna* via the trophic route is likely to be less significant compared to bioconcentration from the aqueous medium.

Copepods are essential trophic links in marine food webs. Therefore, they can be a major source for biomagnification of toxic pollutants in aquatic food webs (Fisher et al., 2000; Watras et al., 1998). Feeding behaviour has long been studied in calanoid copepods and their mode of feeding can be passive or active. They can switch between the two modes at intervals depending on the composition of their food (Kiørboe et al., 1996; Schnack, 1989). Copepod feeding involves generating feeding currents by the beating of locomotory

appendages, and capturing of food items that arrive with this current (suspension feeding) (Fenchel, 1986; Kiørboe et al., 1996; Strickler, 1982), or ambush feeding, where passing preys are detected and captured in surprise attacks (Jonsson and Tiselius, 1990; Svensen and Kiørboe, 2000), or when food particles collide with feeding appendages (Costello et al., 2008). During feeding, the first three mouth appendages (antennae, mandibular palps and maxillules) create a backward motion of water with a metachronal beating pattern, and an asymmetrical vortex system is created on the ventral side of the animal (Van Duren and Videler, 2003). The motion and feeding behaviour shown in the foraging tactic of *Clausocalanus furcatus* explores small volumes of water rapidly (Mazzocchi and Paffenhöfer, 1999). Koehl and Strickler (Koehl and Strickler, 1981), showed that calanoid copepods do not strain algae out of the water as previously reported (Barnes, 1980; Lam and Frost, 1976; Lehman, 1976; Russell-Hunter, 1979). Rather, they flap four pairs of feeding appendages to propel water past itself and use its second maxillae to capture selectively parcels of that water containing food particles, which are then, pushed into the mouth by the endites of the first maxillae.

The study of copepod behaviour and feeding strategies has been of ecological importance for understanding the role of zooplankton in carbon and energy transfers through the aquatic food web and how these behaviours enable them to utilize different ecological niches. Since feeding pattern of copepods could involve water intake (Koehl and Strickler, 1981), the present study demonstrated that oral intake of water by two calanoid copepods, *Pseudodiaptomus annandalei* and *Eurytemora affinis* takes place and has implications for their ecotoxicology. Our hypothesis is that metal uptake from water is a more important route in the bioaccumulation of metals than through dietary route because of oral intake.

5.2. Materials and Methods

In order to demonstrate the oral intake of water by copepods, we first added a local food dye solution (blue colour) dissolved (see Supplementary Movies in publication) in water with male and non ovigerous female of *Pseudodiaptomus annandalei* and

Eurytemora affinis copepods. They were unfed for more than 24 hours before the start of the experiment. The blue dye was composed of distilled water, propylene glycol, FD&C Blue 1, FD&C Red 40, and 0.1% propylparaben (preservative). This allowed us to visually examine the possibility of water taken up by the copepod in an attempt to better understand the process of metal bioaccumulation in copepods. After introducing the dye, they were left for five minutes to allow dyed water to be taken up. The copepods were then rinsed to remove the food dye and placed in another clean medium to examine the intake and ejection rate of the dye. The copepods were observed live under the microscope (Olympus BX51 and SZX10, Tokyo, Japan) and the behaviour was recorded by video.

In the second experiment, we investigated the implication of oral intake of water by copepods in metal bioaccumulation. Both *E. affinis* and *P. annandalei* copepods were filtered through 200 µm mesh filter (contained large copepodids and adults) from a large stock culture, and acclimated to the experimental conditions. *E. affinis* were cultured at 19°C ±1 and *P. annandalei* at 26°C ±1 and in a medium of salinity 15 for both copepod species. The copepods were concentrated in a beaker at a fixed volume. After homogenizing, equal amounts were distributed randomly into 5-liter beakers containing 4 litres of the treatment medium in duplicates.

Both copepod species were exposed to a mixture of 3 metals, copper (Cu), nickel (Ni) and cadmium (Cd) in the water and through their diet. *E. affinis* and *P. annandalei* were both exposed to the same sublethal concentrations, approximately $1/5^{th}$ 96hr LC50 of each metal (Zidour et al., 2019) in the water (analysed concentration: Cd, 17 µg/L; Cu, 13.8 µg/L; Ni, 29.3 µg/L). The algae used was *Rhodomonas salina*, cultured with the mixture of 10x the 96hr LC50 (Zidour et al., 2019) of each of the above metals in Conway media (without EDTA) for 4 days before they were fed to the copepods, in other to achieve similar amount of metal as in the water exposure. The bioaccumulated concentration of the metals analysed in the algae were Cd: 18.8 µg/g; Cu: 35.3 µg/g; Ni: 32.5 µg/g (Table 5.1). Both copepod species were exposed to the mixture of metals in water only (and fed uncontaminated algae) and also to a mixture of metal contaminated algae only. They were sufficiently fed in both conditions twice at 1 hour intervals. The algae were centrifuged and

rinsed with distilled water to remove all the metals in the culture water, to allow for metal uptake only from the contaminated diets. Approximately 4 hours later, half of the culture volumes were collected and filtered through 200µm mesh filter to remove unwanted particles and then filtered through high quality filter papers to retain the copepods. The other halves were filtered through 200 µm mesh filter and transferred to new media without food and without contamination for approximately 2 hours. After which, the copepods were collected as before. Filter papers containing copepods and algae were dried in the oven at 70°C for 72 hours and water samples were preserved with pure nitric acid for further analysis. Copepod samples were mineralized with 3 ml of ultrapure nitric acid (HNO_3) at 105°C for 2 hours in a hotplate. After dilution with pure water, inductively coupled plasma optical emission spectrometry (ICP-OES) was used to analyse the metal concentrations in the copepod, algae and water samples. Data were expressed as the mean ± standard deviation (SD). Multiple comparisons between means were made by one-way ANOVA for identification of the statistically distinct groups within each copepod species. Then, Student t-test was applied to compare the uptake of metals and the residual metals after depuration between both copepod species. Significant differences were accepted for $p<0.05$. The statistical analyses were performed using SPSS, v.18.0 (SPSS Inc., Chicago, IL, USA).

Table 5. 1. Metal concentrations (µg/L) in water before exposure, after 4 hours' exposure to mixture of dissolved metals (Water) (Cadmium (Cd); Copper, Cu; Nickel (Ni)) and dietary metals (Diet) and after 2 hours' depuration in uncontaminated water

	Water (µg/L)			Diet (µg/L)		
	Initial conc. in water	After 4hrs. Uptake	After 2hrs. Depuration	Initial conc. in algae (µg/g)	After 4hrs. Uptake	After 2hrs. Depuration
E. affinis						
Cd	16.90±0.14	16.50±0.21	0.20±0.07	18.82±2.02	0.20±0.14	0.20±0.00
Cu	12.70±1.48	10.90±0.64	1.50±0.28	35.27±7.22	1.60±0.00	1.70±0.14
Ni	29.10±0.31	26.83±0.85	< DL	32.45±1.09	0.85±0.35	0.50±0.14
P. annandalei						
Cd	17.10±0.07	16.80±0.11	0.30±0.04	18.82±2.02	0.25±0.07	0.15±0.07
Cu	14.80±0.74	10.00±0.32	1.10±0.14	35.27±7.22	1.25±0.35	1.80±0.42
Ni	29.53±0.15	28.03±0.42	0.90±0.30	32.45±1.09	1.00±0.14	0.40±0.42

5.3. Results and Discussion

Oral intake was tested in both males and females of *P. annandalei* and *E. affinis* copepods. However, only the videos of females of each copepod species were presented. Moreover, the observed results of oral intake of water were the same in both sexes of the two copepod species.

P. annandalei copepod kept unfed for more than 24 hours showed a clear gut (Fig. 5.1a) and after few minutes of adding dye to the medium, we observed that the dye was taken up and kept in the midgut. With increased water intake, the gut dilated (Fig. 5.1b). *P. annandalei* was observed to move the dye toward the hindgut for excretion (see Supplementary Movie in publication). Figure 5.2 shows the dye contained in the copepod gut after the dye medium was replaced by clear water, although some amount of dye solution was excreted in the process. Approximately 30 minutes after the transfer of copepods to clear medium, further movement of the dye towards the hindgut was observed. The movements were aided by peristaltic contractions and forward and backward movements. Figure 5.3 shows large amounts of dye solution being excreted from the anus of the copepod following the egestion of a faecal pellet. It appeared that more ambient water was taken in orally (indicated by the increased size of the gut and lighter colour of the dye in the gut) (see Supplementary Movies in publication).

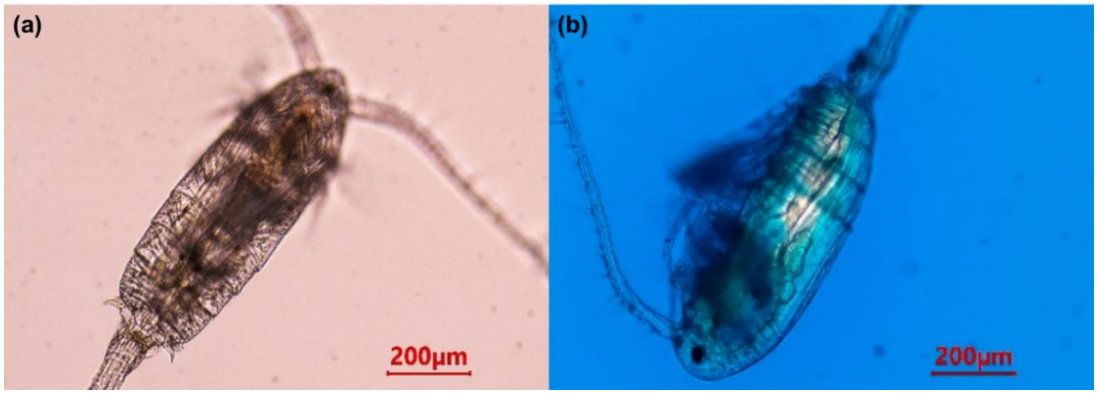

Fig. 5. 1. Non-ovigerous female of *Pseudodiaptomus annandalei* before exposure (a), showing a clear gut and during exposure to dye (b).

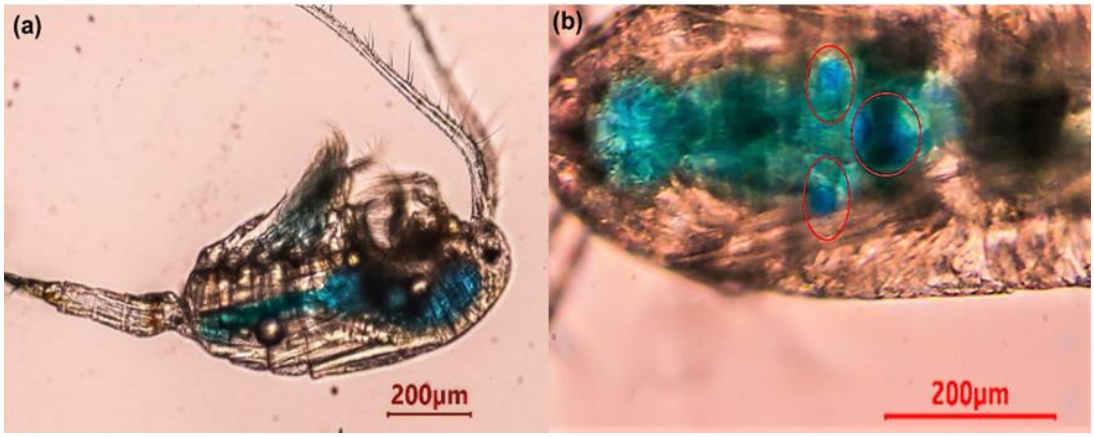

Fig. 5. 2. Non-ovigerous female of *P. annandalei* after exposure to dye, showing dye color in the gut (a), and patches of the dye concentrated around the midgut (b).

Fig. 5. 3. Non-ovigerous female of *P. annandalei* after exposure to dye, showing movement of the dye towards the urosome and excretion from the anus after ejection of a fecal pellet.

Several studies showed that copepods create feeding currents for trapping and selectively taking in food particles into their mouth opening (Dhanker et al., 2012; Goncalves and Kiørboe, 2015; Mazzocchi and Paffenhöfer, 1999). Fox (1952), thought that a continuous rhythmic swallowing of water through the mouth was part of the feeding mechanism of filter-feeding or vortex-feeding Crustaceans. Since the limbs are continuously collecting unicellular algae or detritus suspended in the water, it must be swallowed continuously. Food particles were not present in the medium in the first experiment and even though they did not often employ the use of their appendages, the dye solution was still taken in with each movement of the gullet. To observe them under the microscope without the copepod moving too fast away from the field of view, the surrounding water was reduced after few minutes to a sufficient volume; hence, less movement of the appendages were observed. Therefore, the peristaltic contractions of the gut and the gulping movement of the labrum during the vibratory movement of their appendages and even without their movement, brought water into the copepod as indicated by the dye. This implies oral intake of water by the copepod may not only be a feeding mechanism. Fox (1952), observed that in most crustaceans that he studied, the uptake of water was continuous, rapid and vigorous and the gulps of water were large. In the first experiment, the coloured water could easily be seen in the gut because the copepod is transparent enough to show the differences before, during and after staining (Fig. 5.1 and 5.2). Fox (1952), also observed that the rhythmic oral intake of water by the cladocerans *Daphnia* and *Limnadina* showed each gulp been passed down the gullet corresponding to a movement of the jaws and muscle contractions. Weismann (1874), described the function of oral drinking as a respiration process. Moreover, Fox (1952), described it as a mechanism of feeding, stretching the muscles of the gut wall. The contractions maintained by the hydrostatic pressure of water pumped into midgut mixes food and digestive enzymes. Defecation occurs when this pressure rises to a certain level, forcing the food in the midgut back towards the rectum. In the present study, similar rhythmic oral water uptake was observed (see Supplementary Movies in publication).

A dye test by Fox (1952), demonstrated that *Daphnia* after exposure to a dilute solution of bromo-thymol blue or nigrosin for a few hours, the dye was concentrated about 250-fold

in the intestine and the process of accumulation was rapid. Similarly, we observed dye-accumulation in our test (Fig. 5.2b (red circles)). The explanation for this phenomenon was that water was withdrawn through the gut wall from the solution. However, the dye concentrating around the midgut indicates adsorption by the gut epithelium. In experiment 1, another test was done to check the rate of dye evacuation in *P. annandalei* and was compared with *E. affinis*. Following their exposure to the dye solution, they were placed in clear medium and left for approximately 1 hour. When observed under the microscope, the dye solution in the gut of *P. annandalei* was cleared out except for the dye colour concentrated around the midgut (Fig. 5.4a, see Supplementary Movie 5.1). Whereas in *E. affinis* large remnants of dye solution could still be observed (Figure 5.4b, see Supplementary Movie 5.2). *P. annandalei* is a tropical species that is cultured in the laboratory at temperatures ranging from 25° to 28°, whereas *E. affinis* is a temperate species cultured in the laboratory at temperatures ranging from 18° to 20°. It has been reported that temperature is positively correlated with gut evacuation rate (Booth, 1990; He and Wurtsbaugh, 1993), this could explain why *P. annandalei* showed a faster rate of dye evacuation from the gut than did *E. affinis*. Gut contamination is a major source of variation in measured whole-body concentrations of several elements (Elwood et al, 1976). It has been demonstrated that assimilation efficiency of trace elements increased with longer gut passage time (Chong and Wang, 2000; Wang and Fisher, 1996; Xu, 2001; Xu et al., 2001). Moreover, the efflux rate of metals was higher following uptake from food than uptake from the dissolved phase (Wang and Fisher, 1998). In an egestion study with *Daphnia magna* and *Chironomus riparius,* Scherer *et al*. (2017), found that an exposure to food led to a shorter gut evacuation period of polystyrene spheres. This could imply that with a shorter gut evacuation time, the possibility of reducing or removing toxic pollutants is high. Therefore, the longer the organisms are exposed to contaminants especially from the dissolved phase, the more the contaminants are accumulated (Wallenstein et al., 2009), consequently increasing their toxicity (Ensibi and Yahia, 2017; Javanshir, 2012; Kadiene et al., 2017).

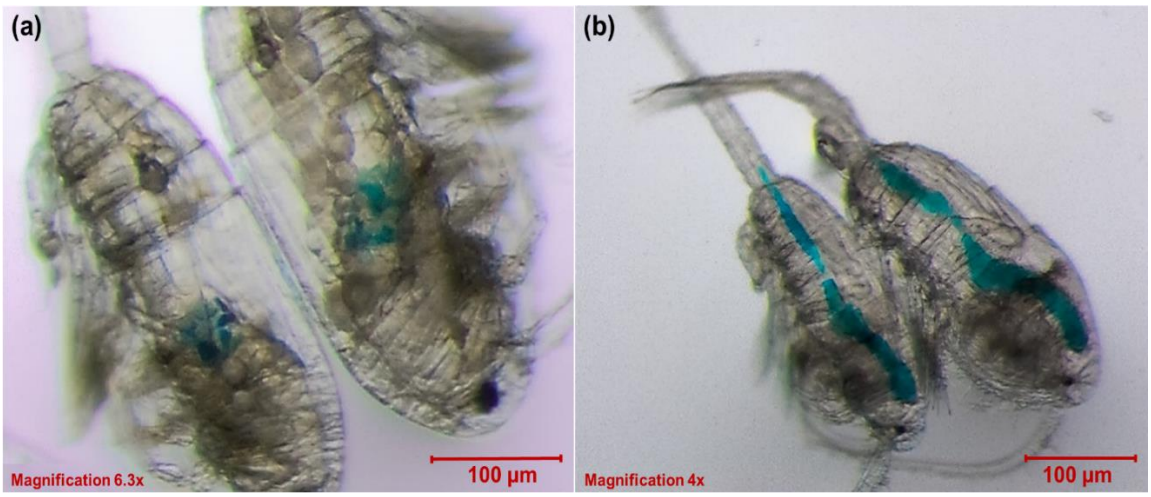

Fig. 5. 4 *Pseudodiaptomus annandalei* (a) and *Eurytemora affinis* (b) male and female copepods showing the dye stain (blue) concentrated in their midgut after 1hr in clear water, following dye exposure.

Figure 5.5a and c shows *E. affinis* female and *P. annandalei* male copepods with algae *Rhodomonas salina* in their guts in the second experiment few minutes after feeding. If copepods were to selectively take in food particles into their mouth as previously reported (Dhanker et al., 2012; Goncalves and Kiørboe, 2015; Mazzocchi and Paffenhöfer, 1999), then it may take a longer time for this amount of algae, indicated by red colour, to fill their guts. However, because the density of the algal cells was high in the medium, the resulting coloration (volume) of algae in their gut within few minutes of feeding (Fig. 5.5a and c) together with the dye intake in the first experiment without the presence of algae implies that water is taken into the copepods orally. In addition, after few hours of feeding *R. salina* to the copepods, discoloured food particles were observed in the gut (foregut). However, there were concentrations of red pigments possible digested or absorbed from the fed algae located in the midgut (Fig. 5b and d), similar with the dye concentrated in the copepods from the first experiment (Fig. 5.2b (red circles) and 5.4a). Similar absorption might also take place in the case of metals (Carpene and George, 1981; Clausen et al., 1993; Sidoumou et al., 1997).

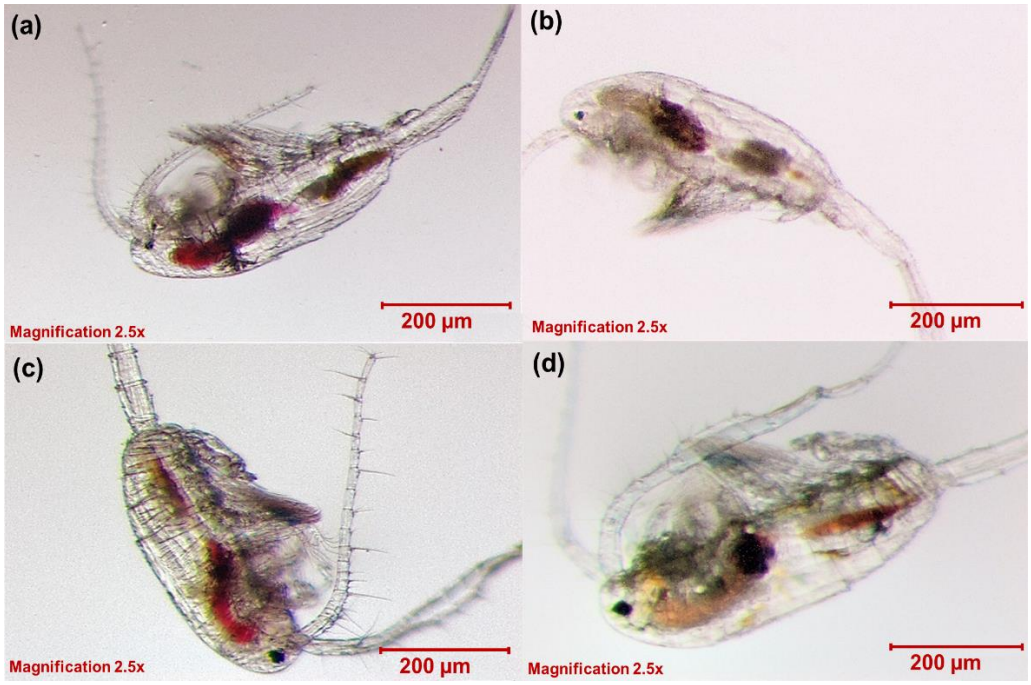

Fig. 5. 5. *Eurytemora affinis* female copepod few minutes after feeding with *R. salina* (a) and few hours later after last feeding (b). *Pseudodiaptomus annandalei* male copepod few minutes after feeding with *R. salina* (c) and few hours later after last feeding(d).

The toxicity of environmental pollutants to aquatic organisms depends among others, on route of exposure or entry (Tchounwou et al., 2012). It is commonly assumed that heavy metals enter copepods passively through diffusion across biological membranes (Bienvenue et al., 1984; INAP, 2002; Li et al., 2017; Mason et al., 1996). Our first experiment demonstrated that dye was taken in by the copepods orally in large amounts. Therefore, the implication of oral intake of water by copepods in this study shows that dissolved metals besides taken in through membranes, could also be actively taken in larger amounts orally. We demonstrated this in the second experiment, by exposing the copepods to a mixture of metals in water only and through their diet with similar concentrations. We observed that metal uptake from the dissolved phase was significantly higher (p<0.05) than metal uptake from the contaminated diet in both copepod species, even when the exposed concentrations in water was lower than those bioaccumulated in the diet (Fig. 6). Similar findings have also been reported (Borchardt, 1983; Kadiene et al., 2019; Riisgård, 1987; Wang and Fisher, 1996; Wang et al., 1996; Wang and Fisher, 1998). This could be a result

of oral intake of the medium, since this intake is frequent and in large gulps, they are constantly being exposed to the metals in the water.

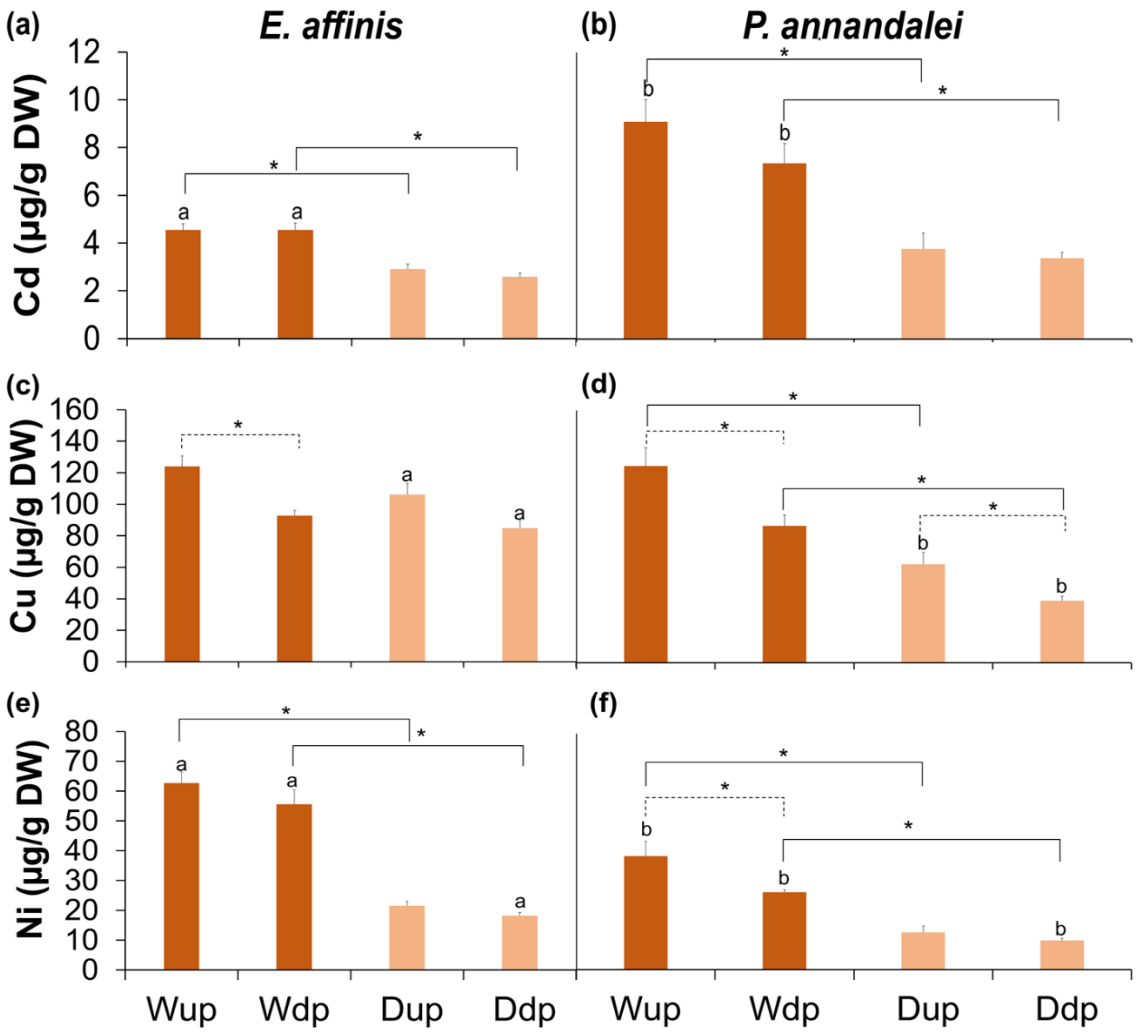

Fig. 5. 6. Concentration of metals in copepod after 4 hrs. uptake (Up) and after 2 hrs. depuration (Dp) from water (W) and diet (D: contaminated algae). Significant differences at $p< 0.05$ after uptake from water vs uptake from diet and after depuration from water vs depuration from diet exposures are presented as smooth brackets and asterisks (*) in *E. affinis* and *P. annandalei*. Significant differences at $p< 0.05$ after uptake from water vs depuration from water and after uptake from diet and vs depuration from diet exposures in *E. affinis* and *P. annandalei* are represented as broken brackets and asterisks (*). Significant differences at $p< 0.05$ after uptake from water and diet and after depuration from water and diet between *E. affinis* and *P. annandalei* are represented as alphabet a and b.

In the first experiment, it took longer for *E. affinis* to clear out the dye solution and in the second experiment, during depuration, metals excreted from *P. annandalei* copepods were more than those excreted from *E. affinis* copepods exposed to dissolved metals. However, metals excreted from both copepod species exposed to dietary metal were similar (Table 5.2). Since oral intake of water is continuous, after been transferred to a clean medium, metal concentration decreased because clean water is exchanged with the contaminated water. Nevertheless, the amount of metals retained after 2 hours of depuration from exposure to water were still significantly higher than the metals retained from dietary exposure (Fig. 5.6). Uptake and excretion of metals by copepods can be specific to the kind of metals they are exposed to (Fang et al., 2014; Hsiao et al., 2010; Luoma and Rainbow, 2005). In this study, the order of the concentration of the metal mixture in dissolved phase that the copepods were initially exposed were Ni>Cd>Cu, however, in both copepod species, the order of highest metal uptake were Cu>Ni>Cd. And the order in which the mixed metals were bioaccumulated in the algae fed to the copepods were Cu>Ni>Cd, and the same order was taken up by both copepod species in terms of concentrations. Although, the concentration of copper (Cu) in the dissolved metal mixture at the beginning was the lowest (13 µg/L) among the 3 metals, however, it was the metal with the highest uptake from the dissolved phase. Moreover, the same concentration order of metals in the diets was in both copepod species. In addition, Cu was considerably the most excreted in both copepod species exposed to metals in water, and Cd the least. The lower gut evacuation rate of *E. affinis* could possibly make them more sensitive to metal toxicity. Kadiene *et al*. (2017), reported that *P. annandalei* was more tolerant to metal toxicity than *E. affinis* exposed to cadmium. This could further support the idea that physiological characteristics of copepods could affect the tolerance levels of pollutant toxicity.

Table 5. 2. Difference between the concentration of metals taken up by *Eurytemora affinis* and *Pseudodiaptomus annandalei* copepods (µg/g DW) after 4 hours' exposure to mixture of dissolved metals (water) (Cadmium (Cd); Copper, Cu; Nickel (Ni)) and dietary metals (Diet) and the concentration left in both copepods (µg/g DW) after 2 hours' depuration in uncontaminated water. Values are mean ± s.d.

	Water		Diet	
	E. affinis	*P. annandalei*	*E. affinis*	*P. annandalei*
Cd	0.03±0.00	1.74±0.01	0.34±0.02	0.40±0.01
Cu	31.20±3.37	37.92±4.80	21.29±1.18	23.27±4.57
Ni	7.08±0.06	12.25±4.23	3.37±0.30	2.75±0.50

In conclusion we state here that both copepod species, *P. annandalei* and *E. affinis*, take in water orally. We suggest that this biological characteristic has an implication for the active accumulation of dissolved metal. In addition, the higher rate of gut evacuation shown by *P. annandalei* could be an adaptive mechanism of excretion of toxic pollutants. Our study showed that metal uptake depends on the exposure routes and the uptake and excretion rates are dependent on the type of metals, amounts and the species.

Chapter 6.

Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to cadmium exposure

Esther U. Kadiene, Baghdad Ouddane Hong-Yi Gong, Min-Sub Kim, Jae-Seong Lee, Yen-Ju Pan, Jiang-Shiou Hwang, Sami Souissi. (In Prep.). Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to cadmium exposure.

6.1. Introduction

Cadmium is known to cause oxidative stress and DNA and cellular damages at prolonged exposure and elicits other biochemical responses in fish and crustaceans (Almeida et al., 2009; Drąg-Kozak et al., 2019; Hamilton et al., 2015; Jia et al., 2011; Wang et al., 2013) and in copepods (Ensibi and Yahia, 2017; Wang and Wang, 2009). However, at concentrations that exceed the physiological threshold, these metals may cause disease. In addition, toxic transition metals, such as cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg), accumulate in the body that leads to neurological and physiological dysfunction and disease (Harrington et al., 2012). Cadmium (Cd) generally has no known biological function in many organism and is of concern as an environmental contaminant due to its extreme toxicity, ability to cause mutations (McMurray and Tainer, 2003), and potential carcinogenicity in higher animals (Beyersmann, 2002.). Although Cd is not a redox active metal, it has been speculated that Cd causes damage to cells primarily by the generation of reactive oxygen species (ROS) (Stohs and Bagchi, 1995), which causes single-strand DNA damage and disrupts the synthesis of nucleic acids and proteins (Mitra and Bernstein 1978). Cd is also an inhibitor of the DNA mismatch repair system (Jin et al., 2003, McMurray and Tainer 2003).

Acute exposure to cadmium resulting to stress in copepods has commonly more striking effects than chronic exposure, and it is due to induced adaptation mechanisms under prolonged exposure (Patra et al., 2011). The goal of ecotoxicology is to understand how pollutants disrupt normal biological processes, and how regulatory measure can be devised to reduce or prevent adverse or compromising effects on the ecological fitness of individual organisms or populations. In other to achieve this goal, integration of studies involving the analyses of toxicity responses at the transcriptomic, proteomic and metabolomic levels are important and this approach is referred to as ecotoxicogenomics (Snape et al., 2004). Molecular tools including the use of environmental gene expression techniques enable the evaluation of transcriptional changes in copepods in order to better understand the mechanisms of action of toxic metals.

Cellular stress response (CSR) of organisms to toxic chemicals involves the induction of stress proteins to prevent, reduce or repair damages to cellular macromolecules, regulation of energy budgets or metabolic resources, the delay or arrest of the cell cycle; and apoptosis (Logan and Buckley, 2015; Roncalli et al., 2016). Generally, exposure to xenobiotics triggers similar genes which are typical of the cellular stress response leading to the activation or an increase in their expression, thereby engaging in the metabolism and elimination of these xenobiotics and/or other cellular stresses arising from the presence and toxicity of such xenobiotics (Xu et al., 2005). Understanding the transcriptomic response of an organism to an environmental stressor provides information on the extent to which stress responses are induced and which physiological functions are induced by such stress responses, indicating whether they are components of the universal stress response or specific to that stressor, species or sex (Bailey et al., 2017; Legrand et al., 2016). Altered gene expressions can indicate stress but also the ability to deal with stressors, these responses could be beneficial or maladaptive (Bailey et al., 2017). Several differentially expressed genes in response to a stressor maybe synergistic, complementary or sequential (De Nadal et al., 2011; De Wit et al., 2015; Evans, 2015; Gibney et al., 2013; Giaever and Nislow, 2014).

Genes responding as a defence or a result of damage caused by toxic chemicals can be used as molecular biomarkers in the field of ecotoxicogenomics. Only two studies could be found as yet on molecular responses of *Pseudodiaptomus annandalei* to metal toxicity. Differentially expressed gene profiles were reported for a strain of *P. annandalei* (Jiang et al., 2013) The suppression of subtractive hybridization (SSH) was used to elucidate the response of *P. annandalei* to nickel exposure at the gene level (Jiang et al., 2013). In addition, transcriptome analysis was performed to investigate responses of *P. annandalei* to a low dose of mercury chloride ($HgCl_2$) (Wang et al., 2017). However, no study has been reported for whole transcriptome profiling for *P. annandalei* male and female copepods at cadmium exposure.

In this study, we exposed one generation of the copepod *P. annandalei* to sublethal concentrations of cadmium with the aim to compare the sex-specific molecular response

mechanisms of *P. annandalei*. Functional enrichment analysis was performed to investigate critical processes/ pathways in response to Cd exposure. The emphasis on male and female response was a result of the different reactions to metal contaminants observed in previous studies (Kadiene et al., 2017; 2019).

6.2. Materials and Methods

Experimental design

60 ovigerous females randomly selected from the large culture, were incubated in a false bottom mesh in polypropylene beakers (5 L) and the media containing the nauplii were contaminated with a sublethal concentration, 40µg/L of cadmium (Cd) equivalent to an LC10 of *P. annandalei* adults (Kadiene et al., 2017). Treatments were prepared in triplicates and kept in the culture conditions as described above. The copepod cultures were maintained by feeding them once every 2 days with uncontaminated algae (*I. galbana* and *N. oculata*) until they became adults. Culture media were renewed once at the copepodid before they attained adulthood. At the end of the first generation, male and non-ovigerous female (NOF) copepods were selected separately for RNA extraction and transcriptome assays.

6.2.1. RNA extraction, library preparation and sequencing

Copepods were separated into males and non ovigerous females. They were kept separately for about 48 hours for them to empty their guts. They were thoroughly screened afterwards to ensure purely male and female copepod samples. This was followed by the removal of faecal pellets and other suspended particles. After transferring the copepod into sterilized Eppendorf tubes, the remaining water was completely removed. The copepod RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was quantified by using a Nanodrop spectrophotometer (UV absorbance at 260nm), and an agarose gel was run for quality control. Reverse transcription to cDNA was performed on the RNA samples for each group using Applied Biosystems Master Mix composed of 10x RT Buffer, 10x RT Random Primers, 25x dNTP Mix (100mM),

MultiScribe Reverse Transcriptase, 50 U/µL, without RNase Inhibitor. And cDNA samples were used for library preparation.

Library preparation

High-throughput transcriptome (RNASeq) sequencing of *P. annandalei* male and female copepod samples (control male, control female, cadmium (Cd) male and cadmium female) was performed by Genomics BioSci & Tech Co., Ltd. Company (Taipei, Taiwan) using an Illumina HiSeq™ 2000 platform.

Quality control and de novo assembly and annotation

MultiQC v1.2 was used to evaluate read quality. Trinity v2.3.2 was used as transcriptome de-novo assembly tool. The alignment tool used was "bowtie2 v2.3.2", and the read count quantification tool used was "RSEM v1.2.31". The alignment QC report used "Qualimap v2" for evaluation. Raw reads in FASTQ format were processed using in-house Perl scripts before assembly. Clean reads were obtained after removing reads with an adapter, reads with poly-N (> 5%) and low-quality raw reads. At the same time, Q20, GC content and sequence duplication level of the clean data were calculated. Transcriptome assembly was accomplished based on all of the clean reads using a Trinity *de novo* assembler with min_kmer_cov set to 2 by default and all other parameters were set to the default values (Grabherr et al., 2011).

6.2.2. Gene function annotation

TransDecoder (http://transdecoder.sourceforge.net/) was used to identify putative coding regions from the assembled contigs. Gene functions were analysed and annotated via Basic Local Alignment Search Tool (BLAST) against the NCBI non-redundant (NR) protein database and the Swiss-Prot database with a threshold e-value $\leq$ 1e−5. The conserved domains present in the assembled transcripts were identified and annotated using InterProScan5 (Zdobnov and Apweiler, 2001). For functional annotation, Gene Ontology and KEGG pathway analysis of the contigs were performed using Blast2GO (Conesa et al., 2005).

Differentially expressed gene (DEGs) comparison between the control and 40 µg/L Cd exposure were calculated using DEGseq (2010) R package. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR) (Benjamini and Hochberg, 1995). A corrected P value < 0.1 was selected as the threshold for significant enrichment (Nätt et al., 2011). A FDR < 0.05, p-value<0.05 and 1>logFC>-1 data was used to compare DE by heatmap in all comparisons, and normalized by z-score.

6.2.3. Validation of the DEGs by quantitative real-time PCR (qRT-PCR)

To validate the RNA-seq data, qRT-PCR was performed for several DEGs in the copepods. Copepod samples from the treatment groups were used for qRT-PCR. RNA extraction and cDNA synthesis of the copepod samples were prepared as described above. qRT-PCR was carried out using SYBR Green as a probe on a 7500 Real-Time PCR system (Applied Biosystems, Singapore). Thermocycling was processed as the followings: 95 °C/min, 55 °C/min, and 40 cycles beginning at 55 °C/10 s with a 0.5 °C increase per cycle. qRT-PCR analysis was performed for Myohemerythrin-1, Myohemerythrin isoform, heat shock 70 kDa protein, heat shock protein 90, heat shock protein beta-1-like/Protein lethal (2) essential for life, lysosome membrane protein 2/ Sensory neuron membrane protein 2, Microsomal glutathione S-transferase 3-like and Myosin heavy chain, muscle-like. The relative expression of the DEGs was calculated based on the $2^{-\Delta\Delta CT}$ relative response method (Livak and Schmittgen, 2001), Elongation factor 1-alpha (EF1α), was used as an internal reference gene.

6.3. Results

6.3.1. RNA-Seq analysis and de novo assembly

Statistics of RNA-Seq and assembly data are summarized in Table 6.1. Trinity assembled 291,603 contigs with a total length of 311,386,569 bp and the largest contigs were 26,347 bp. TransDecoder found 32,625 putative open reading frame (ORF) contigs containing candidate coding regions without stop codons in the nucleotide sequences (Table 6.1). The total length of the final contigs was 41.1 Mb with an N50 of 1,881 bp.

Table 6. 1. Statistics of RNA-Seq and assembled contigs.

Trinity (de Novo)	Value
Number of contigs	291,603
Length of contigs (bp)	311,386,569
Mean contig length (bp)	
N50 (bp)	2,335
Largest contig (bp)	26,347
GC content (%)	40.25
Contigs after TransDecoder (CDS)	
Number of contigs	32,625
Length of contigs (bp)	41,084,901
Mean contig length (bp)	1,259
N50 (bp)	1,881
Largest contig (bp)	23,130
GC content (%)	50.59

6.3.2. Functional annotation of the assembled transcriptome

We performed functional annotation by BLAST analysis using several databases including NCBI non-redundant (nr) protein database and Swiss-Prot. BLAST analysis showed that out of 32,625 contigs, 25,793 in nr database and 19,722 in Swiss-Prot database had positive matches to homologous genes of other species.

The distribution of BLAST top-hit species from Swiss-Prot and NR database are shown in Fig. 6.1. *Homo sapiens* from Swissprot db and *Eurytemora affinis* from NR DB showed sequence similarity with more than 800 and more than 2400 transcripts of *P. annandalei* respectively. The 33 most abundant InterPro domains are listed in supplementary file (Table 6.2). The most hit InterPro domains was Zinc finger C2H2-type (IPR013087) followed by Immunoglobulin domains (IPR007110, IPR003599, IPR003598, IPR013098) and Protein kinase domain(IPR000719). Those top 33 domains were related to GO terms

of protein, metal ion, and nucleic acid binding (GO:0005515, GO:0005509, GO:0005524, GO:0003676, and) and of transferase, protein kinase, and ion channel activity (GO:0016772, GO:0004672, GO:0005230, and GO:0005216) (Table 6.3).

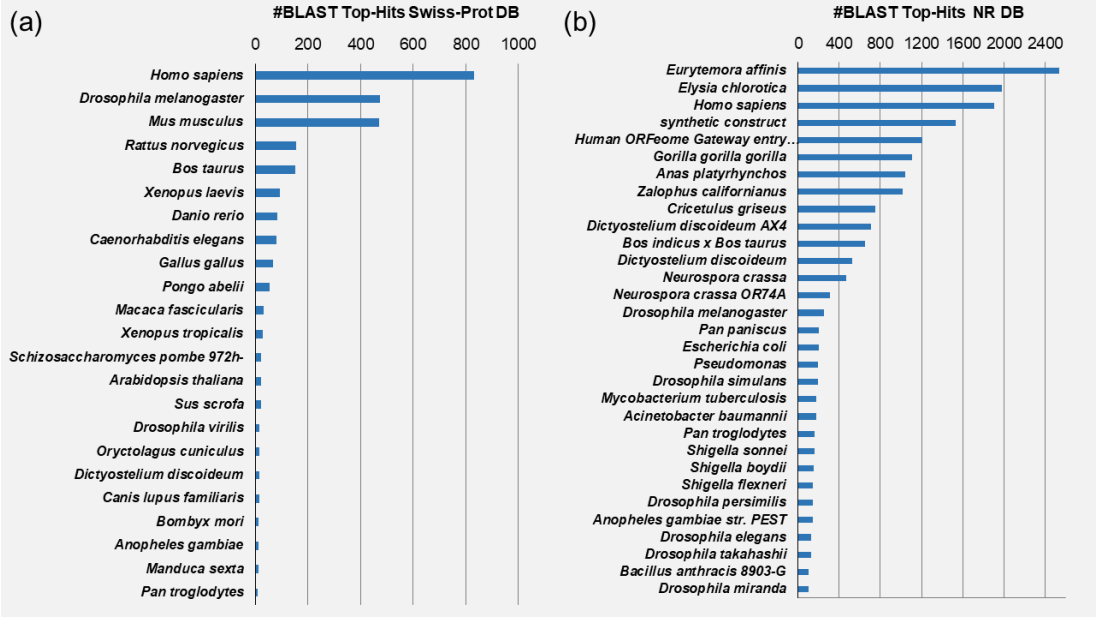

Fig. 6. 1. Number of BLAST Top-Hits Swiss-Prot (a) and non-redundant databases (b)

Table 6. 2. The list of most abundant InterPro domains by the InterProScan annotation of *P. annandalei* transcript contigs.

IPS Domain	No. of contigs
(IPR013087) Zinc finger C2H2-type	3046
(IPR007110) Immunoglobulin-like domain	438
(IPR003599) Immunoglobulin subtype	359
(IPR000719) Protein kinase domain	350
(IPR000477) Reverse transcriptase domain	319
(IPR000210) BTB/POZ domain	312
(IPR002048) EF-hand domain	312
(IPR003598) Immunoglobulin subtype 2	295
(IPR017452) GPCR, rhodopsin-like, 7TM	235
(IPR001254) Serine proteases, trypsin domain	229

(IPR000504) RNA recognition motif domain	224
(IPR020683) Ankyrin repeat-containing domain	221
(IPR020846) Major facilitator superfamily domain	217
(IPR013098) Immunoglobulin I-set	214
(IPR017986) WD40-repeat-containing domain	212
(IPR001841) Zinc finger, RING-type	191
(IPR002156) Ribonuclease H domain	175
(IPR006202) Neurotransmitter-gated ion-channel ligand-binding domain	172
(IPR005135) Endonuclease/exonuclease/phosphatase	160
(IPR001304) C-type lectin-like	159
(IPR001214) SET domain	151
(IPR003961) Fibronectin type III	150
(IPR005821) Ion transport domain	140
(IPR001478) PDZ domain	128
(IPR001849) Pleckstrin homology domain	121
(IPR003593) AAA+ ATPase domain	117
(IPR005225) Small GTP-binding protein domain	116
(IPR001452) SH3 domain	115
(IPR000008) C2 domain	112
(IPR000742) EGF-like domain	111
(IPR001356) Homeobox domain	110
(IPR013026) Tetratricopeptide repeat-containing domain	106
(IPR014001) Helicase superfamily 1/2, ATP-binding domain	101

Table 6. 3. Summary of Gene Ontology related to the Top 20 abundant InterPro Domains

GO terms	Description	Categories
GO:0003676	Nucleic acid binding	MF
GO:0004672	Protein kinase activity	MF
GO:0005524	ATP binding	MF
GO:0006468	Protein phosphorylation	BP
GO:0005515	protein binding	MF
GO:0005509	Calcium ion binding	MF
GO:0016021	Integral component of membrane	CC
GO:0006508	Proteolysis	BP
GO:0004252	Serine-type endopeptidase activity	MF
GO:0004523	RNA-DNA hybrid ribonuclease activity	MF
GO:0006811	Ion transport	BP
GO:0005230	Extracellular ligand-gated ion channel activity	MF
GO:0016021	Integral component of membrane	CC
GO:0055085	Transmembrane transport	BP
GO:0016020	Membrane	CC
GO:0005216	Ion channel activity	MF
GO:0005525	GTP binding	MF
GO:0003677	DNA binding	MF

6.3.3. Functional annotation based on Gene Ontology analysis

The final transcript sequences were functionally annotated based on Gene Ontology (GO) analysis. All results of GO categories including Biological process, Molecular Function, and Cell component were considered at the second level (Fig. 6.2). Under Biological Processes, 81% of the annotated sequences were similarly distributed to 10 GO terms (Fig. 6.3a): cellular process (GO:0009987,14%), metabolic process (GO:0008152, 12%), developmental process (GO:0032502, 10%), response to stimulus (GO:0050896, 8%), cellular component organization or biogenesis (GO:0071840, 7%),

biological regulation (GO:0065007, 7%), signaling (GO:0023052, 6%), regulation of biological process (GO:0050789, 6%), localization (GO:0051179, 6%), multicellular organismal process (GO:0032501, 5%). Based on Molecular Function, about half of the annotated sequences were related to binding function (GO:0005488, 46%) followed by catalytic activity (GO:0003824, 25%), then 14% were involved in transcription regulator activity (GO:0140110) (Fig. 6.3b). Under cellular component, sequences were equally related to cell (GO:005623, 21%), and cell parts (GO:0044464, 21%), followed by organelle (GO0043226, 19%) (Fig. 6.3c).

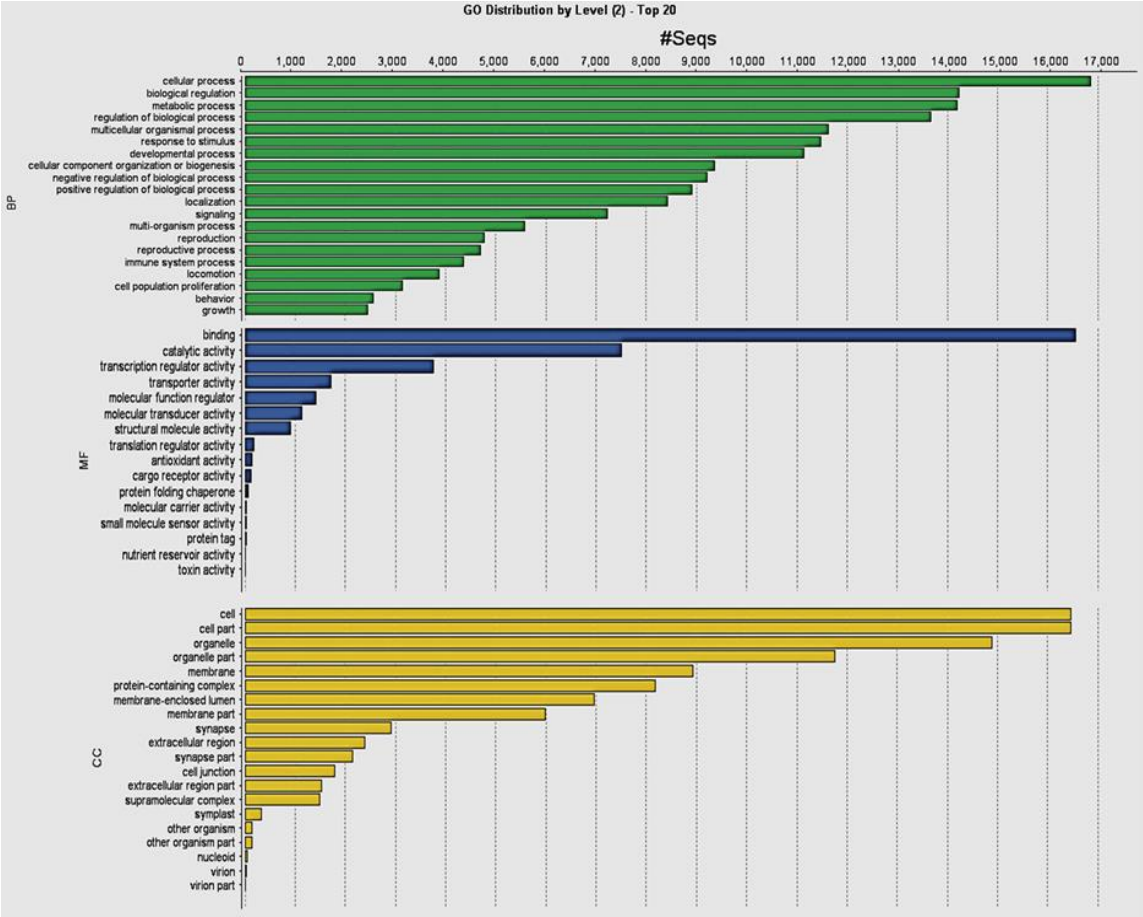

Fig. 6. 2. Top 20 GO distribution at level 2; Biological process (BP), Molecular Function (MF), and Cell component (CC).

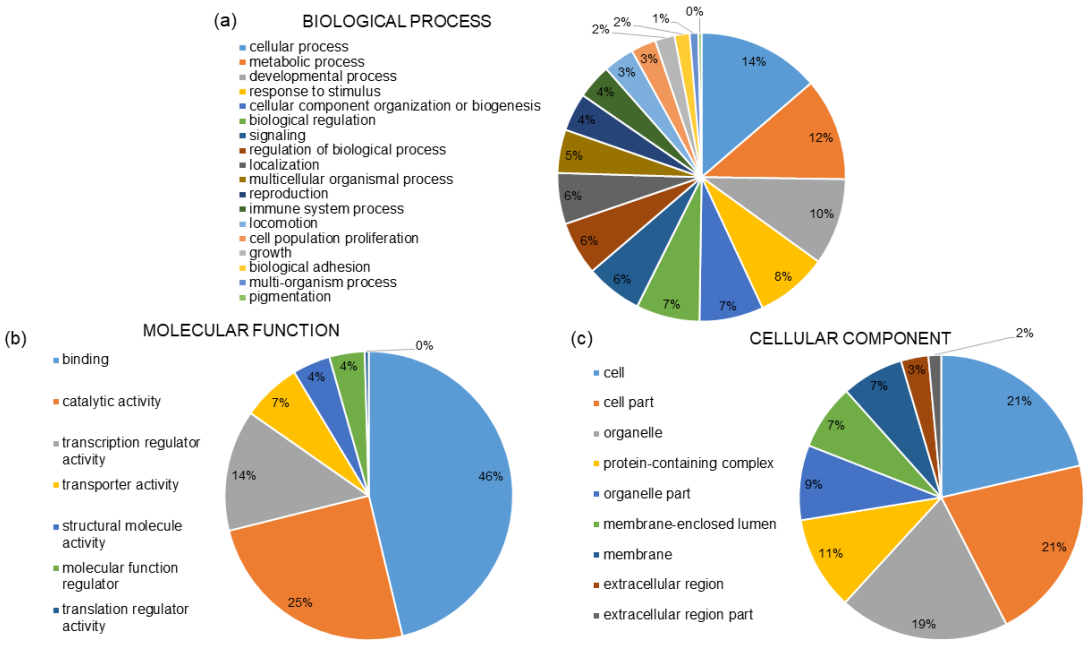

Fig. 6. 3. Percentage of GO categories including Biological process (a), Molecular Function (b), and Cell component (c).

6.3.4. KEGG pathway analysis

KEGG pathway analysis of *P. annandalei* transcripts identified several enzymes with metabolic pathways. The pathways with the highest number of sequences hit to enzyme were the thiamine metabolism and purine metabolism. The pathways with the highest number of sequence hit to enzyme proteins involved in xenobiotic biodegradation and metabolism was Drug metabolism of other enzymes (map00983) followed by Nitrotoluene degradation (map00633), Caprolactam degradation (map00930), Drug metabolism - cytochrome P450 (map00982) and metabolism of xenobiotics by cytochrome P450 (map00980) (Table 6.4). The analysis of KEGG pathways showed that most annotated sequences were related to metabolism pathways (Fig. 6.4).

Table 6. 4. KEGG analysis of enzymes involved in xenobiotics biodegradation and metabolisms.

Pathway	Pathway ID	#Seqs of Enzyme	Enzyme
Drug metabolism - other enzymes	map00983	354	ec:2.7.1.48 - kinase ec:3.5.2.2 - hydantoinase ec:3.1.1.1 - ali-esterase ec:1.17.4.1 - reductase ec:1.3.1.2 - dehydrogenase (NADP+) ec:3.5.1.6 - N-carbamoyl-beta-alanine amidohydrolase ec:3.2.1.31 - beta-glucuronide glucuronohydrolase glucuronidase ec:3.5.4.5 - deaminase ec:2.4.2.3 - phosphorylase ec:1.17.3.2 - oxidase ec:2.7.4.14 - kinase ec:1.1.1.205 - dehydrogenase ec:2.4.2.4 - phosphorylase ec:2.4.2.10 - phosphoribosyltransferase ec:3.6.1.23 - diphosphatase ec:6.3.5.2 - synthase (glutamine-hydrolysing) ec:2.3.1.5 - N-acetyltransferase ec:2.4.1.17 - 1-naphthol glucuronyltransferase ec:2.4.2.8 - phosphoribosyltransferase ec:2.7.1.21 - kinase

			ec:2.7.4.6 - kinase
			ec:2.5.1.18 - transferase
Toluene degradation	map00623	7	ec:1.2.1.28 - dehydrogenase (NAD+)
			ec:1.1.1.35 - dehydrogenase
Benzoate degradation	map00362	16	ec:2.3.1.16 - C-acyltransferase
			ec:1.1.1.157 - dehydrogenase
			ec:1.1.1.35 - dehydrogenase
			ec:1.2.1.10 - dehydrogenase (acetylating)
			ec:1.3.8.6 - dehydrogenase (ETF)
			ec:2.3.1.9 - C-acetyltransferase
			ec:4.2.1.17 - hydratase
Aminobenzoate degradation	map00627	11	ec:1.2.1.28 - dehydrogenase (NAD+)
			ec:3.5.5.1 - acetonitrilase
			ec:4.2.1.84 - hydratase
			ec:3.5.1.4 - acylamidase
			ec:4.2.1.17 - hydratase
Xylene degradation	map00622	4	ec:1.2.1.28 - dehydrogenase (NAD+)
			ec:1.2.1.10 - dehydrogenase (acetylating)
Dioxin degradation	map00621	3	ec:4.5.1.1 - DDT-ase
			ec:1.2.1.10 - dehydrogenase (acetylating)
Steroid degradation	map00984	3	ec:1.1.1.51 - 17)beta-hydroxysteroid dehydrogenase
			ec:1.3.99.5 - 4-dehydrogenase (acceptor)
Styrene degradation	map00643	6	ec:3.5.5.1 - acetonitrilase
			ec:5.2.1.2 - isomerase
			ec:4.2.1.84 - hydratase
			ec:3.7.1.2 - beta-diketonase
			ec:3.5.1.4 - acylamidase

			ec:1.13.11.5 - 1,2-dioxygenase
Nitrotoluene degradation	map00633	154	ec:2.3.1.5 - N-acetyltransferase
Ethylbenzene degradation	map00642	3	ec:2.3.1.16 - C-acyltransferase
Drug metabolism - cytochrome P450	map00982	32	ec:1.1.1.1 - dehydrogenase
			ec:1.2.1.5 - dehydrogenase [NAD(P)+]
			ec:1.2.3.1 - oxidase
			ec:2.4.1.17 - 1-naphthol glucuronyltransferase
			ec:1.14.13.8 - monooxygenase
			ec:2.5.1.18 - transferase
Metabolism of xenobiotics by cytochrome P450	map00980	32	ec:1.1.1.1 - dehydrogenase
			ec:1.1.1.184 - reductase (NADPH)
			ec:1.2.1.5 - dehydrogenase [NAD(P)+]
			ec:2.4.1.17 - 1-naphthol glucuronyltransferase
			ec:2.5.1.18 - transferase
Caprolactam degradation	map00930	39	ec:1.2.1.4 - dehydrogenase (NADP+)
			ec:1.1.1.35 - dehydrogenase
			ec:1.1.1.2 - dehydrogenase (NADP+)
			ec:4.2.1.17 - hydratase

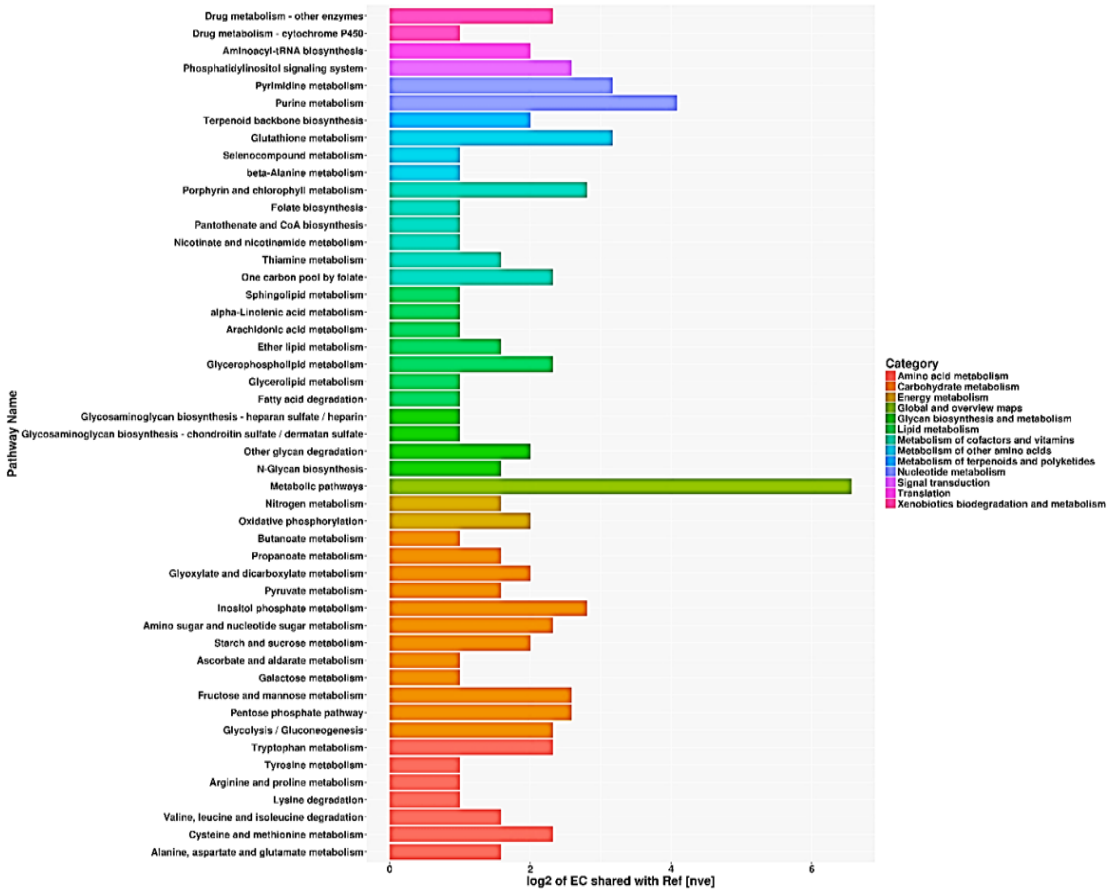

Fig. 6. 4. KEGG pathway

6.3.5. Transcriptional gene regulation in response to Cd

The mRNA responses of *P. annandalei* copepod to Cd toxicity are presented in a heat map (Fig. 6.5) and an MA plot after normalization (Fig. 6.6). With a significance of <0.001 p-value, a total of 4756 DEGs (2216 up-regulated and 2540 down-regulated genes) were found in male copepods, and a total of 2879 DEGs (2007 up-regulated and 872 down-regulated genes) were found in female copepods (Fig. 6.7). When genes at $P < 0.05$ showing log fold change < -1(downregulated genes) and log fold change > 1 (up-regulated genes) were compared based on sex, the highest number of upregulated genes were observed in female copepods (2041 DEGs) and the highest numbers of downregulated genes were observed in male copepods (2275 DEGs) (Fig. 6.8).

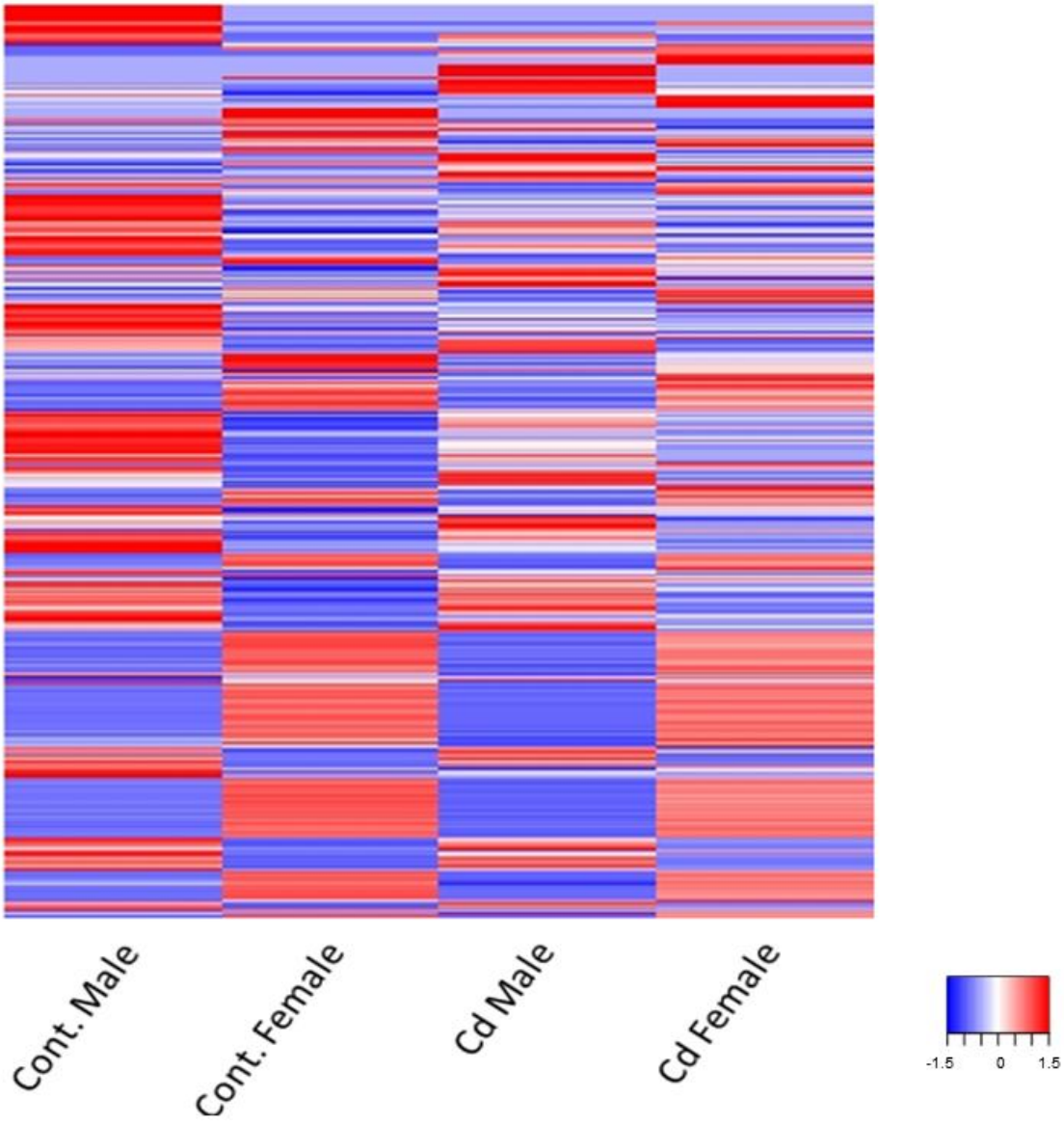

Fig. 6. 5. Heatmap of samples (normalized values).

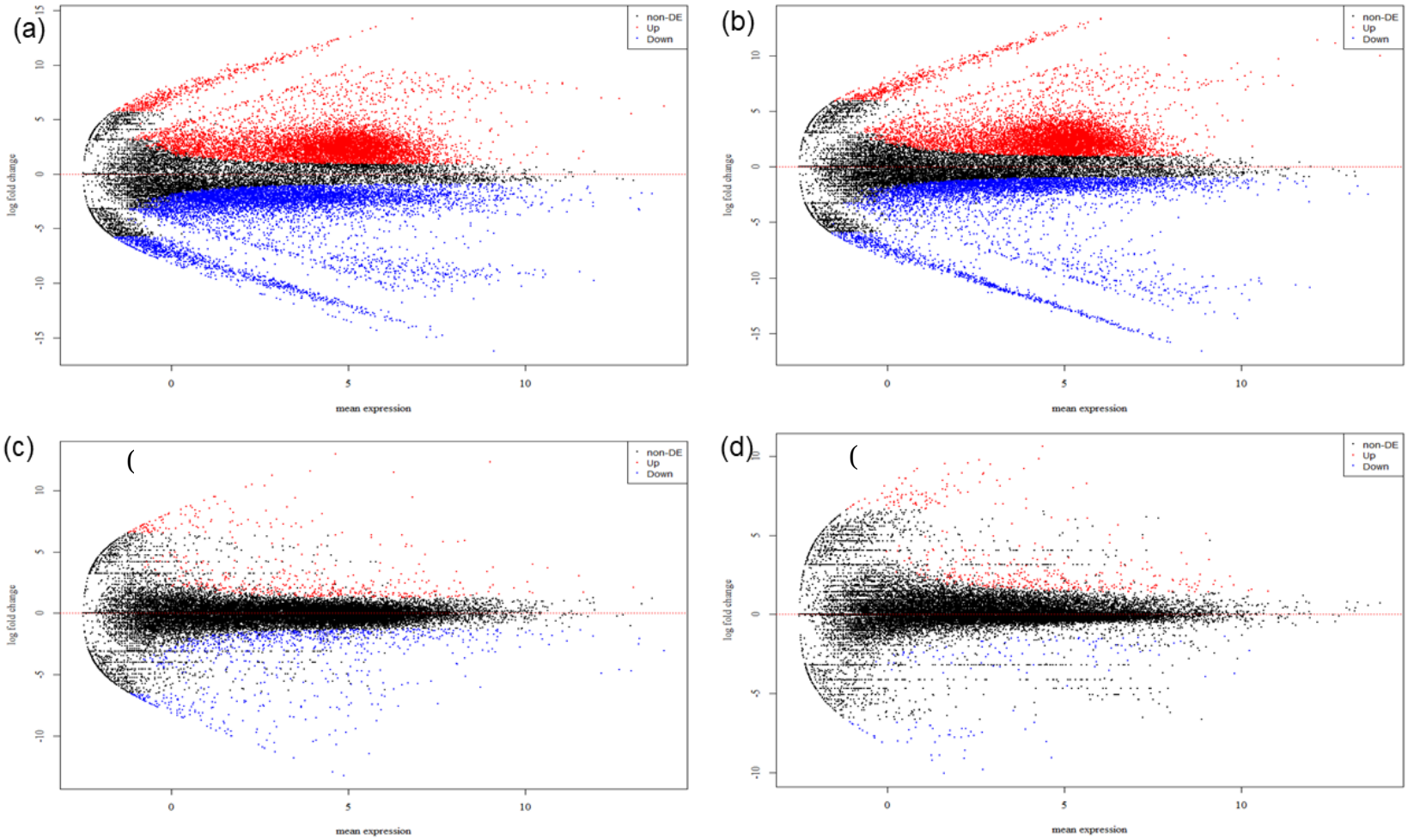

Fig. 6. 6. MA-plot of gene transcription between Control Male vs Control Female (a), Cd Male vs Cd Female (b), Control Male vs Cd Male (c) & Control Female vs Cd Female (d). Red dots represent significantly differentially expressed genes as established by Fisher's exact test at 1%; Blue dots represent genes with similar expression.

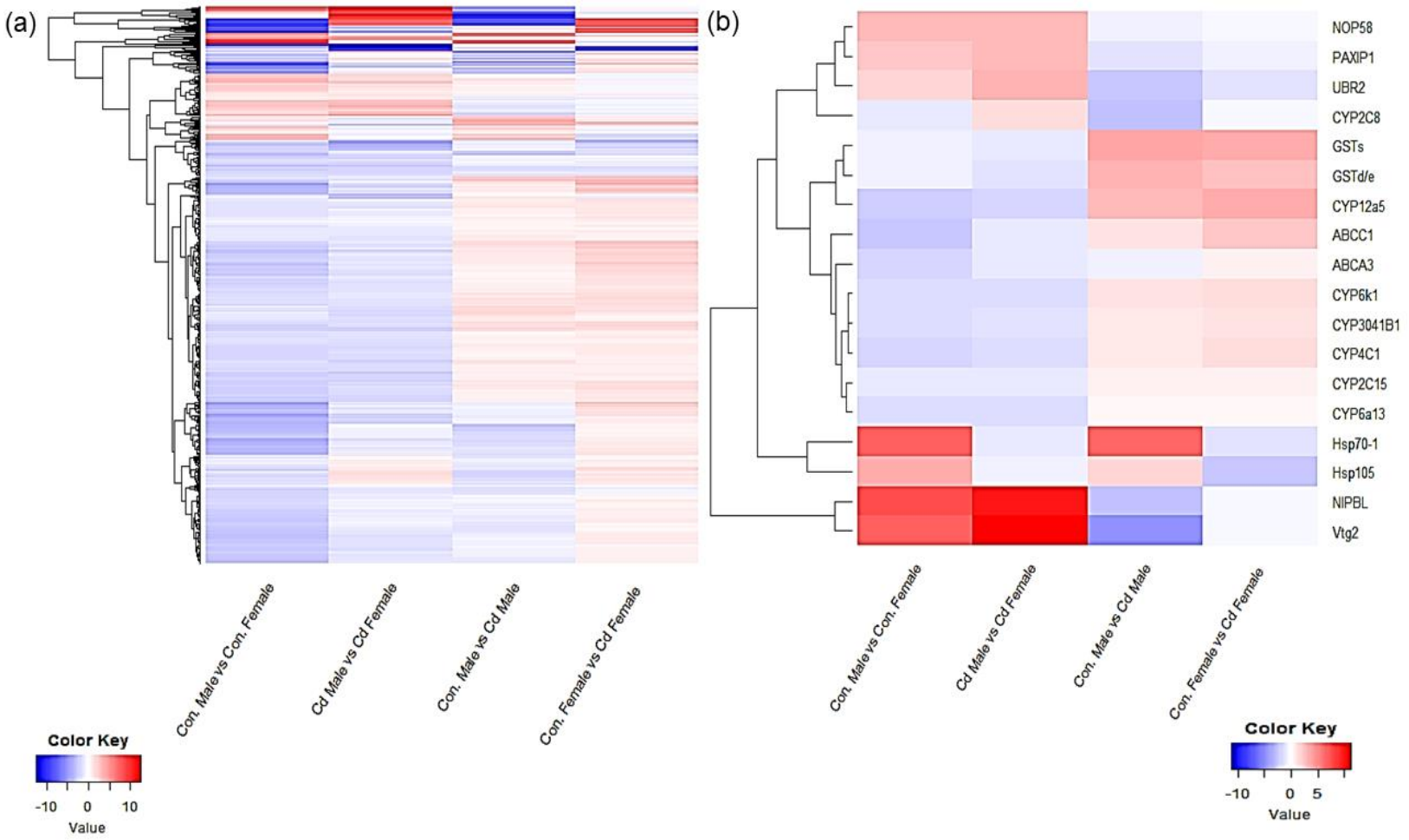

Fig. 6. 7. Heatmap of DEG (*P*-value < 0.001) (a) and selected genes (b)

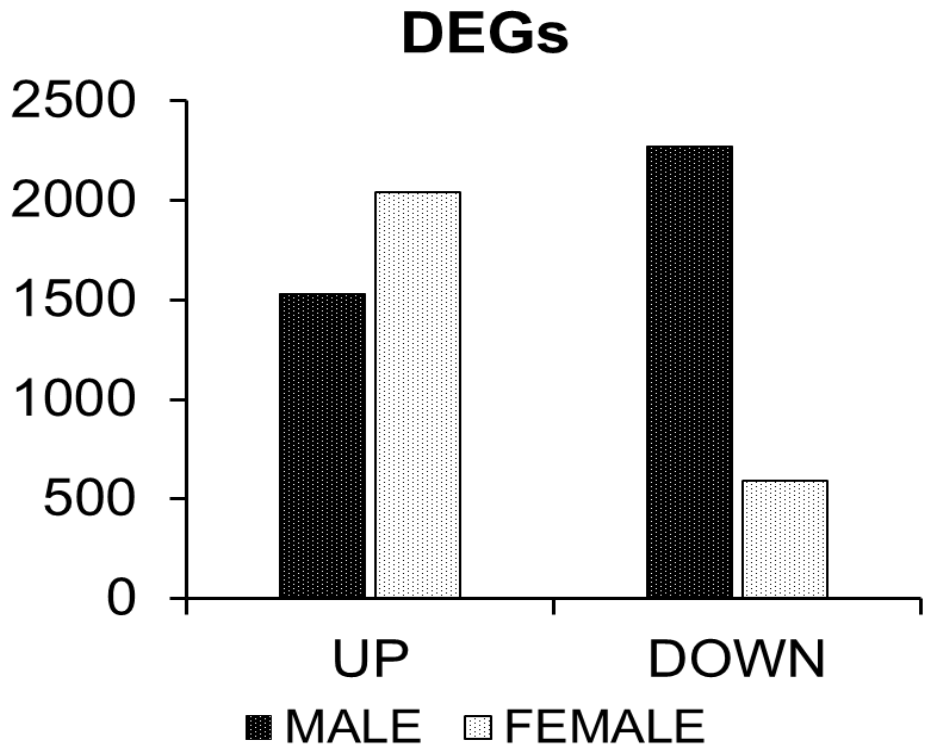

Fig. 6. 8. Up- and down-regulated differentially expressed genes (DEGs) observed in male and female copepods in response to Cd exposure.

6.3.6. QRT-PCR validation

Real-Time qRT-PCR analysis of selected mRNA genes of *P. annandalei* copepod in response to Cd toxicity showed similar magnitude and direction of expression when compared to those observed in the transcriptome microarray (Table 6.5).

Table 6. 5. QPCR validation of selected gene expressions

	Transcriptome Log2FC		QPCR Log2FC	
Gene Description	CdM	CdF	CdM	CdF
Myohemerythrin-1	12.3	5.1	9.10	6.15
Myohemerythrin	3.98	0.17	3.58	1.40
heat shock 70 kDa protein	6.57	-2.48	5.42	-0.82
heat shock protein 90	3.71	-3.56	2.61	-3.26
heat shock protein beta-1-like/Protein lethal(2)essential for life	3.49	-3.50	3.16	-2.76
lysosome membrane protein 2/ Sensory neuron membrane protein 2	0.99	2.42	0.28	2.67
Microsomal glutathione S-transferase 3-like	1.19	1.70	1.62	1.92
Myosin heavy chain, muscle-like	-1.73	0.90	-1.63	1.14

6.3.7. Selected differentially expressed genes

Relevant up- and downregulated DEGs in response to Cd exposure at $p < 0.05$ are listed in the supplementary file (Table 6.6). A large number of genes showed a highly significant difference in both upregulated and downregulated genes between male and females. Also, those with similar up- and downregulated expression levels in male and female copepods include Myohemerythrin-1, Neurohemerythrin, glutathione S-transferase 1-like, probable cytochrome P450 12a5, mitochondrial. However, a number of genes were upregulated in males but down regulated in females, few of them include heat-shock protein Hsp70, heat shock protein 90, cuticle protein 7-like.

Table 6. 6. Relevant differentially expressed genes

	Transcript ID	Description	Log_2FC		p-value	
			CdM	CdF	CdM	CdF
Stress response						
Chaperone	DN1404_c0_g1_i1.p1	Heat shock 70 kda protein 1-like	5.6	-3.0	1E-60	6E-110
	DN1404_c1_g1_i1.p1	Heat shock 70 kda protein 1-like	5.7	-2.5	6E-16	2E-26
	DN539_c0_g1_i1.p1	Heat shock 70 kda protein 1-like	5.8	-2.6	1E-177	2E-114
	DN539_c0_g1_i6.p1	Heat shock 70 kda protein 1-like	6.4	-2.5	1E-118	2E-89
	DN539_c1_g1_i1.p1	Heat shock 70 kda protein 1-like	6.4	-1.2	7E-130	3E-21
	DN70110_c0_g1_i1.p1	Heat shock 70 kda protein 1-like	5.4	-1.3	6E-164	1E-28
	DN89_c0_g1_i3.p1	Heat shock 70 kda protein 1-like	5.8	-1.8	2E-250	1E-61
	DN89_c0_g1_i4.p1	Heat shock 70 kda protein 1-like	5.6	-1.3	5E-162	1E-28
	DN89_c0_g3_i1.p1	Heat shock 70 kda protein 1-like	5.5	-2.9	2E-83	1E-123
	DN8040_c0_g1_i1.p1	Heat shock protein 105 kda-like	1.8	-2.3	3E-44	9E-92
	DN3519_c0_g1_i1.p1	Heat shock protein 90	0.8	-0.7	1E-12	1E-10
	DN85_c0_g1_i1.p1	Heat shock protein 90	3.7	-3.6	4E-11	3E-156
	DN85_c0_g1_i2.p1	Heat shock protein 90	5.9	-1.7	4E-221	8E-57
	DN143_c0_g1_i5.p1	Heat shock protein beta-1-like	0.7	-0.5	4E-10	1E-05
	DN5121_c0_g1_i1.p2	Heat shock protein beta-1-like	2.1	-1.5	4E-29	5E-31
	DN549_c0_g1_i7.p1	Heat shock protein beta-1-like	3.5	-3.2	7E-10	2E-72
	DN89_c0_g1_i6.p1	Heat-shock protein Hsp70	6.6	-2.5	7E-28	2E-63
Detoxification	**Phase I**					
	DN23784_c0_g1_i1.p1	Cytochrome P450 2C15-like	0.6	0.6	7E-08	1E-06
	DN11026_c0_g2_i2.p1	Cytochrome P450 2C31-like	1.4	0.8	2E-02	5E-02
	DN4756_c0_g1_i1.p1	Cytochrome P450 2C8-like	-2.6	-0.4	5E-105	9E-05
	DN5578_c0_g1_i1.p1	Cytochrome P450 3037B1	0.5	1.5	3E-03	1E-10
	DN4828_c0_g1_i2.p1	Cytochrome P450 3041B1	0.9	1.1	2E-10	3E-10
	DN6131_c1_g1_i1.p1	Cytochrome P450 4C1-like	0.9	1.4	1E-09	2E-11
	DN14261_c0_g2_i1.p1	Cytochrome P450 6k1-like	1.3	1.4	7E-11	1E-06

DN2041_c0_g1_i2.p1	Cytochrome P450 6k1-like	0.4	1.1	5E-04	4E-15
DN14195_c0_g1_i1.p1	NADPH--cytochrome P450 reductase-like	-0.2	1.0	4E-02	3E-16
DN13915_c0_g1_i1.p1	Probable cytochrome P450 12a5, mitochondrial	3.1	3.5	2E-58	1E-28
DN25452_c0_g1_i2.p1	Probable cytochrome P450 12a5, mitochondrial	1.1	2.7	7E-14	5E-25
DN3426_c0_g1_i2.p1	Probable cytochrome P450 12a5, mitochondrial	0.6	1.0	1E-04	3E-13
DN8334_c0_g1_i1.p1	Probable cytochrome P450 12a5, mitochondrial	0.4	3.6	1E-03	1E-03
DN50597_c0_g3_i2.p1	Probable cytochrome P450 49a1	-1.0	-0.9	3E-02	3E-02
DN623_c0_g1_i1.p1	Probable cytochrome P450 6a13	0.4	0.4	4E-05	6E-05
DN623_c0_g2_i1.p1	Probable cytochrome P450 6a13	-0.4	1.2	6E-03	2E-09
DN810_c0_g1_i1.p1	Probable cytochrome P450 6a13	-0.8	0.7	9E-14	3E-09
DN12066_c0_g2_i1.p1	Probable cytochrome P450 6a14	0.5	0.6	1E-02	1E-02
DN1868_c0_g2_i2.p1	Myohemerythrin-1	12.3	5.1	0.00	0.00
DN18699_c0_g3_i1.p1	Myohemerythrin-1	4.0	0.2	0.00	0.10
DN206_c0_g1_i10.p1	Myohemerythrin	3.1	0.3	0.00	0.01
DN206_c1_g1_i2.p1	Neurohemerythrin	2.1	0.3	0.00	0.00
DN206_c1_g2_i1.p1	Neurohemerythrin	1.2	0.7	0.00	0.00
DN206_c1_g3_i1.p1	Neurohemerythrin	11.5	4.5	0.00	0.00
DN26880_c0_g1_i3.p1	Ferritin heavy chain isoform X1	3.0	0.9	0.04	1.00
DN3003_c1_g1_i3.p2	Ferritin	0.8	0.7	0.00	0.00
DN3003_c1_g1_i5.p1	Ferritin	0.8	0.8	0.00	0.00
DN3446_c0_g1_i1.p1	Ferritin, lower subunit-like	1.5	1.3	0.00	0.00
DN7689_c0_g1_i1.p1	Ferritin	0.9	0.9	0.00	0.69
Phase II					
DN11993_c0_g1_i1.p1	UDP-glucuronosyltransferase 2B19	-0.2	1.5	0.64	0.00
DN8462_c0_g1_i1.p1	Superoxide dismutase	0.1	0.4	0.31	0.00

DN8462_c0_g1_i4.p1	Superoxide dismutase	-0.5	0.6	0.00	0.07
DN15439_c0_g1_i1.p1	Glutathione S-transferase 1-like	3.7	3.5	7E-226	2E-181
DN19849_c0_g1_i1.p1	Glutathione S-transferase 1-like	0.3	0.5	3E-03	6E-04
DN307_c0_g1_i1.p2	Glutathione S-transferase delta-epsilon 2	3.2	2.6	3E-124	4E-71
DN307_c0_g1_i2.p1	Glutathione S-transferase delta-epsilon 2	-0.8	-0.3	6E-09	4E-02
DN1096_c0_g1_i1.p1	Glutathione S-transferase Mu 2-like isoform X1	1.6	1.3	2E-21	6E-08
DN8354_c0_g3_i1.p1	Glutathione S-transferase Mu 5-like	0.6	0.3	9E-09	1E-02
DN9343_c0_g1_i12.p1	Microsomal glutathione s-transferase	0.5	0.8	0.00	0.00
DN512_c0_g1_i5.p1	Microsomal glutathione S-transferase 3-like	1.2	1.7	0.00	0.00
Phase III					
DN11082_c0_g1_i2.p1	ATP-binding cassette sub-family A member 9-like	-0.6	-0.3	0.00	0.04
DN13758_c0_g1_i7.p1	ATP-binding cassette transporter sub-family A member 1	-1.1	0.7	0.00	0.00
DN21380_c0_g1_i1.p1	ATP-binding cassette domain-containing protein	1.4	0.7	0.00	0.21
DN21384_c0_g2_i1.p1	ATP-binding cassette sub-family G member 8-like	-1.2	0.7	0.01	0.22
DN2149_c1_g1_i4.p1	ATP-binding cassette transporter sub-family G 125057	0.1	1.2	0.67	0.00
DN2316_c0_g1_i1.p1	ATP-binding cassette transporter sub-family B member 1	-0.4	0.3	0.00	0.01
DN2554_c0_g1_i1.p1	ATP-binding cassette sub-family E member 1-like	0.3	-0.1	0.01	0.31
DN5430_c2_g1_i1.p1	ATP-binding cassette sub-family D member 2-like	0.5	1.3	0.02	0.00

	DN5772_c1_g1_i5.p1	ATP-binding cassette transporter sub-family G member 1	0.4	0.6	0.07	0.02
	DN5929_c0_g1_i1.p1	ATP-binding cassette transporter sub-family F member 3	-0.5	-0.1	0.04	0.47
	DN6125_c0_g1_i2.p1	ATP-binding cassette sub-family A member 2-like	-1.0	0.3	0.00	0.10
	DN6885_c0_g1_i4.p1	ATP-binding cassette sub-family B member 7, mitochondrial-like isoform X2	-0.4	-0.3	0.08	0.01
	DN70749_c0_g1_i1.p1	ATP-binding cassette sub-family G member 5-like	-0.8	1.8	0.04	0.00
	DN7737_c0_g1_i1.p1	ATP-binding cassette transporter sub-family A member 3 isoform X2	-0.6	0.5	0.00	0.00
	DN9467_c0_g1_i10.p1	ATP-binding cassette transporter sub-family H 139282	-1.1	0.2	0.02	0.17
	DN5329_c0_g1_i1.p1	Multidrug resistance-associated protein 1-like	-0.5	0.1	0.01	0.64
	DN5499_c0_g2_i3.p1	Multidrug resistance-associated protein 1-like	-1.1	1.1	0.00	0.00
	DN8979_c1_g1_i4.p1	Multidrug resistance-associated protein 1-like isoform X1	-1.3	1.0	0.00	0.00
	DN8985_c1_g1_i1.p1	Multidrug resistance-associated protein 1-like	-1.6	0.1	0.00	0.55
	DN9759_c0_g1_i1.p1	Multidrug resistance-associated protein 1-like	1.1	2.5	0.00	0.00
Apoptosis	DN15025_c0_g1_i1.p1	Caspase-1-like	0.7	1.1	0.01	0.04
	DN15025_c0_g1_i2.p1	Caspase-1-like	7.2	5.6	0.00	0.03
	DN78975_c0_g2_i1.p1	Caspase-6	2.1	3.5	0.00	0.00
Protein turnover	DN1374_c0_g1_i1.p1	Putative serine protease F56F10.1	1.0	0.4	4E-10	2E-02
	DN5962_c0_g1_i3.p1	Serine protease 27-like	1.0	0.6	3E-21	9E-09
	DN10865_c0_g1_i1.p1	Serine protease 29-like	1.5	2.1	1E-04	5E-03

	DN1384_c0_g1_i2.p1	Serine protease 55-like	0.4	1.4	6E-05	2E-39
	DN2809_c0_g1_i1.p1	Serine protease 7-like isoform X1	0.4	0.8	3E-05	2E-12
	DN44987_c0_g1_i1.p1	Serine protease 7-like isoform X1	1.3	0.7	3E-29	1E-08
	DN10711_c0_g1_i3.p2	Serine protease inhibitor Kazal-type 1-like	0.6	1.2	7E-04	3E-05
	DN21075_c0_g3_i3.p1	Transmembrane protease serine 6-like	1.2	1.2	1E-02	2E-02
	DN1519_c0_g3_i1.p1	Transmembrane protease serine 9-like	0.7	-0.6	8E-08	2E-03
	DN2333_c0_g4_i1.p1	Transmembrane protease serine 9-like	2.0	0.7	2E-28	2E-03
	DN2630_c0_g2_i1.p1	Transmembrane protease serine 9-like	2.1	1.2	2E-85	4E-30
	DN2664_c0_g1_i1.p1	Transmembrane protease serine 9-like	1.1	1.2	3E-26	2E-29
	DN3490_c0_g1_i16.p1	Transmembrane protease serine 9-like	0.6	-0.3	3E-08	3E-02
	DN1675_c0_g1_i1.p1	Trypsin	-1.4	-1.4	1E-24	8E-14
	DN5113_c0_g2_i1.p1	Trypsin-1	1.2	1.7	3E-08	6E-06
	DN7171_c0_g1_i1.p1	Trypsin	0.7	0.4	0.00	0.04
	DN934_c0_g1_i1.p1	Trypsin	-0.4	-0.6	0.00	0.00
	DN934_c0_g2_i1.p1	Trypsin	-0.1	0.7	0.32	0.00
	DN934_c0_g2_i2.p2	Trypsin	-0.2	0.8	0.11	0.00
	DN12237_c0_g1_i1.p1	Trypsin-1-like	1.2	2.1	2E-29	1E-75
	DN2125_c0_g1_i1.p1	Trypsin-1-like	0.4	0.6	9E-04	5E-06
	DN3867_c0_g2_i1.p1	Trypsin-1-like	1.1	1.9	6E-25	3E-64
	DN65036_c0_g2_i2.p1	Trypsin-1-like isoform X1	0.9	1.7	2E-03	8E-06
Energy metabolism and growth related genes						
Lipid metabolism						
Degradation	DN9880_c0_g1_i1.p1	Pancreatic triacylglycerol lipase	0.4	2.4	0.048	0.000
	DN3108_c0_g1_i1.p1	Alkylglycerol monooxygenase-like	1.0	0.8	0.00	0.00
	DN18132_c0_g3_i1.p1	Lipase 3-like	1.6	3.5	0.00	0.00
	DN7411_c0_g1_i1.p1	Lipase	0.4	1.0	0.25	0.02
	DN7411_c0_g1_i1.p1	Lipase	0.4	1.0	0.25	0.02
	DN2824_c0_g2_i1.p1	Lipase 3-like	0.1	0.5	0.40	0.00

	DN5410_c0_g1_i5.p1	Phospholipase D alpha 2-like isoform X1	0.5	0.8	0.00	0.00
	DN9891_c0_g1_i11.p1	Phospholipase D2	-0.9	0.9	0.00	0.00
	DN47671_c0_g1_i2.p1	Phospholipase DDHD1-like	-1.3	1.7	0.01	0.01
	DN57_c0_g1_i4.p1	Phytanoyl-coa dioxygenase, peroxisomal-like	0.3	1.7	0.01	0.00
	DN2413_c0_g1_i1.p1	Putative phospholipase B-like 2 isoform X1	0.6	0.9	0.00	0.00
	DN428_c0_g4_i1.p1	1-acylglycerol-3-phosphate O-acyltransferase ABHD5-like	0.5	1.2	0.00	0.00
Transport	DN8012_c0_g1_i1.p1	Putative lipoprotein	0.7	1.7	0.00	0.00
	DN8012_c0_g1_i3.p1	Putative lipoprotein	0.5	2.1	0.00	0.00
	DN7145_c0_g1_i1.p1	Apolipoprotein D-like	1.1	1.2	0.00	0.00
	DN7424_c0_g1_i14.p1	Low-density lipoprotein receptor-related protein 2-like isoform X1	-0.4	0.3	0.00	0.00
	DN14492_c0_g1_i4.p1	Low-density lipoprotein receptor-related protein 8-like	-1.4	1.6	0.02	0.00
	DN2568_c0_g1_i2.p1	Elongation of very long chain fatty acids protein 6-like	-0.2	0.8	0.30	0.02
	DN2801_c0_g1_i2.p1	Elongation of very long chain fatty acids protein AAEL008004-like isoform X1	0.6	1.1	0.00	0.00
	DN28189_c0_g1_i1.p1	Elongation of very long chain fatty acids protein AAEL008004-like	2.1	3.0	0.38	0.00
	DN3487_c5_g2_i1.p1	Elongation of very long chain fatty acids protein AAEL008004-like	-0.4	0.6	0.01	0.00
Carbohydrate metabolism						
Degradation	DN10446_c0_g1_i5.p1	Acidic mammalian chitinase-like isoform X3	-0.4	-0.1	0.02	0.62
	DN24580_c0_g1_i6.p1	Chitinase 6	-1.5	2.2	0.00	0.00
	DN7521_c0_g1_i1.p1	Acidic mammalian chitinase-like	-0.7	0.3	0.00	0.00

	DN2401_c0_g1_i2.p1	Alpha-amylase-like	-0.8	-0.5	0.00	0.00
	DN1373_c0_g1_i3.p1	Basic endochitinase B-like	0.6	0.4	0.00	0.02
	DN36789_c0_g2_i2.p1	Chitinase 2	1.3	5.3	0.01	0.06
	DN4782_c0_g1_i1.p4	Chitinase-3-like protein 1	0.3	0.9	0.01	0.00
	DN9492_c0_g1_i1.p1	Endoglucanase-like	0.3	1.3	0.02	0.00
	DN715_c0_g1_i2.p1	6-phosphogluconolactonase	1.4	1.7	0.00	0.00
	DN1269_c0_g1_i1.p1	Glucoamylase ARB_02327-1-like	-1.0	0.2	0.00	0.04
	DN1269_c0_g2_i1.p1	Glucoamylase ARB_02327-1-like	-0.3	0.9	0.00	0.00
	DN22516_c0_g1_i1.p1	Probable chitinase 10	-7.1	0.0	0.00	1.00
	DN23710_c0_g2_i2.p1	Probable chitinase 10	1.2	1.7	0.01	0.00
	DN80038_c0_g1_i1.p1	Probable chitinase 10	-4.3	0.0	0.00	1.00
	DN36690_c0_g1_i3.p1	Probable chitinase 10	-2.6	0.5	0.00	0.76
	DN94143_c0_g1_i1.p1	Probable chitinase 2	-1.5	0.3	0.00	0.31
	DN4416_c0_g1_i2.p1	Probable maltase-glucoamylase 2	0.4	0.9	0.02	0.00
Transport	DN14770_c0_g1_i2.p1	Sodium-dependent glucose transporter 1A-like	1.4	0.9	0.00	0.01
	DN7027_c0_g1_i1.p1	Sodium/glucose cotransporter 4-like	-0.5	1.0	0.00	0.00
	DN8818_c0_g1_i1.p1	Sodium-dependent glucose transporter 1-like isoform X1	1.1	0.1	0.00	0.68
	DN9916_c0_g1_i1.p1	Sodium-dependent glucose transporter 1A-like isoform X1	-1.6	0.5	0.00	0.15
	DN9969_c0_g1_i2.p1	Solute carrier family 2, facilitated glucose transporter member 1-like	0.9	1.9	0.00	0.00
	DN79649_c0_g1_i10.p1	Sodium/myo-inositol cotransporter-like	-0.5	-0.1	0.01	0.50
	DN9553_c0_g1_i1.p1	Proton myo-inositol cotransporter-like	0.4	0.7	0.01	0.00
Biosynthesis	DN24332_c0_g1_i1.p1	Fructose-bisphosphate aldolase	-0.7	-0.5	0.00	0.00
	DN3201_c0_g1_i1.p1	Fructose-bisphosphate aldolase	0.8	0.4	0.00	0.00
	DN34870_c0_g1_i1.p1	Fructose-bisphosphate aldolase	0.6	-0.1	0.00	0.31

Reproduction and Growth						
	DN8905_c0_g1_i5.p1	Peroxidase-like	-1.0	-0.9	0.00	0.00
	DN4382_c0_g1_i1.p1	Chorion peroxidase-like isoform X1	0.3	-5.4	0.01	0.06
	DN376_c0_g1_i4.p1	Peroxidase-like	0.8	0.7	0.00	0.00
	DN27265_c0_g1_i1.p1	Peroxidase-like	3.4	-2.7	0.01	0.07
	DN208_c0_g1_i4.p1	Chorion peroxidase-like	-0.7	0.7	0.00	0.00
	DN2534_c0_g2_i2.p1	Chorion peroxidase	0.5	-0.5	0.00	0.00
	DN2534_c0_g2_i3.p1	Chorion peroxidase-like	0.8	-0.5	0.00	0.12
	DN17272_c0_g1_i2.p1	Chorion peroxidase	-0.5	-1.4	0.00	0.00
	DN14522_c0_g3_i1.p1	Peroxidase-like	-2.7	3.2	0.00	0.00
	DN10244_c0_g1_i1.p1	Vitellogenin 2	-4.7	0.8	0.00	0.00
	DN1963_c0_g1_i4.p1	Vitellogenin 2	-4.6	-0.4	0.00	0.00
	DN1963_c0_g1_i5.p1	Vitellogenin 2	-4.9	-0.5	0.00	0.00
	DN6766_c0_g1_i1.p1	Vitellogenin-like isoform X2	0.4	1.1	0.01	0.00
	DN97_c0_g1_i1.p1	Vitellogenin-like isoform X2	-3.1	0.7	0.00	0.00
	DN9932_c0_g1_i1.p1	Vitellogenin-2-like	-3.3	0.9	0.00	0.00
	DN14417_c0_g1_i1.p1	Cuticle protein 7-like	4.1	-1.9	0.00	0.38
	DN15103_c0_g1_i3.p1	Cuticle protein 5-like	-3.7	-4.1	0.00	0.50
	DN15103_c1_g1_i1.p1	Cuticle protein 5-like	-7.2	0.0	0.00	1.00
	DN160_c0_g1_i1.p2	Cuticle protein 5-like	-11.8	0.0	0.00	1.00
	DN160_c0_g1_i3.p2	Cuticle protein 5-like	-6.9	0.0	0.00	1.00
	DN160_c0_g1_i4.p2	Cuticle protein 5-like	-5.6	6.3	0.00	0.00
	DN18219_c0_g1_i1.p1	Cuticle protein 19.8-like	-9.4	-1.0	0.00	1.00
	DN18757_c0_g1_i1.p1	Cuticle protein 19-like	2.6	0.3	0.01	1.00
	DN22_c0_g1_i4.p2	Cuticle protein 5-like	-5.0	-0.8	0.00	0.21
	DN22751_c0_g1_i2.p1	Larval cuticle protein LCP-22-like	6.5	4.6	0.00	0.25
	DN23515_c1_g1_i1.p2	Cuticle protein 7-like	3.1	-3.4	0.00	0.03
	DN29243_c0_g1_i1.p1	Cuticle protein 16.5-like	-2.8	1.6	0.00	0.00
	DN29251_c0_g1_i1.p1	Cuticle protein 7-like	7.6	-4.1	0.00	0.50

	DN42291_c0_g1_i1.p1	Cuticle protein 16	-7.4	2.4	0.00	0.00
	DN4880_c0_g2_i1.p1	Cuticle protein 16.5-like	-1.9	1.1	0.00	0.00
	DN5491_c0_g1_i1.p1	Putative cuticle protein	-2.8	1.9	0.00	0.00
	DN6114_c0_g1_i1.p1	Cuticle protein 16.5-like	-8.8	5.6	0.00	0.03
	DN78289_c0_g1_i1.p1	Cuticle protein 16.5, isoform A-like	6.8	-4.7	0.00	0.25
	DN83_c0_g1_i2.p1	Cuticle protein 18.6-like	-7.9	-0.9	0.00	0.27
	DN83_c0_g1_i3.p1	Cuticle protein 18	-13.0	0.0	0.00	1.00
	DN85072_c0_g1_i1.p1	Cuticle protein 7-like	-1.3	-0.5	0.00	0.78
	DN932_c0_g1_i1.p1	Cuticle protein 6	-7.4	1.6	0.00	0.02
	DN1035_c1_g1_i1.p1	Myosin heavy chain, muscle-like	-3.2	0.7	0.00	0.00
	DN1035_c1_g1_i5.p2	Myosin heavy chain, muscle-like	-2.5	1.2	0.00	0.00
	DN10570_c0_g1_i1.p1	Myosin heavy chain, muscle-like	-2.5	1.3	0.00	0.23
	DN1128_c0_g1_i12.p1	Myosin heavy chain, non-muscle-like	-0.7	0.4	0.00	0.00
	DN1128_c0_g1_i2.p1	Myosin heavy chain, non-muscle-like	-0.8	0.3	0.00	0.00
	DN1128_c0_g1_i5.p1	Myosin heavy chain, non-muscle-like	-0.3	1.1	0.02	0.00
	DN12794_c0_g1_i1.p2	Alpha-tubulin	-2.0	0.5	0.00	0.33
	DN12794_c0_g2_i1.p1	Tubulin alpha-1 chain-like isoform X2	0.9	1.0	0.00	0.09
	DN12863_c0_g1_i1.p1	Tubulin alpha-1 chain-like	-0.5	0.3	0.00	0.17
	DN1367_c0_g2_i1.p1	Tubulin beta-1 chain	0.4	-0.3	0.00	0.00
	DN137_c2_g1_i3.p1	Tubulin alpha-3 chain-like	-0.4	0.5	0.01	0.01
	DN13815_c0_g1_i2.p1	Tropomyosin	-0.6	0.2	0.00	0.22
	DN14710_c0_g1_i1.p3	Tubulin A	-7.6	0.0	0.00	1.00

6.4. Discussion

Studies on the toxicity of metals, particularly cadmium toxicity in copepods is still insufficient. In addition to other studies previously reported, we found that toxicity of metals in copepods is specific to their sexes. In *P. annandalei*, male copepods were more sensitive to cadmium than the females (Kadiene et al., 2017). This could be a result of their different physiology, influencing their responses to xenobiotics. Some studies have reported alterations in the genetic expression of copepods exposed to different concentrations of xenobiotics (Hansen et al., 2008; Lee et al., 2008; Jiang et al., 2013). The aim of this study was to understand differences in gene expressions of male and female *P. annandalei* in response to cadmium exposure.

6.4.1. Transcriptome analysis of *P. annandalei*

In recent years, sequencing of copepod transcriptomes has increased with the development of NGS through Illumina technologies (Gallardo-Ecárate et al., 2014; Lenz et al., 2014; Ning et al., 2013; Valenzuela-Muñoz et al., 2015). Most recently, *P. annandalei* has shown much potential for use in aquaculture and ecotoxicology. We generated whole transcriptome data in the calanoid copepod *P. annandalei* using RNA-seq technology and bioinformatics tools. In this study 32, 625 contigs were identified in *P. annandalei* after trimming with a mean length of 1,259 bp. Similar results of transcriptome sequencing have been obtained from *Trigriopus japanicus* with a sequenced transcriptome composed of 26,946 contigs with an average contigs length of 532 bp (Ki et al., 2009). Also, Lee et al. (2015; 2018), identified 67, 179 contigs with average length of 1443 bp in *Paracyclopina nana* and 48, 480 contigs with an average mean of 963 bp in *Eurytemora affinis*, Legrand et al. (2016), identified 19,721 contigs with an average mean of 865 bp in *Eurytemora affinis* and Semmouri et al., (2019), identified 44,985 contigs with an average length of 540 bp in *Temora longicornis*. Contigs have been found to be mostly aligned up to 80% with arthropod sequences (Legrand et al., 2016; Lee et al., 2015; 2018). In our study, contigs mostly aligned with arthropod (*Eurytemora affinis*) followed by mollusk (*Elysia chlorotica*) sequences using non-redundant database (nr). However, most

Eurytemora affinis sequences with significant similarity to *P. annandalei* sequences were annotated as hypothetical proteins or uncategorized proteins. Using Swiss-Prot databases *P. annandalei* contigs were mostly aligned with chordates (*Homo sapiens*) and followed by arthropod (*Drosophila melanogaster*). This resulted in the annotation of those uncategorized proteins particularly those with significant differential expressions. The differential expression analysis was performed independently in both males and females in order to investigate the sex-specific molecular responses to cadmium. Although, the experimental exposure of *P. annandalei* in this study was setup in triplicates, we pooled large number of individual males (900) and females (600) separately (equally per replicate) from each treatment for sequencing. Similar approach was used in Legrand et al. (2016) and other studies (Hook et al., 2014; Jung et al., 2011; Santure et al., 2011; Traylor-Knowles et al., 2011; Zeng et al., 2011).

The result of the KEGG pathway analysis identified a group of pathways related to xenobiotics biodegradation and metabolism in *P. annandalei*. This is important as *P. annandalei* is becoming an emerging model species in aquatic ecotoxicology.

6.4.2. Differential gene expression

The increasing influx of persistent pollutants like non-essential metals in the aquatic environment is of ecological concern, because they may alter some physiological processes of the organisms (Calabrese et al. 1977; Poynton et al., 2011; Spurgeon et al. 1994; Zhang et al. 2009). Exposure to elevated levels of cadmium have been reported to cause cellular damage through the induction of intracellular reactive oxygen species (ROS) (Chelomin et al., 2005; Kim et al., 2014; Rico et al., 2009; Stohs and Bagchi 1995). Excess production of ROS causes oxidative stress which induces a cellular redox imbalance and protective stress response (Kim et al., 2011; Kim et al. 2014; Pestana et al. 2016; Rhee et al. 2009).

Drug metabolizing enzymes (DMEs) play major roles in the metabolism, elimination and detoxification of xenobiotics. This process is often divided into three phases: - Phase I, modification (Oxidation, Reduction, Hydrolysis), Phase II, conjugation (with glutathione, sulphate, glucuronic acid and with amino acids), and Phase III, excretion.

KEGG pathway analysis of the transcript sequences identified several enzymes that could directly or indirectly be involved in the phases of xenobiotics metabolism (Table 6.4). Similar pathways were identified and described in Lee et al. (2015; 2018). Upregulation of the genes involved in detoxification is usually an oxidative stress response to metal exposure (Martinez-Finley and Aschner, 2011; Roh et al., 2006; Valko et al., 2005), and similar response to Cd exposure was observed in this study. Few molecular biomarker genes have been used in ecophysiological and ecotoxicological investigations of *P. annandalei* (Jiang et al., 2013; Low et al., 2018; Wang et al., 2017).

Generally, xenobiotics directly or indirectly cause harmful effects in copepods. For example, cadmium can directly interfere with the physiological activity of specific, particularly susceptible proteins, either by forming a complex with functional side chain groups or by displacing essential metal ions such as zinc, iron and calcium ions. Cadmium interferes with protein folding, and inhibiting mismatch repairs (Banerjee and Flore-Roza, 2005; Chrestensen et al., 2000; Faller et al., 2005; Hartwig, 2001; Jin et al., 2003). Moreover, accumulation of misfolded proteins increases ROS production (Gregerson and Bross, 2010; Scheuner and Kaufman, 2008).

6.4.2.1. Heat shock protein expressions

Heat shock proteins (hsp) were shown to play important roles in the intrinsic immune system and stress responses of crustaceans (Kim et al. 2014; Pestana et al. 2016; Qian et al. 2012). Heat shock protein 90 assists in the folding and function of proteins that are vulnerable or easily prone to misfolding, and ensures their timely and proper reaction and allocation (Buchner, 1999; Mayer and Bukau, 1999; Young et al., 2001). Hsp90 chaperone system is central to cellular regulation and function as a result of the important role they play in eukaryotic cell signalling pathways (Taipale et al. 2014), and contributes to various biological activities under non-stress conditions (Bohen et al., 1995). The expression of heat shock protein 70 (hsp70) is mostly regulated by environmental and physiological stressors (Bedulina et al. 2017; Chichester et al. 2015; Henry et al. 2017; Pestana et al. 2016), but also by non-stressful conditions, such as cell growth, development,

and pathophysiological conditions (Behnke and Hendershot, 2014; Tiroli-Cepeda et al., 2014).

Heat shock proteins are generally up-regulated when exposed to Cd (Beyersmann, and Hechtenberg, 1997; Ferianc et al., 1998). Modulation of heat shock proteins have been reported in the calanoid species *Calanus finmarchicus* after copepod exposure to pollutants (Hansen et al., 2008). In this study, hsp70 and hsp90 were up-regulated about 13 to 97 folds in *P. annandalei* males and were down-regulated about 3 to 12 folds in females in response to Cd stress (Table 6.6). Kumar et al. (2015), reported a 65-fold increase of hsp70 upon exposure to the MC (metal cocktail including cadmium). Upregulation of hsp70 was observed in adult copepods after 3 days of exposure to NiNPs (nano particles) (Zhou et al., 2016) and at UV-B exposure, expressions of four isoforms of Hsp genes (i.e., Hsp10, Hsp40, Hsp60, and Hsp70) were significantly increased (Won et al., 2015). Agell et al. (2004), reported that Hsp70 protein was significantly induced by Cu exposure (15 µg/L for 5 days) in the Ascidian *Pseudodistoma crucigaster*. Planelló et al. (2010), demonstrated that hsp70 gene was significantly increased 12 h and 24 h after 10 mM Cd exposure in the aquatic midge, *C. riparius,* indicating that this gene is useful as a potential biomarker for Cd detection. Boulangé-Lecomte et al. (2014), demonstrated that basal hsp transcript levels were lower in male *E. affinis* than in females, suggesting sex-specific tolerance to stress. However, this transcript levels increased at the female reproductive stage. In this study we observed over-expression of hsp in *P. annandalei* female in the control. Moreover, when both male and female of *P. annandalei* copepods were exposed to cadmium stress, hsps were up-regulated in the male and downregulated in females. In contrast, Legrand et al. (2016), observed that hsp70 was over-regulated in exposed *E. affinis* female copepods and no induction was observed in exposed male copepods. Although, *E. affinis* were exposed to endocrine disruptor pesticides for just 48h. However, in this study, *P. annandalei* was exposed to Cd from naupliar to adult stage. The different responses observed in *P. annandalei* males and females expose to Cd stress, for example down regulation of hsp in female after exposure to Cd in one generation as opposed to up regulation observed in male might be because, the genes were overly expressed earlier as a result of the combined stress of reproduction and exposure to Cd in the females and was later depleted. The direction of

gene regulation (up- or downregulation) can change with time after initial exposure to a stressor, whereby some genes that were initially upregulated is later downregulated in response to stress (Ogawa et al., 2013; Meistertzheim et al., 2007). For example, in response to stress, *Daphnia pulex* showed an initial upregulation and then a longer-term downregulation of stress-related genes (Heckmann et al., 2008), which may be the case for *P. annandalei* female in this study. That is an upregulation of the same stress-related genes may have occurred earlier at the adult stage. Bailey et al. (2017), suggested that an early upregulation of stress genes, depending on the half-life of the proteins they code for, could have produced enough protein that the transcription of more mRNA had been negatively regulated by the time samples were collected, thus explaining the general downregulation of the hsp in *P. annandalei* female copepods in this study. Qian et al. (2012), reported that the expression of hsps in white shrimp, *Litopenaeus vannamei* increased gradually to the highest expression level and then decreased when exposed to Cd in a time dependent manner. In addition, Wang and Crowley (2005), observed that genes for stress proteins were mostly up-regulated in the early phase of Cd exposure to *Escherichia coli* and subsequent decline in the later phase. This may indicate that the resulting downregulation observed is a result of the long-term stress exposure prior. Also, it has been reported in oysters that hsp70 can be downregulated at the transcription level after Cd treatment (Boutet et al. 2003). In the study of Luan et al. (2010), no expression of hsp70 cloned from *Fenneropenaeus chinensis* was detected when shrimp were exposed to Cd. Which could be caused by the depression from Cd treatment. When an organism recognizes the presence of a toxic substance, they respond by producing proteins to metabolize the toxic chemicals.

6.4.2.2. Cadmium metabolic processes (Phase I)

Drug metabolizing enzymes (DMEs) including phase I, phase II metabolizing enzymes and phase III transporters, are abundantly present at basal levels under unstressed conditions, and/or induced at elevated levels in the presence of stressors such as xenobiotics (Meyer, 1996; Rushmore and Kong, 2002; Wang and LeCluyse, 2003). Phase I metabolic process involves converting the xenobioic, in this case Cd, to a form that can be eliminated or to a form that is non-toxic to the organism.

Cytochrome P450s (CYPs) constitute a superfamily of heme proteins (Nelson et al., 1996). Each CYP subfamily has a potentially substrate specific roles in endogenous metabolism and phase 1 biotransformation (Morel and Barouki, 1998) into a form that can easily be eliminated. They play an important role in the detoxification of xenobiotics (Berndtson and Chen, 1994; Rhee et al., 2013). Upregulated expression of CYP genes has been reported in response to the water accommodated fraction (WAF) of crude oil and alkylated forms of two PAH (phenanthrene and fluorine) (Han et al., 2015). In this study, all the differentially expressed CYP genes were significantly upregulated in *P. annandalei* females except for CYP49a1 and CYP2C8-like, also, CYPs were mostly upregulated in *P. annandalei* males exposed to Cd. However, CYPs expression levels were mostly higher in females than in males (Table 6.6). This could imply that in phase I, *P. annandalei* females show more potential in metabolizing Cd than males. In contrast, Musasia et al. (2013), showed that expressions of CYP genes were significantly lower in *Anopheles gambiae* mosquito females than in males when exposed to cadmium. Therefore, tolerance to cadmium in copepod can influence response of cytochrome p450 genes in a sex-specific manner.

Hemerythrin (HMET) is an oligomeric protein responsible for oxygen (O_2) transport in the marine invertebrate phyla of sipunculids, priapulids, brachiopods, and in a single annelid worm genus, *Magelona*. Myohemerythrin (MHEMT) is a monomeric O_2-binding protein found in the muscles of marine invertebrates. Hemerythrin holds the O_2 as a hydroperoxide (HO_2, or -OOH−). The site that binds O_2 consists of a pair of iron centres. The iron atoms are bound to the protein through the carboxylate side chains of a glutamate and aspartates as well as through five histidine residues. Hemerythrin and myohemerythrin are often described according to oxidation and ligation states of the iron centre (Roat-Malone, 2007). Hemerythrins are multi-functional non-heme-iron oxygen-transport proteins (Bates et al., 1968) and members of the HMET gene family, including MHEMT, participate in respiration, heavy metal detoxification and aspects of innate immunity in some annelids (e.g., the leeches *Theromyzon tessulatum* and *Hirudo medicinalis* and the polychaete *Neanthes diversicolor*) (Baert et al. 1992; Demuynck et al. 1993; Vergote et al. 2004: Yang et al., 2012). Hemerythrins transport oxygen using two Fe^{2+} ions that bind

directly to the polypeptide chain (Bailly et al. 2008; Terwilliger 1998). Hemerythrin, act as potent immune effectors under certain physiological conditions (Terwilliger 1998). Several studies have shown that the use of HMET as a defence strategy is not only employed by metazoan hosts. Kao et al. (2008), indicated that a hemerythrin-like protein was over-produced when *Methylococcus capsulatus* (Bath) was grown at high copper concentrations. In this study, several genes responding to metal toxicity were mostly up regulated, some at several folds more than others. The over-expression of hemerythrin was observed. Particularly, the myohemerythrin isoform was significantly upregulated in *P. annandalei* exposed to Cd. In addition, their expression pattern was sex-specific, in that, male expression levels were significantly more than the females (Table 6.6). A result of higher concentration of Cd reported in male than in female copepod possibly due to slower excretion rate of Cd in *P. annandalei* males (Kadiene et al., 2019) may give reason to the over-production of HMET observed in male copepods, considering its function as Cd-binding.

6.4.2.3. Effect of cadmium on cellular mechanisms

Cadmium interacts with the iron metabolism (Crowe and Morgan,1997; Moshtaghie et al., 1994), by reducing the uptake of iron. Because hemerythrin is also an iron binding protein, when it binds to cadmium, it displaces iron, which may result in excessive iron in the system. Excessive iron is toxic when it binds to O_2 to produce ROS, which are extremely powerful oxidizing agents capable of causing cell damage.

Ferritin (Frt) is a protein responsible for iron homeostasis and Iron is required for life since it plays a crucial role in essential biological processes. Frt, plays a very vital role in protecting the cell from iron catalysed ROS formation (Kumar et al., 2015; Orihuela et al., 2011). Ferritin expression is increased when an organism experiences oxidative damage caused by ROS, supporting its role in responding to oxidative stress (Berg et al., 2002). In this study, ferritins were equally upregulated in male and female copepods when exposed to Cd (Table 6.6). In addition, because the role of ferritin is majorly for iron storage, their role here could also be a response to excessive iron that may be released when hemerythrin binds to Cd.

Cadmium indirectly causes oxidative stress from the excess production of ROS. Also, ROS is excessively produced as by-products of the various metabolic processes in an attempt to detoxify Cd. Therefore, as a way to regulate ROS, copepods produce enzymes that reduces or deactivates excess ROS. Oxidative stress activates enzymic (e.g. SOD, CAT) and non enzymic (e.g. GSH) antioxidants.

6.4.2.4. Cadmium metabolic processes (Phase II)

In phase II metabolic process, we identified several genes that were either induced or inhibited by cadmium (Cd) exposure. However, the magnitude and direction of expression was regulated in a sex-specific manner. In the Phase II conjugation reaction, enzymes play important roles in the biotransformation of endogenous compounds and xenobiotics to more easily excreted forms mostly by transferases such as glutathione S-transferases (GST) among others (Jancova et al., 2010). GSTs contributes indirectly by catalysing the conjugation of glutathione (GSH) to electrophilic substrates, producing compounds that are generally less reactive and more soluble in other to facilitate their excretion from the cell via membrane-based glutathione conjugate pumps (Danielson et al., 1987; Hayes and Pulford, 1995; Hubatsch et al., 1998; Lee et al., 2005; Rushmore and Pickett, 1993; Veal et al., 2002;). The broad substrate specificity of GSTs allows them to protect cells against a range of toxic chemicals (Salinas and Wong, 1999). It was reported that heavy metal exposure increases gene expression of glutathione S-transferase (GST) in a concentration-dependent way in Tigriopus up to 24 h (Lee et al., 2008). GST isoforms are known to play an important role in the antioxidant defence. As in the present study, GST enzyme activity increased with Cd exposure (Jemec et al., 2008). In this study, GSTs were similarly upregulated in males and females. One isoform of SOD was downregulated in male and another SOD isoform was upregulated in females ($p<0.05$). UDP-glucuronosyltransferase 2B19 was significantly ($p<0.05$) upregulated in female (Table 6.6). Similarly, GST activity was increased in the liver of the roach *Rutilus rutilus* exposed to Cu (Paris-Palacios et al., 2000). Wang and Wang (2009), also showed a similar induction pattern of GST with 100 μg/L of Cd exposure for 96 h in *T. japonicus*. Superoxide dismutase (SOD), together with GST are involved in resistance to oxidative stress.

6.4.2.5. Cadmium excretion processes (Phase III)

Phase III transporters, including the ATP-binding cassette (ABC) transporters, regulation of drug metabolism and drug transport (Brinkmann and Eichelbaum, 2001), multidrug resistance associated proteins (MRP) (Kerb et aL, 2001) provide a formidable barrier against drug penetration, and play crucial roles in drug absorption, distribution, and excretion (Brinkmann and Eichelbaum, 2001; Kim, 2003; Mizuno et al, 2003; Staudinger et al, 2003; Xu, et al., 2005). In this study, these proteins which may be responsible for the excretion of Cd were mostly downregulated in male copepods and upregulated in females (Table 6.6). This implies that the female copepods have the ability to better excrete Cd from their system than the males, hence their high tolerance to Cd exposure when compared to the male copepods.

6.4.2.6. Induction of apoptosis

Cd indirectly activates apoptosis by inducing intracellular peroxide production (Wang et al. 2012). The increasing ROS generation, subsequently triggers pathways that leads to cell death. Caspase proteins are protease enzymes they play a major role in programmed cell death either as initiators or effectors of apoptosis (Chowdhury et al., 2008). Specific responses of caspase to pollutants have been reported in *Mytilus galloprovincialis* (Romero et al., 2011). In this study, caspase-1 and -6 were upregulated in both male and female copepods (Table 6.6). Cd was able to induce apoptosis in the gills of freshwater crab through increased production of caspase activity (Wang et al., 2012).

6.4.2.7. Cadmium effect on energy processes

In this study, several DEGs associated with digestion including endoglucanases, chitinase, trypsins, lipase and Phosphogluconolactonase (Table 6.6), were mostly up-regulated in both male and female copepods. Some digestive enzymes like alpha-amylase, and a number of trypsin isoforms were downregulated in both male and female copepods. Moreover, few digestive enzymes were downregulated in males. Also, only few lipid transport proteins were down regulated in male copepod. Poynton et al. (2011), found that genes involved in lipid transport were down-regulated by Cd exposure. In this study,

similar response was observed only in male, lipid transport genes were mostly downregulated and those that were upregulated, their expression levels were lower than the females. Lipid transport genes were upregulated in female copepods exposed to Cd. Previous studies have shown a decrease in the expression level of fatty acid binding proteins, which are important for the absorption of fatty acids (Poynton et al., 2007). Soetaert et al. (2007), observed a decreased internal lipid reserves of *D. magna* after Cd exposure as a result of decreased absorption and/or increased breakdown of fatty acids through fatty acid oxidation. This could imply to some level that Cd could interfere with the copepod digestive system and the regulation of energy budget in *P. annandalei* male copepods. However, in the female copepods, lipid metabolism was not affected.

The upregulation of digestive enzyme could be a physiological response which is of primary importance for the generation of energy resources in *P. annandalei* copepods. Members of the elongase family are involved in lipid biosynthesis. Few of them were significantly differentially expressed. Elongases (ELOV4) were upregulated in females and were either downregulated in males or their expression levels were lower in males than in females when exposed to Cd (Table 6.6). Poynton et al. (2011), reported that several genes involved in carbohydrate and protein metabolism were downregulated by Cd exposure due to decreased expression levels of digestive enzymes, and decreased feeding rate in Cd-exposed daphniids. This was also observed by De Coen and Janssen (1998). Cd exposure causes a depletion in lipid and protein reserves, which would likely lead to decreased fitness and survival during prolonged exposure (Poynton et al., 2011).

6.4.2.8. Cadmium effect on growth and reproduction

It is commonly known that the heavy metals together with environmental factors induce toxicity, significantly generate oxidative stress, and inhibit growth and development in aquatic organisms (Huang et al., 2012; Kodrík et al., 2015; Suganya et al., 2016). Chorion peroxidase Pxt is a cyclooxygenase-like protein involved in the formation of a rigid and insoluble egg chorion by catalysing chorion protein cross-linking through dityrosine formation and phenol oxidase-catalysed chorion melanisation (Tootle and Spradling, 2008). It is required for both the actin filament bundle formation and the cortical

actin strengthening during oogenesis (Groen et al., 2012). In this study, this enzyme was mostly downregulated (Table 6.6), meaning, that exposure to Cd could have a negative impact on egg production or formation in *P. annandalei* copepods. Michibata (1981), have reported considerable amount of Cd accumulated in the chorion of *Oryzius latipes*, which supports the possible effect of Cd on egg production.

Vitellogenin (Vtg) is a precursor of egg yolk proteins (vitellin), and plays a key role in reproduction. As a result of their expression patterns when exposed to stress factors, they have been widely used as a biomarker in fish and copepods exposed to stressors (Hwang et al., 2010b; Lee et al., 2008; Matozzo et al., 2008; Sumpter and Jobling, 1995). In this study, vtg mRNA was mostly downregulated by Cd in males and in females (Table 6.6), similar results were reported by Legrand et al. (2016), when *E. affinis* male and female copepods were exposed to a pesticide (PXF). Vtg is normally expressed in female copepods during the reproductive process. However, under Cd stress, vtg production was affected. This implies that Cd could exact a negative effect on reproductive performance of female copepods. Jiang et al. (2013), reported downregulation of vtg 2 after 24h exposure of *P. annandalei* to nickel. Similarly, Vandenbrouck et al. (2009), reported a downregulation of vtg mRNA after nickel exposure in *Daphnia magna*. These studies indicated that the repression of vtg mRNA may be a secondary response to lack of sufficient energy reserves after metal exposure. It is possible that vitellogenins in females exposed to Cd are temporarily accumulated in the fat body during the first day after exposure, because receptors for this protein are not developed yet, thus, the transcription of vitellogenin, and consequently the effect on protein level could be a result of Cd exposure (Płachetka-Bożek et al. 2018).

Cuticle-related genes are known to be widely regulated by chemical exposures (Chávez-Mardones et al., 2016; Valenzuela-Munoz et al., 2015; Zhu et al., 2010). In our study, some genes involved in growth and development, were regulated by Cd exposure in *P. annandalei*. Cuticle protein were downregulated in male and mostly up regulated in female copepods (Table 6.6). Cuticle alterations may contribute to the higher sensitivity of

males to environmental stressors. Cuticle has already been demonstrated to be a target of pesticides, particularly for growth regulator insecticides such as PXF (Aribi et al., 2006).

Chitinase was both down- and upregulated in female copepods exposed to Cd suggesting an inhibition of chitinhydrolysis (Table 6.6), similarly observed in Legrand et al. (2016). Chitinase is not only involved in molting but also in defence and digestion in insects (Tetreau et al., 2015).

6.4.3. Sex-specific responses

Legrand et al. (2016), reported that *E. affinis* male copepods were mostly impacted by chemical exposures than females with twice as many DEGs. We found similar response in male *P. annandalei* compared to the females. We have previously reported that *P. annandalei* males are more sensitive to Cd toxicity than the females (Kadiene et al., 2017) and this was associated with the higher Cd concentration observed in males than in female copepods (Kadiene et al., 2019). In addition, several other studies have reported male copepods to have lower survival rate under environmental stress or chemical exposures than females (Beyrend-Dur et al., 2009; Chen et al., 2006; Hagopian-Schlekat et al., 2001; Legrand et al., 2016; Medina et al., 2002; Saiz et al., 2015).

Many calanoid species males showed a significantly higher moving activity than females (Dur et al., 2010; Kiørboe and Bagøien, 2005; Kiørboe, 2007; Michalec et al., 2010; Uttieri et al., 2007; Van Duren and Videler, 1995). Specific oxygen consumption was found to double in male copepods than in female copepods, probably due to higher swimming activity of males. Both in terms of total and basal metabolism, males have higher metabolic status (Svetlichny et al., 2012). Also, Lehette et al. (2016), reported that respiration rate of *P. annandalei* male copepod was significantly higher than non ovigerous copepods but not significantly different from ovigerous copepods. Protein synthesis maybe upregulated as part of the biological processes. However, in this study many genes that encode for the synthesis of proteins were downregulated. Protein synthesis is both energetically costly (Bailey et al. 2016; Rolfe and Brown, 1997; Wieser & Krumschnabel, 2001; Sokolova et al., 2012) and produces reactive oxygen species (Tomanek, 2014). Thus,

downregulation of protein synthesis in the case of hsps in female copepods may be a strategy to conserve energy and minimize oxidative damage from the excess production of ROS (Bailey et al. 2016). Significant regulation of gene expression can be associated with tolerance to stress (Bailey et al., 2016). Several studies show both up- and downregulation of cellular stress response-related genes to several stressors (Anderson et al., 2015; Goncalves et al., 2016).

It is shown that the effect of Cd exposure on the protein synthesis in vivo depends on duration of exposure (Ivanov et al., 2003). Franssen et al. (2011), proposed that the quick return to normal gene expression after exposure to a stressor is a sign that an organism can tolerate environmental changes. Cd in low concentrations can activate both the rate and the level of translation but in high concentrations it inhibits those parameters (Ivanov et al., 2003). Goncalves et al. (2016), found that one population had developed tolerance to low pH by downregulating genes associated with stress response, however, the same genes were upregulated in another population. Downregulation of stress-related genes when exposed to a stressor may be a characteristic of tolerant populations (Goncalves et al., 2016). In our study, the downregulation of the majority of DEGs in this study can reasonably be interpreted as an indication that *P. annandalei* female copepod could be tolerant to Cd exposure to a certain level. Energy budget could be a major difference for how male and female copepods differentially metabolizes and eliminate xenobiotic, since cellular stress response (CSR) is consistent with increased energy requirement (Roncalli et al., 2016).

6.5. Conclusions

The present study represents one of very few analyses done on *P. annandalei* mRNA after chemical exposure. Using a de novo assembly, 7,635 DEGs were identified. The present data provides a transcriptome assay as a contribution to the molecular study of metal toxicity in *P. annandalei*. We focused our discussions on a few cellular stress response genes; genes participating in the 3 phases of xenobiotic metabolism, apoptosis, protein turnover, lipid and carbohydrate metabolism, growth and reproduction. Exposure

to cadmium and the process of its metabolism causes ripple effects of toxicity in copepods. We showed that most of these processes were altered in a sex-specific manner. The sex-specific differences in the regulation of genes expressed in response to pollutants could be the reason for the differences in sensitivity between male and female copepods. Differential response patterns between male and female copepods exposed to stressors reinforces the need to consider sex as a factor in ecotoxicogenomics.

The reason for the sex-specificity of the gene response patterns in *P. annandalei* male and female copepods may lie in the kinetics of uptake, accumulation, and loss of metals and in the specific cellular, subcellular, and physiological responses to these metals. The downregulation or low expression of CSR (cellular stress response) genes at the adult stage of copepod that normally would have been upregulated or overly expressed under stressed conditions, might be because of their physiological adjustments which could have been influenced by the duration of exposure. Moreover, the differences in DEGs observed between male and female copepods in this study, could also be caused by the basic physiological, hormonal and genetic differences between male and female copepods and on how they physiologically respond to cadmium upon incorporation.

Chapter 7.

Multigenerational study on the life traits, bioaccumulation and molecular responses of *Pseudodiaptomus annandalei* to cadmium

Kadiene Esther U., Baghdad Ouddane, Hong-Yi Gong, Jiang-Shiou Hwang, Sami Souissi. (In prep.). Multigenerational study on the life traits, bioaccumulation and molecular responses of *Pseudodiaptomus annandalei* to cadmium

7.1. Introduction

The natural features of copepod such as availability, relevance, ease of handling, culturability, sensitivity, abundance, size (Bron et al., 2011; Cutts, 2003; Devreker et al., 2004; Gilbert and Williamson, 1983; Greenstein et al., 2008; Souissi et al., 1997; 2010; Wang et al., 2014; Zhang and Uhlig, 1993) makes them suitable candidates as reference species in aquatic toxicology. *P. annandalei* exhibit sexual dimorphism (Dur et al., 2011; Katona, 1975), this make it possible to carry out investigations on sex-specific toxicity to chemicals. Their fast growth and short life cycle make it possible for toxicity test on life cycle traits and multigenerational studies to be carried out.

Cadmium (Cd) is highly toxic to copepods and its toxicity is species and sex-specific (Kadiene et al., 2017). Cadmium is known to cause oxidative stress or DNA damage in various tissues resulting in loss of membrane functions (Sarkar et al., 1998) and elicit other biochemical responses in fish (Almeida et al., 2009; Jia et al., 2011) and in copepods (Ensibi and Yahia, 2017; Wang and Wang, 2009). Acute exposure to Cd resulting to stress in copepods are mostly significant than chronic exposure, and it is due to induced adaptation mechanisms under chronic exposure (Patra et al., 2011). The goal of ecotoxicology is to understand how pollutants disrupt the normal biological processes, and how regulatory measure can be devised to reduce or prevent adverse or compromising effects on the ecological fitness of individual organism or populations.

Ecotoxicogenomics describes the studies involved with analysing responses to toxic exposure at the transcriptomic, proteomic and metabolomics levels (Snape et al., 2004). It also involves the use of quantitative polymerase chain reaction (qPCR) or complementary DNA microarrays to analyse sequenced target genes responding to environmental contaminants. Ecotoxicogenomics-based endpoints are becoming increasingly important for the detection of sublethal effects. Environmental gene expression techniques enable the evaluation of transcriptional changes in copepods in order to understand the mechanisms of action of heavy metals.

The multigenerational effects of heavy metals have been shown to include physiological acclimation and genetic adaptation (Kwok et al., 2009; Lilley et al., 2012; Sun et al., 2014; Tsui et al., 2005; Vidal et al., 2003). Adaptation to chronic Cd exposure reduces ROS production, but acquired Cd tolerance with aberrant gene expression plays important roles in chronic Cd toxicity (Patra et al., 2011). Very few literatures are available on transcriptomic study of *P. annandalei* copepod. Differentially expressed gene profile was reported for a strain of *P. annandalei* (Jiang et al., 2013), the suppression subtractive hybridization (SSH) was used to elucidate the response of *P. annandalei* to nickel exposure at the gene level (Jiang et al., 2013). Transcriptome analysis was performed to investigate response of *P. annandalei* to a low dose of mercury chloride ($HgCl_2$) (Wang et al., 2017). However, no study has been reported for molecular response of *P. annandalei* male and female copepods to cadmium under multiple generational exposure. Variations in chemical tolerance between populations are influenced by acclimation and adaptation among others linked to exposure history. Their difference is in how long their effects are maintained. Tolerance to toxicity of chemical changes over multiple generations of exposure. Sun et al. (2014), showed that metals like copper (Cu) exposed to copepod are consistent with acclimation, because of their loss in tolerance in an unpolluted environment after exposure to Cu. However, other types of pollutants like tributyltin oxide (TBTO) exposed to copepods are consistent with adaptation, after several generations, they maintain tolerance when transferred to an unpolluted environment perhaps because of delayed onset of tolerance. Short multigenerational studies with smaller population sizes has shown copepod response to Cu to be strictly plastic (LeBlanc, 1982; Kwok et al., 2009; Sun et al., 2014). Moreover, other environmental factors may influence their rate or pattern of response. The degree to how these conditions affect their biological responses may be dependent on the fitness of the generation. Plastic acclimation response, a selective tolerance that could be switched on and off to cope with a rapidly changing environment can be highly energy demanding (Agra et al., 2010, 2011; Kwok et al., 2009; Lukasik and Laskowski, 2007; Su et al., 2014). The recent development of molecular biomarkers in ecotoxicology involves real-time measurements of changes in specific messenger RNA. The underlying concept is that exposure to a particular chemical (e.g., Cd) will result in the

transcription of messenger RNA sequences specific to proteins involved in the metabolism and/or detoxification of Cd or generally responding to xenobiotic stress. This may provide a chemical specific "fingerprint" for the type and quantity of chemical involved in the exposure (McGeer et al., 2004).

This study was performed to investigate the multigenerational effect of cadmium on copepods. This is important to understand the dynamics of copepod ecology with the persistence of pollutants in their environment. Therefore, we exposed *P. annandalei* to a sub-lethal concentration of cadmium. We aim to compare the molecular responses of selected differentially expressed genes (DEGs) in male and female *P. annandalei* copepods under multigenerational exposure to cadmium.

7.2. Materials and Methods

Experimental design

Pseudodiaptomus annandalei copepods were exposed to a sublethal concentration, 40µg/L of cadmium (Cd) equivalent to LC10 of *P. annandalei* (Kadiene et al., 2017). Treatments were prepared in triplicates and kept in the culture conditions as described in chapter 1. The protocol for multigenerational study used here was according to Souissi et al. (2016), with major modifications. Polypropylene beakers (5 L) were used, after cleaning, they were pre-contaminated with Cd for about 3 days (to prevent further absorption of the metal to the walls of the beakers, which may reduce Cd availability) (Clason and Zauke, 2000). Because when metal is introduced into water, they tend to precipitate or adsorb to suspended particulate matter and colloidal or dissolved organic materials (Salomons and Foster, 1984). 60 ovigerous females randomly selected from the large culture, were incubated in a false bottom mesh and the medium containing the nauplii was spiked with 40µg/L of Cd. The culture media were changed when they became copepodid and were maintained until they became adults. They were fed every 2 days with uncontaminated algae (*I. galbana* and *N. oculata*). When a large number of first generation female with egg sacs were observed, 60 ovigerous females (OVF) were selected and placed in already contaminated medium to produce the next generation. This process was repeated

for up to 10 generations. At the end of each generation, ovigerous females were preserved to estimate fecundity, more than 300 males and non-ovigerous female (NOF) copepods were separately selected for RNA extraction and another set of copepods were selected for bioaccumulation analysis. The remaining copepods were preserved to estimate the copepod population. Selected differentially expressed genes were analysed using real time qPCR quantification in male and non-ovigerous females of *P. annandalei* to evaluate treatment effect (control versus Cadmium) and sex-specific effect (Male versus female) from multiple generations (F1-F4 and F9).

Bioaccumulation analysis of cadmium in copepod samples

Copepod samples collected were filtered with high quality membrane filter papers, the filtrate (water) was collected in an acid washed water-sampling bottle and preserved with ultrapure HNO_3 until further analysis. Copepod samples filtered were rinsed with milli-Q water to remove the salt, and then placed in the labelled cell culture plate and dried in an oven at 60 °C until constant weight. Dried and weighted samples were carefully folded and placed at the bottom of the digestion tubes. The mixture of ultrapure HNO_3 and HCl at a ratio of 1:3 was added, tightly covered and then placed in a digestion hot block at 120°C for 3 hours. After digestion, samples were let to cool to room temperature and diluted with milli-Q water to 10 ml. Digested samples were analysed with ICP- OES/MS. The quality of the data analysed was validated using the certified reference materials.

RNA extraction and Real-time qPCR

The male and female copepod samples were kept separately for 48 hours to prevent further mating and to empty their guts, and for ovigerous females present in the selection to release their eggs. After 48 hours, the males and females were thoroughly screened to ensure purely males and female. Which was followed by the removal of faeces and debris. The medium was siphoned out and re-suspended several time with clean medium to completely remove all debris. They were transferred with glass pipette into autoclaved Eppendorf tubes. RNA was extracted using TRIzol and purified using Invitrogen PureLink kits. DNA contaminants were removed with DNase (QIAGEN). The purified RNA was

quantified by using Nanodrop spectrophotometer (UV absorbance at 260nm). In addition, an aliquot of the RNA sample was run in agarose gel for quality control. Reverse transcription of RNA sample to cDNA was carried out with Applied Biosystems Master Mix composed of 10x RT Buffer, 10x RT Random Primers, 25x dNTP Mix (100mM), MultiScribe Reverse Transcriptase, 50 U/µL, without RNase Inhibitor. Real-time PCR (qPCR) was used to measure variations in transcriptome levels between samples. 3 genes (Table 7.1) where selected from a large number of differentially expressed genes (chapter 6) as target genes in the QPCR analysis of male and female copepod samples from F1-F4 and F9, to estimate differences in gene expression across generations. Elongation factor 1-alpha (EF1a) was used as the reference gene. Primers were designed using *Primer3* Software (Table 7.1). Beacon Designer^{TM} (Free Edition) was used to check for primer secondary structures. The Real-Time PCR was carried out on an Applied Biosystems 7500 model, featuring a reaction mixture with SYBR Green as fluorescent dye. 1 µg of cDNA samples was prepared as template (50 ng/µl). A master reaction mix was first prepared and contained (per tube): 10 µl of SYBR Green mix, 1µl of forward primer (4 µM final), 1µl/ of reverse primer (4 µM final) 3 µl of DEPC water. For a 20 reaction volume, 15 µl of the master mix was first pipetted into the reaction plate (96-well), 5 µl of cDNA template (2 ng/µl) was pipetted and added to 15 µl of master mix already in each reaction plate. Air bubbles in the reaction plates were removed but vortex or by finger tapping and centrifuged at 3000 xg for 2 minutes at 4^{o}C before starting the reaction.

Statistical analysis

Comparison between groups was made by one-way ANOVA and independent sample t-tests, followed by a Tukey test for identification of the statistically distinct groups. Significant differences were accepted for $p< 0.05$. The statistical analyses were performed using SPSS 18 software and Microsoft excel 2016. Experiments were performed in triplicates. Fold change for the gene expression relative to controls was determined by the $2^{-\Delta\Delta CT}$ method of Livak & Chmittgen (2001).

Table 7. 1. Target genes of interest for qPCR analysis of copepod *Pseudodiaptomus annandalei* exposed to cadmium

Protein Code	Protein name	F and R Primers
MHEMT1	Myohemerythrin-1	>>TGTGCTCCTCGTCAAGATTT <<TGAGTTTGCTGTGTTTGTGAG
MHEMT	Myohemerythrin	>>GATTATAGCCTAACACCTCTCAAT <<GGTTACAGGGTTTACAGTCTATT
HSP70	Heat shock protein 70	>>ACTCTCTGGCTGAAGGTATTG <<TCGAGTGTGCCCTTGAAC
EF1a	Elongation factor 1-alpha (Reference gene)	>>TTCAAGTACGCCTGGGTTT <<AAACTTCCAGAGAGCAATGTC

7.3. Results

7.3.1. Bioaccumulation of Cd in *P. annandalei*

Figure 7.1a shows the bioaccumulation of cadmium (Cd) in *P. annandalei* copepods (mixture of males and females) from the 1st generation (F0) to the 7th generation (F6) and the 10th generation (F9). Cd accumulation in copepod was about 43 µg/g in F0 then increased to F4, followed by a slight decrease from F5 to F9. Figure 1b shows the sex specific accumulation after 10 generations (F9). The concentration of Cd accumulated in males was significantly ($p<0.05$) higher those in females, however, not significantly different ($p>0.05$) than the concentration in the male and female copepods mixture (here the number of females was more than males).

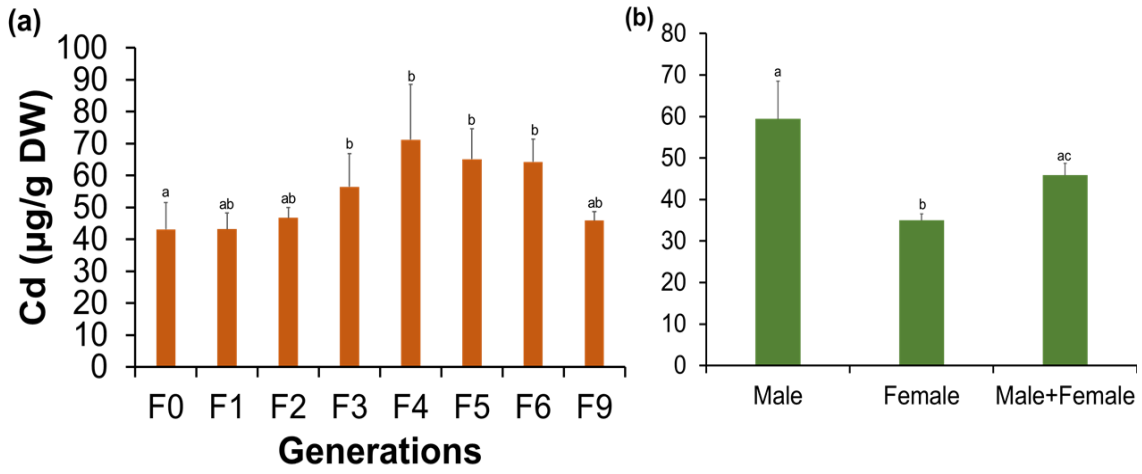

Fig. 7. 1. Cadmium accumulation in *P. annandalei* adults (male + female) at the end of each generation **(a)** and sex-specific Cd accumulation of *P. annandalei* at the end of F9 **(b)**. n = 3, mean ± SD.

7.3.2. Fecundity and population density

Clutch size (number of eggs in a pair of egg sacs) of copepod exposed to Cd was significantly lower than control in F0 (Figure 7.2). However, clutch size increased significantly in the subsequent generations more than the control groups except in F5. Moreover, the population density of Cd treated copepods were lower than controls except in F2, F7 and F8 (Figure 7.3). In addition, negative effect of Cd on the population density of copepods where statistically significant ($p<0.05$) in F0, F3, F4 and F9.

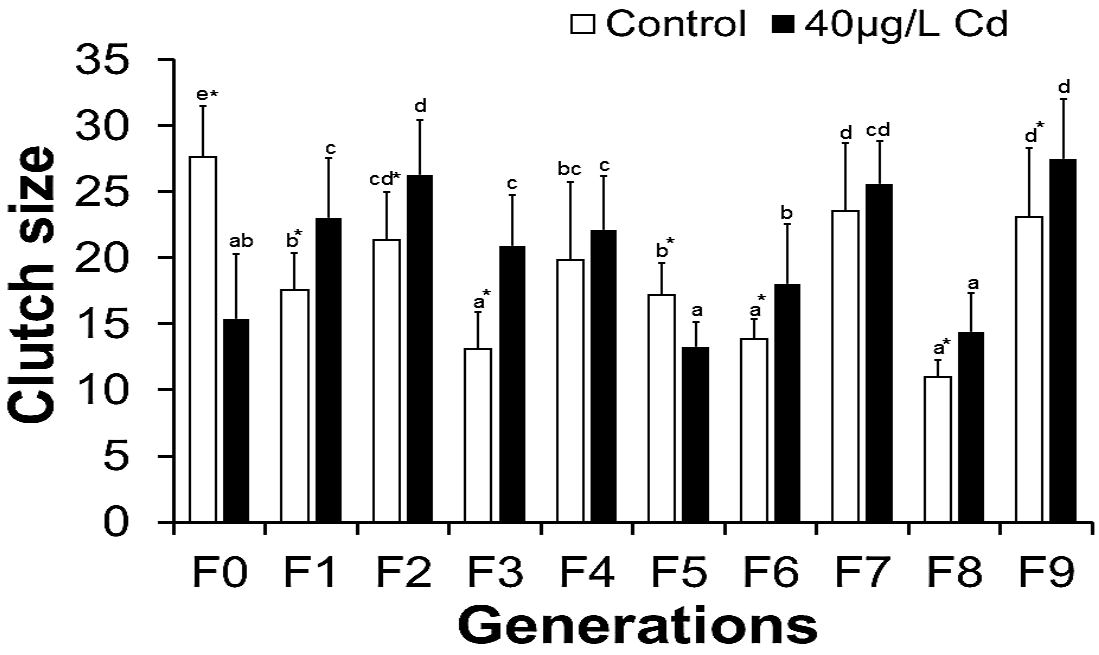

Fig. 7. 2. Clutch size (number of eggs per female) of *P. annandalei* copepod with and without exposure to cadmium at the end of each generation. Mean ± SD.

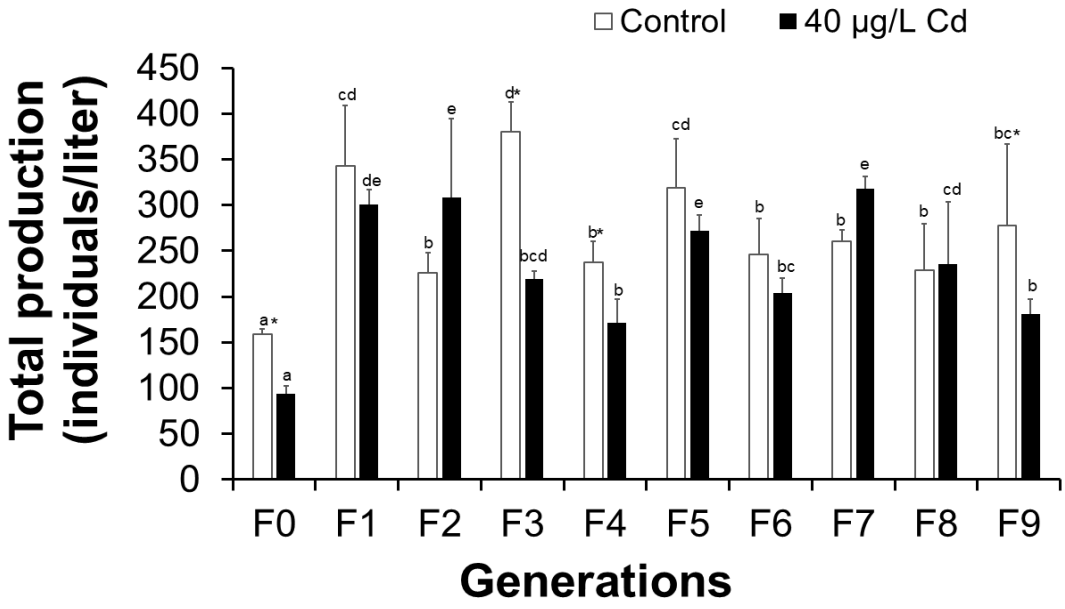

Fig. 7. 3. Total population density of *P. annandalei* copepod with and without exposure to cadmium at the end of each generation. Mean ± SD.

In the control, female prosome length (PL) of copepods in F0 was significantly ($p<0.05$) higher than those in F1 – F9 and those in F8 was significantly ($p<0.05$) lower. Female prosome length (PL) of copepods exposed to Cd were significantly ($p<0.05$) lower in F9. Moreover, PL of females in F0 were not significantly different ($p>0.05$) from those in F1- F7, however, they were significantly higher than those in F8 and F9. Student ttest analysis showed statistical significance ($p<0.05$) between PL of females in control and those exposed to Cd in F0 and F9. Similar statistical significance was also observed in prosome width (PW) and prosome area (PA). In the control, PA of females in F0, F1 and F7 were significantly different from those in F8. However, PA of females exposed to Cd in F8 and F9 were significantly lower than those in F0 – F7 (Fig. 7.4).

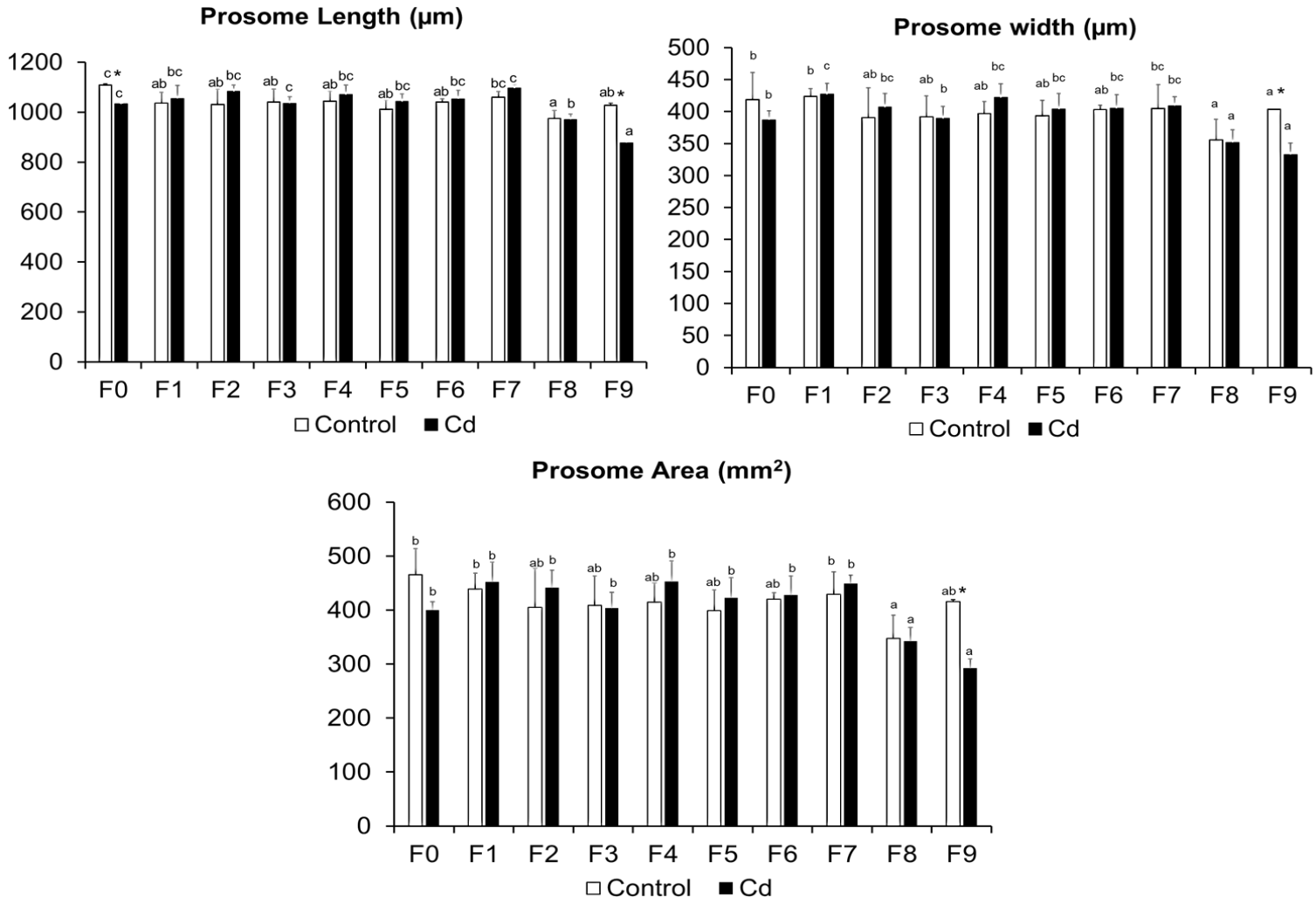

Fig. 7. 4. Prosome length (a), prosome width (b), Prosome area (c) of *P. annandalei* female copepod with and without exposure to cadmium (Cd) at the end of each generation. Mean ± SD. Alphabets represents statistical significance ($p<0.05$) between generations of each treatments and asterisks (*) represents statistical significance ($p<0.05$) between control and Cd.

Table 7.2 shows the Pearson correlation (R) between population (Pop) vs. prosome length (PL), population (Pop) vs. clutch size (CS) and prosome length (PL) vs clutch size (CS) of *P. annandalei* under control and cadmium (Cd) treatments. The linear relationship between each comparison for control and Cd treatment was carried out first in the whole generation (F0 – F9) then for the first 3 generations (F0 – F2), intermediate generations (F3 –F6), and last 3 generations (F7 – F9), also, for the first 5 generations (F0 – F4) and the last 5 generations (F5 –F9).

Table 7. 2. Relationship (R) between population (Pop) vs. prosome length (PL), population (Pop) vs. clutch size (CS) and prosome length (PL) vs clutch size (CS) of *P. annandalei* under control and cadmium (Cd) treatments.

		F0-F9		F0-F2		F3-F6		F7-F9		F0-F4		F5-F9	
		Control	**Cd**	**Control**	**Cd**	**Control**	**Cd**	**Control**	**Cd**	**Control**	**Cd**	**Control**	**Cd**
Pop vs. PL	R	-0.236	0.239	-0.496	0.44	-0.127	-0.519	0.213	0.803**	-0.483	0.255	0.212	0.527*
	p value	0.202	0.195	0.174	0.236	0.664	0.069	0.612	0.009	0.068	0.359	0.449	0.043
Pop vs. CS	R	-0.361*	0.168	-0.764*	0.871**	-0.319	-0.706**	0.401	-0.121	-0.793**	0.780**	0.258	-0.248
	p value	0.046	0.366	0.017	0.002	0.266	0.007	0.325	0.756	0	0.001	0.354	0.372
PL vs. CS	R	0.619**	0.061	0.815**	0.769*	0.196	0.209	0.777*	-0.011	0.636*	0.666**	0.569*	-0.218
	p value	0	0.746	0.007	0.015	0.501	0.494	0.023	0.977	0.011	0.007	0.027	0.435

*. Correlation is significant at the 0.05 level(2-tailed); **. Correlation is significant at the 0.01 level (2-tailed).

7.3.3. Multi-generational expression of target genes in *P. annandalei* exposed to Cd: female vs male

Two isoforms of myohemerythrin proteins (MHMET-1 and MHMET) were significantly expressed in male (CdM) and female (CdF) exposed to cadmium (Cd). MHMET-1 expression in male and female copepods exposed to Cd was 434- and 146-fold change relative to their control respectively in F0 (Figure 7.5a). However, in F1, the expression level of MHMET-1 was significantly reduced in CdM and downregulated in CdF. In F2, MHMET-1 was downregulated in both CdM and CdF. In F3, MHMET-1 was upregulated in both CdM and CdF, however, MHMET-1 was significantly ($p<0.05$) more expressed in CdM than in CdF. In F4 and F9, MHMET-1 was downregulated in both CdM and CdF. MHMET isoform expression in male and female copepods exposed to Cd was 12- and 3-fold change relative to their control respectively in F0 (Figure 7.5b). However, in F1, the expression level of MHMET decreased in CdM and CdF. In F2, MHMET expression was further reduced in CdM, however, it was downregulated in CdF. In addition, MHMET was upregulated in both CdM and CdF from F3 to F9. Upregulation of MHMET was significantly ($p<0.05$) higher in CdM than in CdF in F0 and F1, however, was significantly ($p<0.05$) lower in CdM than CdF in F3 and F4.

Heat shock protein 70 (hsp70) was downregulated in CdF in F0 and was upregulated in F1. However, it was downregulated in F2 and then upregulated in F3, and downregulated in F4 and again upregulated in F9 (Figure 7.5c). In F0, hsp70 was upregulated about 43-fold in CdM, then the expression level was reduced in F1. In F2, hsp70 was downregulated in CdM and then in F3 it was upregulated with further increase in expression levels in F4. However, in F9, hsp70 levels in CdM decreased (Figure 7.5c).

Figure 7.6, shows muscle deformation of *P. annandalei* female copepod when continuously exposed to Cd for 10 generations. In addition, slow swimming speed of male and female copepods after 10 generations of exposure to Cd was visually observed (data not available).

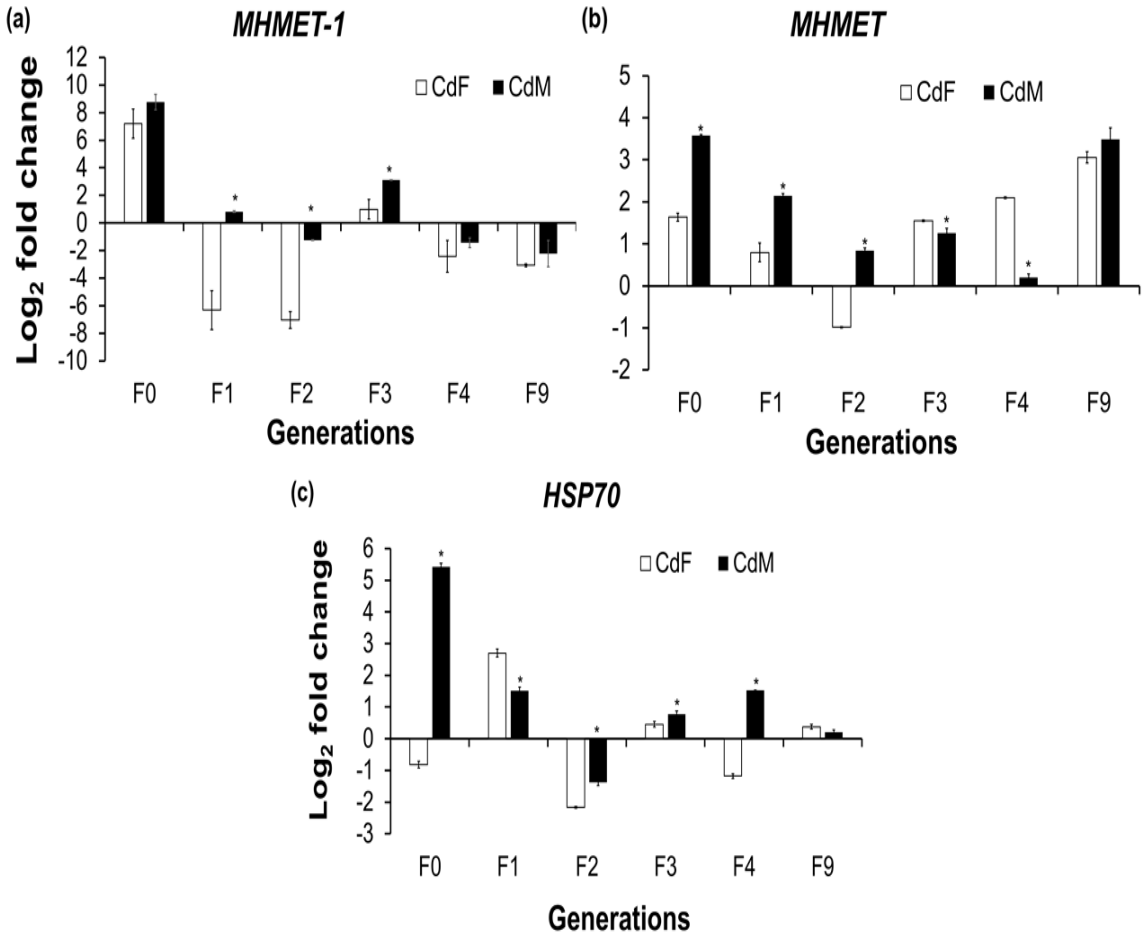

Fig. 7. 5. Myohemerythrin-1 **(a)**, Myohemerythrin **(b)** and Heat shock 70 kDa protein **(c)** expressed (Log_2FC) in males and females of *P. annandalei* copepod exposed to cadmium relative to control after each generation. n = 3, mean ± SD. CdF: female exposed to cadmium; CdM: male exposed to cadmium.

Fig. 7. 6. Normal *P. annandalei* female copepod (a). Muscle deformation (b and c), curved-like shape (red arrows) in *P. annandalei* female copepod exposed to cadmium in the 10th generation.

7.4. Discussion

This study showed high concentration of cadmium (Cd) bioaccumulated in *P. annandalei* copepod in all generations. Zidour et al. (2019), reported an unsaturated increase of Cd in *E. affinis* over time, at the time of sampling. The high concentration of Cd could indicate a low ability of the copepod to excrete Cd. In this study, we observed increasing accumulation of Cd from F1 to F4. This could imply that Cd was accumulated from one generation to the next through maternal transfer, corresponding to similar observations in previous reports (Guan and Wang, 2006; Li et al., 2015; Wang et al., 2018). In addition, we analysed the amount of Cd accumulated in male and female copepods

separately in F9 and observed that, Cd concentration in male was higher than that in the female. This could imply that the female copepods were able to eliminate Cd faster or more efficiently than the males. This may correspond to what was observed in our previous study, where *P. annandalei* male copepods showed more sensitivity towards Cd than the females (Kadiene et al., 2017). It is generally known that metal becomes toxic when the bioaccumulated amount exceeds that which the organism can tolerate. However, some studies show that the change in toxicity of a chemical is not consistently related to bioaccumulation, that is, the fate of the bioaccumulated chemical and its toxicity may depend on the organism's ability to metabolize it (Arunakumara et al., 2008; Gomez et al., 2010; Jennett et al., 1983; Peake et al., 2015). In this study, bioaccumulation of Cd significantly affected egg production in the first generation. Some studies showed that metal pollutant does not affect egg production (Zhou et al., 2016). However, in our study, we observed an increased clutch size in copepods exposed to Cd than the control in the subsequent generations. Higher clutch size in copepod exposed to Cd, could indicate an adaptive trait in *P. annandalei* females as a way to ensure continuity under unfavourable conditions. Although this increase in egg production was observed after the first generation. It is possible that *P. annandalei* female copepods optimizes a trade-off between increasing egg production and the survival of their offspring. Some female copepods under certain environmental stress, produce diapause or resting eggs (Hairston and Olds, 1984). *P. annandalei* females are eggs carriers, and since they are not currently known to produce diapause or resting eggs, in this study, the increase in clutch size could be an adaptation strategy under a long term exposure to Cd stress. Population density followed a similar oscillating pattern as the female fecundity and this regulation could depend on individual or within-generation plasticity of copepods. Similarly, significant toxic effect of Cd was observed in the total population at the end of the first generation as in the clutch size. However, in subsequent generations population density was regulated. Moreover, under Cd treatment, total population density was generally reduced. This may be related to decrease in hatching success or high mortality at the naupliar or early copepodid stages as Cd accumulation increased. Generally, increase in population density (Pop) inversely correlate with prosome length (PL) and clutch size (CS) (Maly, 1973) and prosome length

positively correlate with clutch size (Chow-Fraser and Maly 1991; Maly and Maly. 1998; Souissi et al., 2016). This agrees with our correlation results for control in all generations (see Table 7.2). However, under Cd treatment, Pop positively correlated with PL and CS. Therefore, the higher clutch size observed in copepods exposed to Cd compared to the control may not be due to low density, but a response strategy to Cd exposure. Within the first 3 generations, the same positive correlation between Pop and PL, Pop and CS was observed. Also, positive relationship between Pop and CS was observed. In the following 4 generations, Pop and PL, Pop and CS tend to return to normal as in the control. However, in the last 3 generations, correlation between Pop and PL was positive as in the first 3 generations. A strong positive correlation was generally observed between PL and CS for copepods exposed to Cd. In this study, the normal biological processes in the copepod show an up and down regulatory pattern alternating between each generations, which could indicate biological fitness adjustment mechanism. However, an interesting pattern was observed in copepods exposed to Cd, whereby there was a stepwise increase or decrease in 2, mostly 3 consecutive generations at the beginning and toward the end of the 10 generations. Moreover, the intermediate generations (F3/F4 – F6) tend to regulate up and down more frequently as in the control, in a bid to recover or restore normal processes. The toxicity of Cd was strongly observed at the earlier and/or later generations as observed in the total population, clutch size and female morphology.

There are two main types of low-molecular-mass Cd-binding proteins (Cd-BP) that have been reported in invertebrates, metallothionein-like and non-metallothionein-like proteins (Stone and Overnell, 1985; Slice et al.,1990). Hemerythrin (HMET) is a non-metallothionein cadmium-binding protein, a non-heme iron protein containing a di-iron oxygen binding site attached to a protein. The members of the HMET gene family, including myohemerythrin (MHMET) are multi-functional proteins, they participate in respiration, oxygen transfer and/or storage and may act as a buffer to control the concentration and therefore the toxicity of cadmium and aspects of innate immunity and their functions have effect on oxidation-reduction regulation (Bailly et al., 2008; Coates and Decker, 2017; Liu et al., 2013; Meyer and Lieb, 2010; Stenkamp, 1994; Terwilliger, 1998; Yang et al., 2012).

The physiological response of copepod to metal contamination is very fast particularly under experimental conditions, as we observed in the first generation. This followed a decrease in the production of both MHMET isoforms (MHMET-1 and MHMET) after exposure to Cd in multiple generations. Although, MHMET-1 tends to decrease or was downregulated in 2 generations after F0 and then increased in F3, followed by downregulation in subsequent generations. However, MHMET production increased in the later generations after a significant decrease in a generation. The decrease in expression level or downregulation of one gene responding to Cd and the increase or upregulation of another gene similar in function could be supplementary or complementary as observed in this study.

Heat shock proteins (hsps) are a form of protein expressed under stressed conditions. Hsps promote proper protein folding; prevent misfolding, and protein restoration following toxic cellular stresses such as heavy metals (Kim et al., 2014; Pirkkala et al., 2001). Hsp70 is usually more induced than other hsps, and its expression in response to xenobiotic exposure appears to be a protective response (Rhee et al., 2009; Kim et al., 2014). In an unstressed condition, heat shock proteins have constitutive functions that are essential in various aspects of protein metabolism (Nikinmaa and Rytkönen, 2011). In this study, hsp70 was up regulated in the control female, however, when the females were exposed to Cd (CdF), hsp70 was downregulated and was upregulated in males exposed to Cd (CdM) in the F0. Whereas, hsp70 in CdF became upregulated in the F1, and in subsequent generations, hsp70 was regulated in an up or down manner. For males exposed to Cd, hsp70 was consistently upregulated in all the generations, except when it was downregulated in F2, however, the expression levels in subsequent generations were lower than in F0. Similar modulation of heat shock proteins was reported in the calanoid species *Calanus finmarchicus* after copepod exposure to pollutants (Hansen et al., 2008). Both up- and downregulation of stress related genes have been reported in response to xenobiotic (Anderson et al., 2015; Goncalves et al., 2016). Downregulation of these genes could indicate tolerance in one generation and with further upregulation or increase in expression level, could also indicate a change in sensitivity in another generation. The energy cost for egg production in female copepod and general protein synthesis which also produces

reactive oxygen species (ROS) can be very high (Wieser and Krumschnabel, 2001; Sokolova et al., 2012; Tomanek, 2014). Therefore, downregulating protein synthesis under prolonged exposure to Cd could be a strategy for conserving energy and minimizing oxidative damage from excess ROS production (Bailey et al., 2017). If the low production or downregulation of protein in this study is a strategy to conserve energy, it could also explain the slow swimming speed of the copepods observed in the later generations.

In our previous study (Kadiene et al., 2017), we reported that metal toxicity to calanoid copepods is sex-specific. We observed corresponding molecular response between males and females in the present study. Moreover, gene expression in response to Cd such as MHMET and hsp70 were mostly expressed significantly in the male than in females.

The multigenerational regulation of genes expressed may indicate plasticity or differences in individual fitness. Most genes that were highly upregulated at the F0 generations were either down regulated or their expression levels were reduced in the subsequent generations. This could be attributed to tolerance or suppression, when they become overwhelmed, since the amount of Cd accumulated increased with each generation. Kimberly and Salice (2015), reported that a long-term exposure to cadmium alters tolerance traits in a non-monotonic way. As a result of the effect observed after one-generation removal from Cd, their results indicated that multigenerational exposure may result to transgenerational effect. The fluctuations in the genes responding to metal exposure are usually linked to variation in general protein metabolism than to changes in metal accumulation (Legras et al., 2000). In addition, Vandegehuchte et al. (2010), attributed the differential gene regulation between subsequent control generations after exposing to zinc to possible differences in synchronization of the molting and reproductive cycle of the daphnids in the different generations. Furthermore, high variations and fluctuations in response from multigenerational studies are often observed, resulting from different sampling time per generation, sex specific sensitivity to contaminants, within and trans-generational plasticity, and influence of non-contaminant stressors (handling) at different generations. Based on the type of test carried out, these factors cannot easily be predicted and controlled, underscoring the need to use multiple toxicity methods.

The female copepods exposed to Cd in this study firstly, showed lower cadmium uptake or rather faster excretion rate than male, and secondly, produced more eggs when compared to those in the control. Although, this indicates their level of adaptability when compared to the males, however, other physiological aspects could be compromised, such as the deformation of their muscular structure. Transmission electron micrographs of the muscle of *Pseudodiaptomus annandalei* after 12 days of Cd exposure showed evidence of damage of the myofibril ultrastructure attributed to ROS overproduction caused by oxidative damage (Chen, 2016; Ibrahim et al. (2015). This is consistent with the external muscle contortion or deformation observed in our study.

In conclusion, the disparate physiological changes or molecular responses observed from multiple generations continuously exposed to Cd may represent decreased and/or increased sensitivity or tolerance (Clements, 1999; Klerks and Weis, 1987; Koivisto et al., 1992; Kashian et al., 2007) and could be regulated by each generations plasticity. The increased egg production and slight improvement in the total population density of copepods exposed to Cd compared to the control, in addition to the observed slow swimming speed in both sexes and muscle deformation observed most in the female copepods, indicates that *P. annandalei* copepod females can still be able to strive in such environmental condition. *P. annandalei* showed possible tolerance or fitness-based adjustments to Cd toxicity after several generations in terms of reproduction. Although, the existing data are not sufficient and conclusive enough, however, they have the potential to be used in the development of chronic water quality criteria and estimating the long term effect of persistent pollutants in the ecosystem.

Chapter 8. Summary and Conclusion

8.1. Summary and conclusion

The goal of this study initially was to determine the effect of cadmium (Cd) toxicity comparatively on two copepods, *Eurytemora affinis* from a temperate region (Seine Estuary, France) and *Pseudodiaptomus annandalei* from a subtropical region (Danshuei Estuary, Taiwan). We found that male and female copepods have different response levels to heavy metal toxicity like cadmium. In addition, both copepod species had different levels of sensitivity to Cd. We proposed that the reason for this differences were because they bioaccumulated different amounts of Cd. After testing this theory using *P. annandalei* as the subsequent test species, we found that both males and females had different Cd body burden. Moreover, we discovered that water (dissolved phase) is the major uptake route of metal in copepods, and the reason is partly because of oral intake in addition to external adsorption through exoskeleton or cuticle.

Studies on gene expression profile of both sexes of *P. annandalei* showed that male and females gene expression both in magnitude and directions (up/downregulation) in response to Cd exposure were different. In addition, detoxification and particularly excretion of Cd seemed to be more efficient in females than in male. This could account for the lower body burden of Cd in the females than in the male.

Environmental factors such as temperature and salinity affects the level of chemical toxicity by both influencing their uptake rate and bioavailability. This was demonstrated, by exposing *Diacronema lutheri* to a range of salinity, temperature and increasing Cd concentrations and, one or a combination of low salinity, high temperature or increasing Cd concentrations increased Cd uptake in the algae. These also applied to copepods as shown by other studies. The frequent fluctuations of abiotic factors in estuaries and the increasing presence of persistent pollutants from anthropogenic activities poses a threat to the copepod community. However, the plasticity of copepod observed from different copepod generations exposed to Cd pollution may be an added advantage enabling them to strive in these unfavourable conditions.

Although, copepods could develop adaptive mechanisms to tolerate toxic chemicals, however, an increasing concentration of metals in the aquatic environment in addition to maternal transfers of metals over several generations could increase these concentrations in copepods. Therefore, a long term exposure could reduce their fitness, thereby compromising copepod population structure.

This study showed that mortality, life history traits and molecular responses of model species can provide important bio-indicators for environmental risk assessment.

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