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"Effects of environmental factors on the eco-physiology and life traits of a macro-crustacean (shrimp) and micro-crustacean (copepod)"

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Abstract

The present thesis constitutes two major sections, the first deals with macro crustaceans, mud shrimp *Austinogebia edulis* and the second with micro crustaceans, the copepod *Eurytemora affinis.* The objectives were to 1. Explore the role of *A. edulis* as an ecological engineer, 2. Effects of Cadmium (Cd) on oxidative enzymes and morphology in *A. edulis*, 3. To assess the spatial and temporal variation of persistent organic pollutants (POPs) in the natural habitat of *A. edulis*, 4. To explore the effect of combined heavy metals to *E. affinis* and to depict the effect of sediment in re-suspension to *E. affinis* by following a multigenerational approach.

For the first objective, we highlight the significant differences between burrow walls and burrow lumen. The burrow wall of *A. edulis* showed low permeability and increased sheer strength. Statistically, a significant difference was noticed in the comparison between the sediment composition of the burrow wall and the background. The burrow wall consisted of a 24 times higher organic matter content than one individual of mud shrimp. Thus, they transform the sediment characteristics as an ecological engineer, which is expected to have a significant ecological impact on the ecosystem.

Furthermore, on exposure to Cd the antioxidant enzyme (SOD, CAT, and GPx) activities decreased with increasing Cd concentration and extended exposure time in these organs of *A. edulis*. Significant damage of the hepatopancreas of *A. edulis* was noticed at higher concentrations of Cd, showing damages like the disappearance of epithelial cell boundaries, detachment of cells from the basal lamina, cellular swelling, necrosis, etc. In conclusion, Cd caused oxidative damage by reducing the activities of antioxidant enzymes and by damaging the tissue structure in major organs of *A. edulis*.

For the measured POPs, the spatial distribution showed that the proximity to sources was the most important determining factor for the distribution of these contaminants showing greater concentrations in samples collected near the industrial parks. The analyzed PAH ratios determined an existence of both pyrolytic and petrogenic inputs. PCBs Aroclor 1016 and 1260 were the main sources near the industrial zone, with DDT inputs showing recent addition to the area. The overall study reflected a borderline risk to the benthic organisms.

For *E. affinis*, the acute toxicity LC 50% (96h) for the exposure of Lead (Pb) was found to be 431.99 µg/l for males showing lower sensitivity than females with 394.27 µg/l. The total population became lowest in the 2^{nd} generation (F1) for all the exposure treatments and also the mortality increased in this generation. The bioaccumulation of metals in the copepod *E. affinis* was also higher in this generation; thereby fecundity and survival appeared to be linked to the bioaccumulation of heavy metals and concluding that the sensitivity or fitness of *E. affinis* was directly connected to the trace metal accumulation. The percentage of males was less in the sediment treatment than the heavy metal and control. This observation can slightly indicate the different ways of copepod sensitivity to heavy metals and sediment in re-suspension when exposed for multiple generations.

Keywords- Toxicology, crustaceans, intertidal flats, mud shrimp, copepod, trace metals, organic pollutants.

Résumé

La présente thèse comprend deux grandes sections, la première traitant des macro-crustacés, la crevette de vase *Austinogebia edulis* et la seconde des micro-crustacés, le copépode *Eurytemora affinis*. Les objectifs étaient de 1. Explorer le rôle d'*A. edulis* en tant qu'ingénieur écologique, 2. Effets du Cd sur les enzymes oxydatives et la morphologie d'*A. edulis,* 3. Évaluer la variation spatiale et temporelle des polluants organiques persistants (POP) dans l'habitat naturel d'*A. edulis*, 4. Explorer l'effet d'un cocktail de 4 métaux lourds sur *E. affinis* et décrire l'effet des sédiments en suspension sur *E. affinis* en suivant une approche multigénérationnelle.

Pour le premier objectif, nous soulignons les différences significatives entre les murs des terriers et les lumens. La paroi du terrier d'*A. edulis* présentait une faible perméabilité et augmentait la résistance. Statistiquement, on a remarqué une différence importante dans la comparaison entre la composition des sédiments de la paroi du terrier et le fond. La paroi du terrier se composait d'une teneur en matière organique 24 fois supérieure à celle d'un individu de crevette. Ainsi, ils transforment les caractéristiques des sédiments en tant qu'ingénieur écologique, ce qui devrait avoir un impact écologique important sur l'écosystème.

De plus, lors de l'exposition au Cd, les activités des enzymes antioxydantes (SOD, CAT et GPx) diminuaient avec l'augmentation de la concentration de Cd et le temps d'exposition prolongé dans ces organes de la crevette *A. edulis*. Des dommages importants de l'hépatopancréas d'*A. edulis* ont été remarqués à des concentrations plus élevées de Cd, montrant des dommages comme la disparition des limites des cellules épithéliales, le détachement des cellules de la lamine basale, gonflement cellulaire, nécrose, etc. En conclusion, le Cd a causé des dommages oxydatifs en réduisant les activités des enzymes antioxydantes et en endommageant la structure tissulaire dans les principaux organes de la crevette *A. edulis*.

Pour les POP mesurés, la distribution spatiale a montré que la proximité des sources pollution était le plus important facteur déterminant pour la distribution de ces contaminants, montrant des concentrations plus élevées dans les échantillons prélevés près des parcs industriels. Les rapports des HAP analysés ont permis de déterminer l'existence d'intrants pyrolytiques et pétrogéniques. Les PCB Aroclor 1016 et 1260 étaient les principales sources près de la zone industrielle, avec des entrées de DDT montrant un ajout récent à la zone. L'étude globale a révélé un risque limite pour les organismes benthiques.

Dans le cas d'*E. affinis*, la toxicité aiguë de CL 50 % (96h) pour l'exposition au plomb (Pb) était de 431,99 µg/l chez les mâles présentant une sensibilité inférieure à celle des femelles avec 394,27 µg/l. Les plus faibles effectifs totaux de la population ont été observés durant la deuxième génération (F1) pour tous les traitements d'exposition et, par conséquent, la mortalité a augmenté dans cette génération. La plus forte bioaccumulation des métaux dans le copépode *E. affinis* était également plus élevée dans cette génération ; la fécondité et la survie semblaient donc liées à la bioaccumulation des métaux lourds et concluaient que la sensibilité ou la fitness d'*E. affinis* était directement liée à l'accumulation de métaux traces. Le pourcentage de mâles était moins élevé dans le traitement des sédiments que dans celui des métaux lourds et des témoins. Cette observation peut indiquer légèrement les différentes façons dont les copépodes sont sensibles aux métaux lourds et aux sédiments en suspension lorsqu'ils sont exposés pendant plusieurs générations.

Mots-clés: Toxicologie, crustacés, vasières intertidales, crevettes de vase, copépodes, métaux traces, polluants organiques.

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Chapter 1 – General introduction

1.1 Intertidal zone and its creatures

Intertidal zone or the littoral zone is the area, which is exposed to the air during low tide and under water at high tide. Such tidal flats constitute a transition zone between land and sea (Bearup et al., 2017). This area generally includes different types of habitats, with many types of creatures, such as clam, crab, shrimp, starfish, sea urchins, microalgae and even bacteria. Rocky cliffs, sandy beaches, or mudflats mainly characterize intertidal zones. Organisms in the intertidal zone are adapted to an extreme environment (Walag et al., 2016). Salinity in these areas can vary seasonally, with low salinity during monsoon to highly saline during the summer depending on the tidal inundations. Due to strong wave splashing, often the burrows of these creatures are distorted. Temperature can vary due to high exposure of the sun in the intertidal zone to nearly freezing in colder climates. Nutrients are supplied to the intertidal zone regularly from the seawater that actively moves by the tidal actions. By this rise and fall of tides, a dominant rhythm of plant and animal life is supported.

The ecology of intertidal zone is the study of the ecosystem where the organisms live between the high and the low tide lines. A broad way of classifying the intertidal communities are generally based on the type of substrates, which is, rocky shore and mud flat or soft bottom communities (Raffaelli et al., 1999; Lars et al., 2002). Benthic ecologists therefore study the interaction between intertidal organisms and their environment including the interaction between different species within a specific community. Rocky intertidal organisms like the barnacles, limpets, chitons, mussels, etc., are mainly found on rocky shores, cobble beaches or man-made jetties. These animals face higher wave action and must be adapted to develop means to tightly hold on to the substratum during strong wave actions. To avoid the dislodgement due to strong waves, organisms have developed types of attachments like byssal threads and glues for the mussels, suctioning tube feet for sea stars, hook-like appendages for isopods etc. to hold on to the substrates (Egbert et al., 1987). On contrary, organisms living in the soft bottom sediments or mudflat like crabs, starfish, snails, shrimps, clams, etc., are usually protected from large waves. These mudflat organisms like snails, crabs or mud shrimps, have the potential to move both as juveniles and as adults and can protect themselves in a habitable environment by adapting themselves to burrowing (Kelleher et al., 1995; Simon et al., 2002). During low tide in very high temperatures they usually get into these moist refuges where the temperature is cool and these burrows also helps them to fight with desiccation during the periods of emersion (Burnaford et al., 2004). Depending on various environmental factors, intertidal communities have evolved different adaptations to survive and cope up with these conditions.

Trophic mode of some intertidal organisms is filter feeding where they filter the seawater in the search for planktonic food sources (Paine et al., 1966). Mussels, clams, barnacles, worms and some crabs and shrimps mostly consume these planktons that are carried by the seawater. Wide range of intertidal producers like the benthic diatoms to macro algae like seaweeds can get their primary source of nutrients from the ocean. Some herbivorous grazers like the limpets consume diatoms and kelp crabs feed on the bladelets of the kelp (Fig 1.1). At a higher trophic level, consumers like the starfish eats the grazers like, snails, mussels, etc. and the fishes eat the crabs and the shrimps (Geoffrey et al., 2002). Some intertidal organisms also feed on dead decaying organic matter and are the deposit feeding species like some crabs, shrimps and bivalves, etc.

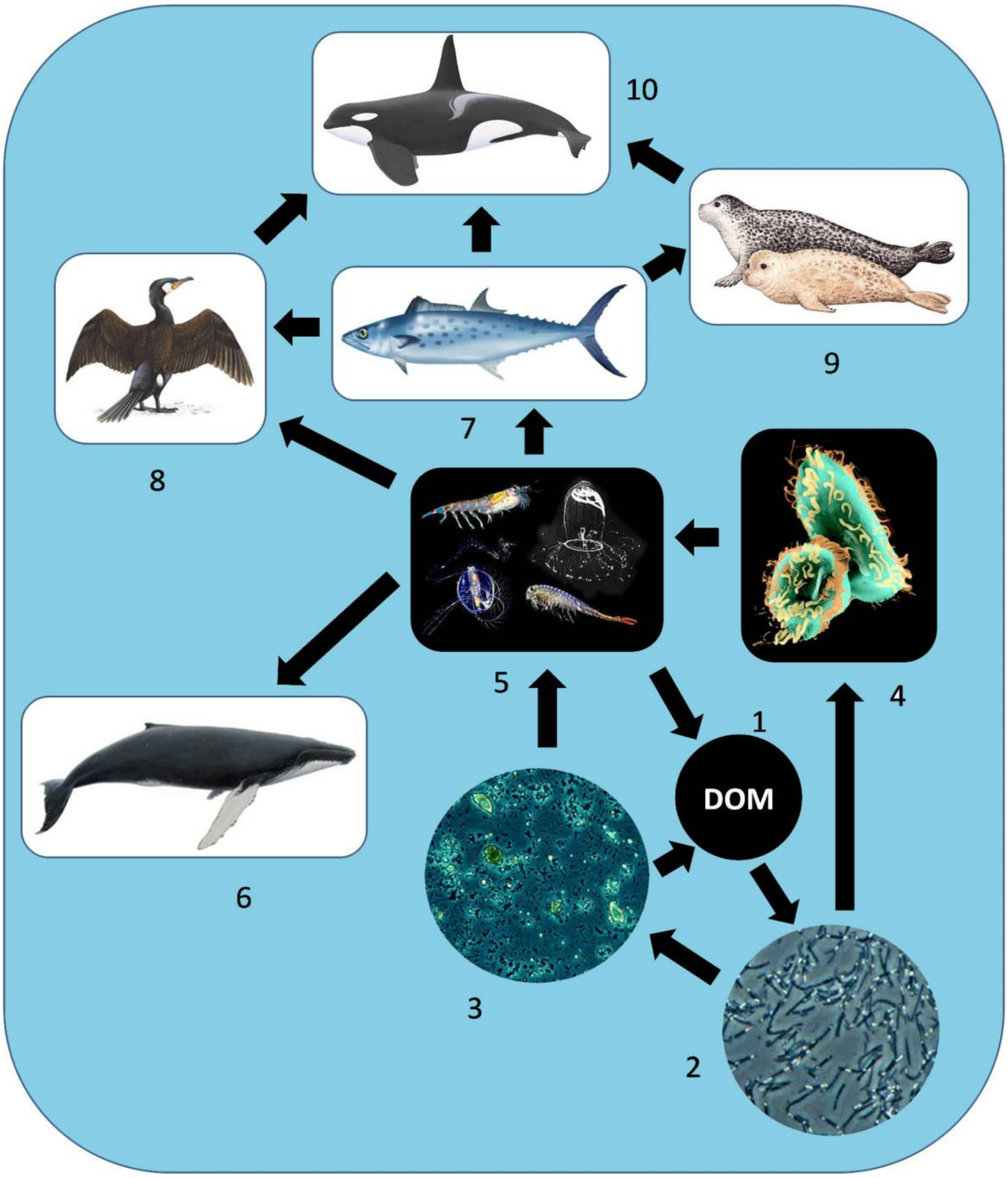

Fig 1.1 The complex food web including the microbial loop Dead organic matter (1), bacteria (2), Phytoplankton (3), protozoa (4), zooplankton (5), large filter feeders (6), fish (7), birds (8), seals (9), killer whales (10) (taken from seos-project.eu).

1.2 Significance of intertidal zones

Intertidal zones are ecologically and economically important and human beings are somewhat dependent on such habitats for food and raw materials (Harley et al., 2006).

In turn, intertidal zones are greatly influenced by human impacts as many human population lives in the coastal area (Manriquez et al., 2001). Therefore, such habitats is challenged by several factors as the effects of global climate change for example increased temperature, sea level rise, ocean acidification, anthropogenic actions, etc. An example is the introduction of non-native species through the shipping traffic by the transport of invasive species in ballast water (Cohen et al., 1998). Human beings have huge impacts on the intertidal zone and its creatures because they tend to exploit these areas for food gathering, for an example, the clam digging in muddy substratum. Due to this, the government of many nations have established many marine protected areas to restrict collection of these creatures ultimately leading to conservation (National Academy Press, Washington, DC, 2001).

1.3 Significance in toxicology

Intertidal areas or mud flats are often addressed as a complex and dynamic environment where massive deposition of numerous amounts of substances takes place. These areas are the sink for several pollutants and as well as nutrients which is carried from freshwater land to the open ocean. From the toxicological view, such intertidal flats are of great significance since they play a major role in providing feeding grounds for certain migrant and native birds, nurseries for fishes, crabs, shrimps etc. Such grounds are also associated with industries and urbanized enterprises that often contribute to a huge amount of toxic substances in these areas (Buggy and Tobin 2008). Such toxic compounds can often get bio-assimilated or bio-accumulated inside the benthic and some benthos-pelagic organisms thereby potentially affecting the entire ecosystem and also posing long term implications on human health (Spencer et al. 2003, Ip et al. 2007). Furthermore, the tidal flats are a compilation of all the physical, chemical and biological interplay and therefore such interactions can have potential influences on the dispensation or distribution of toxic chemicals. Other than natural factors, human activities play a key role in coastal contamination mainly contributing toxic compounds from terrestrial origin through mining industries, construction of harbors, jetties, chemical factories and other commercial and agricultural activities near the vicinity of such tidal habitats. Therefore, a clear understanding of the origin of toxic contaminants along with the spatial and temporal variation within that area is extremely crucial for comprehending the mechanism of accumulation. A major factor controlling the dispersion of trace metals or other lipophilic compounds is the sediment grain size. According to previous literatures, the coarser sediments block the mobilization of toxic chemicals since they are mainly in the crystalline-solid form and present in low concentrations. Whereas, the finer sediment like the silt/clay are usually surface active with the presence of higher total organic carbon and other oxides of Iron (Fe) and Manganese (Mn) (Ip et al., 2007), thereby containing higher toxic compounds in general. Identification of the source of pollutants is an extremely important step in toxicological studies, which allows us to discriminate between natural and anthropogenic sources (Adamo et al. 2005; Lacerda et al. 1988; Ramirez et al. 2005).

1.4 Role of crustaceans as bio-indicators

To be precise, bioindicators are organisms that signify or indicates an overall well-defined environmental condition, which acts as an integrator of contaminant loading on a system (Phillips, 1980; Wilson, 1994). If the precision between the organism and its natural environment or habitat is well specified, then such organisms can be referred to as bioindicators. The entire study to search bioindicators and report the health of the coastal areas is a major part of the integrated coastal management (ICM). Such management strategy tries to embrace areas in near shore to coastal waters including the shoreline and even inland watersheds from the rising disturbances or impacts on the tidal flats from land activities. The subset amongst various other environmental indicators which uses living component of the particular area to address the condition of the ecosystem in response to man- made stresses are known as bio-indicators. This group includes animals as well as plants and definite patterns of their subsequent absence/presence, their behavior, and density can state the condition inside an ecosystem. Pollution from several chemical plants have recently grabbed the attention of many research groups since such toxic compounds mainly contributed by human activities is rapidly growing and is considered to be one of the foremost stressors of the coastal ecosystem. To precisely understand or monitor the negative changes of the environment, bioindicators are used in several aspects of toxicology and as well as monitoring projects. Such studies helps us to assess the entire quality of the environment, be it sediment or water and also find out reasons of the possible threats to the marine organisms especially in the areas closer to industries, sewage discharges, big cities and factories. Zooplankton micro or macro crustaceans are one of the most sensitive groups to the toxic pollutants or chemical stressors (Gutierrez et al., 2012). For the last 10 years, researchers have taken active interest on these groups to monitor or assess the impact of lipophilic compounds, heavy metals, organic pollutants including pesticides, to achieve a holistic view of the individual habitat they belong to (Koivisto and Ketola 1995; Barry and Logan 1998; DeLorenzo et al. 2002; Zauke and Schmalenbach 2006; Bossuyt and Janssen 2005). There exists several standard laboratory and field tests for analyzing the toxicity in crustacean or similar groups. Micro-crustaceans are often used for studying the integration of pollutants to each generation because mostly their life span is short and hence the time of analysis supposedly for an entire or multiple generation is not time consuming. For

Macro-crustaceans, a low level of pollutant even in early exposure or short exposure times along with simpler methods can provide evident and adequate results to comprehend the ecosystem they live in (Rinderhagen et al. 2000). There are very specific behaviors that has been documented as few sensitive responses or parameters for the evaluation of the effect of pollutants on crustaceans or zooplanktons as a whole (Sharp and Stearns 1997; Lopes et al. 2004). The analysis of such responses can help us in gaining a deeper understanding of the consequent changes in the life-traits or other metabolic activities of the zooplankton and as well as its role in determining the health of the environment thereby signifying its importance as a bioindicator.

1.5 Sections of thesis

1.5.1 Macro-crustaceans- mud shrimp

Mud shrimps are a group of mud dwelling crustaceans. They live in the burrows and are referred to as bioturbators (Coelho et al. 2000). They can be called as fine architects as they build their own burrows that are quite stable structures and also facilitated for water exchange (Rodrigues & Hod1 1990). They are well equipped in building their burrows as they can dig extremely complicated passages for very long depths (Ott et al. 1976; Dworschak 1983, 1987a). These shrimps can construct burrows that are shallow (20-50 cm) or very long (up to 1 to 2 meter of depth or even deeper) (Pemberton et al. 1976). Often their burrow structures are preserved and their fossil records have reported their occurrence back to late Jurassic. They are under the infraorder Thalassinidea of decapod crustaceans and has been grouped based on their habitat of muddy substratum (Dworschak 1983). This group was later discovered to have two separate lineages called Gebiidea and Axiidea. However, these are extremely important for the ecological balance and have significant economic importance as they are often used as food for human consumption. These animals are active bioturbators and they unknowingly alter many factors of the associated environment. As a result of increased oxygenation and turnover of the substrate through burrowing and feeding activities, they significantly influence sediment characteristics as well as composition and density of other organisms within the benthic community (Pemberton et al. 1976; Brenchley 1981; Posey 1986). The gebiidean and axiidean mud shrimps have distributions in all oceans from temperate, tropical and subtropical coasts. Burrow morphology varies with different families and genera, from the simple U- or Y-shaped structure for members of the Upogebiidae to complex burrow patterns with chambers and branches in Callianassidae (Dworschak, 1983). Burrows of thalassinidean mud shrimps are one of the most significant structures in marine intertidal and shallow sub tidal sediments because they are stable and can be very deep depending on their body size. The burrows of the genus *Upogebia* have a distinct U or Y (upper U-shaped part and a lower I-shaped part) shape (Ott et al. 1976; Dworschak 1983, 1987; Scott et al. 1988, Griffis & Suchanek 1991; Nickell & Atkinson 1995; Coelho et al. 2000; Kinoshita 2002). The U-shaped part of the burrow is ideal for efficient unidirectional water flow (Dworschak 1981; Allanson et al. 1992), which keeps the burrow wall oxidized and provides suitable conditions for aerobic bacterial metabolic activities, such as nitrification (Aller et al. 1983; Koike & Mukai 1983). Mud shrimps depend on their burrows for shelter, protection from predators, feeding and also reproduction (Coelho et al. 2000).

Filter feeding and deposit-feeding are the main trophic mechanism for thalassinideans. Deposit feeding is also known as 'gardening' (Hylleberg 1975; Dworschak 1987b) and it has been described as filtering phytoplankton or small zooplankton from the seawater. On the other hand mud shrimps that are deposit feeding tend to increase the quantity of organic matter in the sediment by burying plant fragments or other debris in their burrow walls and later grazing on the enriched substrates (Rodrigues 1966; Dworschak 1987b; Griffis & Chavez 1988). Upogebiids have been considered as primarily filter feeders (MacGinitie 1930; Dworschak 1987b; Scott et al. 1988; Nickel1 & Atkinson 1995), although certain species are also capable of deposit feeding like most of the Callianassidae (Dworschak 1987b; Nickel1 & Atkinson 1995).

Mud shrimp burrows often act as trap for organic matter and has high bacterial abundance. Bioturbation by mud shrimp draws water of the upper column of the tidal sand flat into the burrow. Thus along with seawater, it also allows all the suspended particles or materials into its burrow (Kinoshita et al., 2003). This includes particulate matters, plant debris, sea grass, etc. They even reach very long depths and thus increase the organic content of the area. In these places where the organic activity is high, bacterium starts growing profusely (Bird et al., 2000). The burrow of *Upogebia major* has higher bacterial abundance and Electron Transport System Activity (ETSA) than the surrounding non-burrow sediments (Kinoshita et al., 2003). The burrow accumulates considerable organic carbon and nitrogen probably derived from the tidal-flat surface. Fresh detritus forms the labile fraction of the organic matter and is efficiently utilized by bacterial populations in the sediment of the burrow wall. Therefore, the burrow wall of the mud shrimp provides a suitable niche for heterotrophic microorganisms.

1.5.2 Micro-crustacean – copepod

Copepods are a group of micro crustaceans found in the marine, estuarine and some freshwater habitat. Some species are planktonic while some are benthic (living on the ocean floor), and some continental species may live in limno-terrestrial habitats and wet terrestrial places, such as swamps, springs, bogs, ephemeral ponds, streambeds etc. Like other crustaceans, copepods have a larval form and the egg hatches into nauplii form, with a head and a tail but no thorax or abdomen. The larva molts several times until it resembles the adult and then, after more molts, transforms into an adult (Charles et al., 2004).

Copepods can vary considerably, but are typically 1 to 2 mm (0.04 to 0.08 in) long, with a teardrop-shaped body, large antennae (two pairs; first pair is often longer), single median compound eye, which is usually bright red placed in the center of the transparent head with almost totally a transparent body (Johannes et al., 1997). Copepods have no need of any heart or circulatory system (the members of the order Calanoida have a heart, but no blood vessels), and most also lack gills. Instead, they absorb oxygen directly into their bodies. Their excretory system consists of maxillary glands.

Some copepods have extremely rapid escape responses when they can sense a predator, and can jump with high speed over a few millimeters (Laurent et al., 2004). This acceleration (jumping or fast swimming) is crucial in the ecology of copepods and recently Michalec et al. (2017) discovered that this feature could help copepods to reduce diffusion in turbulent flow. This means that copepods develop several evolutionary strategies including their typical swimming behavior. Many species highly evolved neurons surrounded by myelin (for increased conduction speed), which is very rare among invertebrates. Despite their fast escape response, slow-swimming seahorses successfully hunt copepods; however, they possess significant self-locomotion under strong hydrodynamic conditions (BBC news, 2013; Michalec et al., 2017).

Many of the free-living copepods feed on phytoplankton. Some of the larger species are predators of the smaller copepods. Many benthic copepods eat organic detritus or the bacteria that grow in it, and their mouthparts are adapted for scraping and biting. Herbivorous copepods, those in rich, cold seas, store up energy from their food as oil droplets while they feed in the spring and summer on plankton blooms. Many copepods (e.g., fish lice like the Siphonostomatoida) are parasites, and feed on their host organisms.

Planktonic copepods are very important to global ecology and the carbon cycle. They are usually the major food organisms for small fish such as the dragonet, banded killifish, whales, etc. and other crustaceans in the ocean and in fresh water. Some scientists say they form the largest animal biomass on earth (Geoff et al., 2008).

Because of their smaller size and relatively faster growth rates, and because they are more evenly distributed, copepods almost certainly contribute to the secondary productivity of the world's oceans, and to the global ocean carbon sink.

Moreover, copepods being the natural living prey for fish larvae they have a great potential to promote larval cultures in different merging programs of aquaculture. Consequently mastering their mass culture can be not only useful for aquaculture development but also offers good quality batches to perform different experiments. Their molted exoskeletons, faecal pellets, and respiration at depth all bring carbon to the deep sea.

1.6 Overall aim of the doctoral study

The aim of this doctoral study is clearly divided in two sections, section-1 namely macro crustacean - mud shrimp and section-2 namely micro crustacean- copepod. The mud shrimp *Austionogebia edulis* (Fig 1.2) was studied as an indicator of macro-crustaceans, which is a rare species inhabiting in the tidal flats of the Western Taiwan (Fig 1.4), Northern Vietnam and Hong Kong.

In the first chapter, detailed observation of the ability of *A. edulis* to modify their burrow by choosing finer particles was noted. Furthermore, the amount of organic matter inside the burrow was significantly higher than the sediment without any burrows (ambient sediment). *Austinogebia edulis* is both ecologically and economically important in South East Asia and conservation of *A. edulis* is one of the foremost concerns of the Environmental protection agency of Taiwan. This mud shrimp is found along the western Coast of Taiwan and some of these areas are in very close proximity to a proliferating industry known as the Changbin Industrial Park. As a result, many pollutants or trace metals can have a direct effect on the density of the population by several physiological or metabolic damages. Therefore, the second chapter was to analyze the biochemical and histological alterations of *A. edulis* when exposed to Cd. Cd is quite abundant in this area and more readily bioavailable for mobilization to living organisms (Peng, et al., 2006). To highlight the biochemical changes, alteration of oxidative enzymes in different organs of *A. edulis* like hepatopancreas, gills and muscles were studied. Most crustaceans, like crabs, mussels and/ or mud shrimps are highly sensitive to environmental stressors because of their lower response amplitudes. Other than trace metals, they can also be used as bio-indicator of several organic pollutants to notify possible toxicological risks in that particular area. Therefore, the third chapter dealt with the analysis of the concentration of the Persistent Organic Pollutants (POPs) in the sediment and in the mud shrimp. The possible sources of POPs and assessment of the potential ecological risk to the nearby benthic organisms was also accomplished. These above studies had a principle aim to know the effect of toxic substances on this bio-indicator species of mud shrimp, which also has major economic importance. To acquire a finer understanding of the diverse responses of a macro-crustacean (mud-shrimp) in response to different environmental factors was our underlying or tangential aim.

The second section deals with copepod *Eurytemora affinis* (Fig 1.3), which acts as an indicator of micro-crustaceans in this co-tutorial thesis and is abundant in the Seine estuary (Fig 1.5). Siene estuary is known to be the largest mega-tidal estuary along the English Channel, which also serves the purpose of major international shipping routes (Cailleaud, et al., 2007). Siene is highly contaminated with several heavy metals and other lipophilic compounds, with increasing human activities and industrial enterprises. Such continuous commercial activities since the last 50 years along the Siene river basin pose a significant threat to the nearby living organisms (Dauvin, 2008; Zidour, et al., 2019). Decrease in fish population density was noticed in 1970s, which might be a partial consequence of the high toxicity in this area. This also led to a decrease in fishing activities since then in this estuary (Minier et al., 2006). In the Seine River, *E. affinis* is a dominant species representing almost 90% of the entire zooplankton biodiversity (Mouny and Dauvin, 2002). Since copepod are often used as a bio-indicator species in toxicological studies, analysis of the response of *E. affinis* to different environmental factors was acquired (Dauvin, 2008; Zidour, et al., 2019). Copepods also act as a functional link between phytoplankton and bacterioplankton with the other higher trophic levels. Such important relationships with other taxonomic groups aid many researchers to use the population abundance of copepods as crucial indicator of the ecological condition in a particular area (Landa et al., 2007; Santos-Wisniewsky and Rocha, 2007; Silva, 2011; Perbiche-Neves et al., 2016). In the fourth chapter, the role of a mixture of heavy metals (Cd, Cu, Pb and Ni) and its effect on *E. affinis* in terms of total population, morphological structures and bioaccumulation of heavy metals was obtained. Furthermore, to get a detailed effect of toxic substances (mainly heavy metals) on copepods released from sediment re-suspension was executed. Additionally, a comparison between whole sediment toxicity (in re-suspension) and the extract of heavy metals from whole sediment was targeted. This allowed us to partially assess the marine sediment quality in that particular area.

The underlying principle aim of this study was to compare the two crustaceans on various environmental factors and parameters. Interestingly although these two crustaceans (mud shrimps and copepods) have, different habitat with the former being purely benthic but depending on the tidal inundations and the latter being pelagic to epi-benthic but bioaccumulating substances from the sediments. This well connected ecosystem also known as the sediment water interface or the benthos-pelagic column is very interesting in terms of toxicology and a wide exploration is still due.

Fig 1.2 *Austinogebia edulis*

Fig 1.3 *Eurytemora affinis*

Fig 1.4 Map of Taiwan highlighting the Western Coast (Changhua County)

Fig 1.4 Map of France highlighting the Seine estuary

Section-1- Macro-crustaceans Mud shrimp

Chapter 2 – Burrow characteristics of the mud shrimp *Austinogebia edulis*, an ecological engineer causing sediment modification of a tidal flat.

2.1 Introduction

Mudflats are coastal wetlands that are formed by the sedimentation of mud layers during tidal movements (Miththapala, 2013). Generally, these layers are made of sand, silt, or clay. Tidal flats constitute a transition zone between land and sea (Bearup et al. 2017). Tidal flats are habitats to different kinds of organisms like, benthic burrowers, microalgae, and even bacteria. They are important wetlands where numerous biological activities take place. Many species of crabs, clams, shrimps, fish etc. hide there by creating burrows into the sediment (Kogure and Wada, 2005). Among them are mud shrimps that dig complex and deep burrows (Coelho et al., 2000; Kinoshita, 2002; Seike and Goto, 2017). Some mud shrimp species are known to dig more than 2 meter deep burrows, for which it has been always been difficult to acquire a holistic approach to their behavior (Pemberton, 1976; Hong, 2013).

In the coastal wetlands of western Taiwan and northern Vietnam, the mud shrimp *Austinogebia edulis* (Ngoc-Ho and Chan, 1992) (Fig 1) is abundant and of economic importance as seafood. The species *Upogebia edulis* was revised to *Austinogebia edulis* after the re-classification of upogebiid species into the genus *Austinogebia* sp **(**Ngoc-Ho, 2001). Locals of western Taiwan catch and consume this shrimp extensively (Peng et al., 2006) and the ovigerous female shrimps occur only found during the reproductive season and are more expensive than the males or non-ovigerous females. Mud shrimps are cryptic animals that prefer to reside inside their burrows in deeper layers of sediment. Their burrows are only recognizable through small burrow openings (Peng et al., 2006; Suchanek, 1983; Tudhope and Scoffin, 1984; Colin et al, 1986; Sepahvand, 2014). With the advent of the resin casting technique (Shinn, 1968) the interior morphology of mud shrimp burrows received great attention, which improved the understanding of their burrow structures (Pemberton, 1976, Dworschak, 1983; Nash et al., 1984; Atkinson and Nash, 1990; Nickell and Atkinson, 1995; Ziebis et al., 1996; Atkinson and Taylor, 2005; Dworschak et al., 2012; Atkinson and Eastman, 2015). Reports of resin casting in the inner burrow structure of *A. edulis* showed that these are generally Y shaped with an upper U part and a lower shaft (Li et al., 2008; Griffis and Suchanek, 1991). One mud shrimp burrow has usually two openings and the mean distance between them is 21.8 cm (Lin, 1994) and 26.4 cm (Li et al., 2008). A single shrimp generally inhabits it. Studies on the outer morphology of the burrows are still scarce.

We observed that the burrow wall composition was different from ambient uninhabited sediments without the burrow. A previous study on the grain size of the sediments from abundant areas of *A. edulis* revealed that these were mainly composed of fine silt (0.061mm), but a detailed analysis between burrow and areas without burrows was not made as yet (Li et al., 2008). Therefore, we studied here whether mud shrimps could change the sediment characteristics while constructing their burrows acting as ecological engineers with substantial ecological impact.

Previous studies highlights the effect of burrowing behavior of mud shrimps on the ecology of tidal flats, and examines factors such as bacterial abundance, change of oxygen, nutrient fluxes, organic content inside the burrow and its potential to change the environment, etc. (Seike and Goto, 2017, Sepahvand, 2014, Koike and Mukai, 1983; Kinoshita et al., 2003; Kinoshita et al., 2008; Berkenbusch, et al., 2000; Wada et al., 2016; Laverock, et al., 2010). The burrow of *A. edulis* is more than 1 meter deep and this species is abundant in the wetlands of western Taiwan (Li et al., 2008). Therefore, the influence of this thalassinidean shrimp on the alteration of tidal flats must be substantial and they are expected to affect other benthic animals living in the surroundings. Hence, studies on the composition (grain size analysis) of the mud shrimp burrow wall (MSBW), and its comparison with the sediment from the background (a place without mud shrimp burrows) is timely.

While studies have indicated substantial changes of the environment caused by the mud shrimp, it also remains important to calculate the substantial amount of carbon inside their burrows as a food source (Kinoshita et al., 2008; Papaspyrou et al., 2006; D'Andrea and DeWitt, 2009) Amongst thalassinidean shrimps, Upogebiidae are considered mainly as filter feeders but some representatives also show plasticity for feeding behavior (Coelho et al., 2000; Hong, 2013). The burrow provides a steady water flow and a stable carbon source in the burrow for the animals living inside (Nickell and Atkinson, 1995). Therefore, an estimation of the organic carbon in the MSBW that might be utilized by the mud shrimp *A. edulis* would be worthwhile.

The main objective of this study was to test the hypothesis whether the mud shrimp can change its environment by building a burrow. In addition, we addressed the following ecological issues, to study: (1) the outer morphological structure of a mud shrimps burrow wall and its characteristics; (2) the potential of mud shrimps to modify a tidal environment by selecting and fractionating sediments in the process of burrow building; (3) to measure the organic matter in a MSBW.

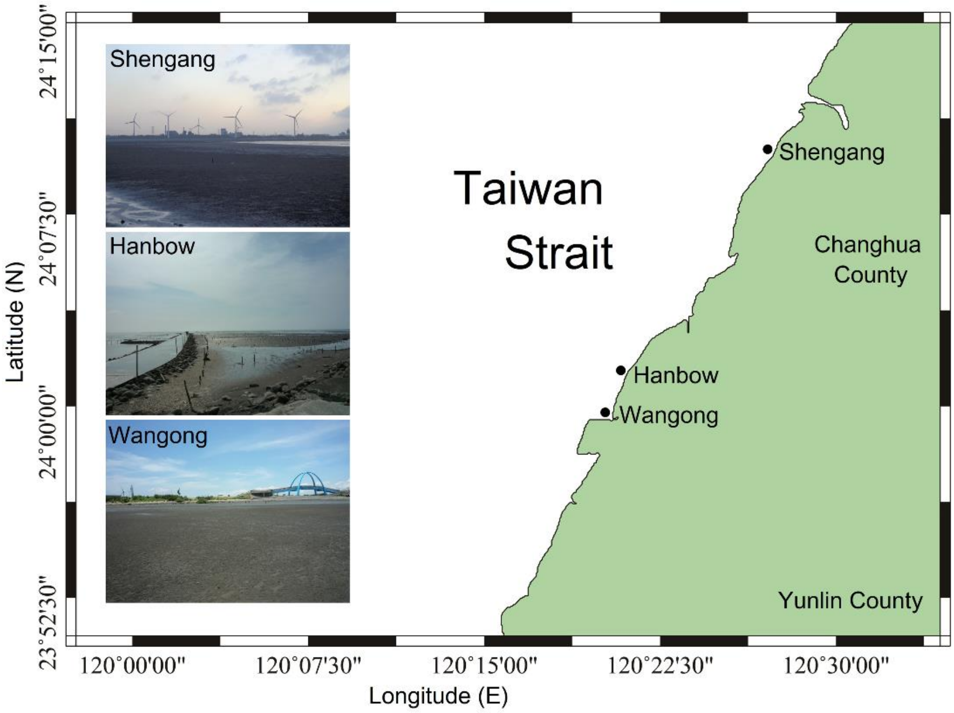

Fig 2.1 Map of the sampling area in western Taiwan.

2.2 Material and Methods

2.2.1 Study area

For sampling, we chose three sampling areas from north to south, which are tourist attractions in Changhua County (Fig 2.1). The areas of investigation were : Shengang in the northern part located close to the industrial park, Hanbow in the central, and Wangong in the southern part along the western coast of Taiwan facing the Taiwan Strait (Table 2.1). Our study was permitted and supported by the Industrial Development Bureau, Ministry of Economic Affairs. This study did not involve specimens; tissue samples or any endangered or protected species. The climate of Taiwan is affected by seasonal monsoons with the air temperature being 12^0C in winter and 30^0C in summer. Ocean currents in this region are influenced by seasonal monsoons. In summer, the Kuroshio Branch Current and the South China Sea surface water enter the Taiwan Strait from the south. In winter, the China Coastal Current enters the Taiwan Strait from the north [Jan et al., 2002].

Table 2.1 Sampling period, location and coordinates of experimental specimen collections from different mudflat environments in western Taiwan.

Sampling period	Sampling location (ca.)		
	Local name	Latitude (Nº)	Longitude (Eº)
September 2015 - November 2016 (3 times within this period)	Shengang	24.168094	120.457894
	Hanbow	24.015691	120.349280
	Wangong	23.968126	120.323173

2.2.2 Sampling strategy

We conducted the field sampling from September 2015 to November 2016 (3 times within this period). Samples of mud shrimp *A. edulis* burrows were collected carefully by using a shovel or small rake or fork. A densely populated area was randomly chosen to be sampled with a shovel; a portion of mud block containing burrows of mud shrimps was scooped out. As the burrows were very distinct from the background sediment in terms of texture, hardness, compactness, and shape, this enabled an easy separation of muddy burrow blocks and loose sandy background sediments.

The samples of burrow and background sediment were both collected from above 30 cm depth. We washed the burrows gently to remove loose sediments attached to it. A total of 50 samples from burrow and 50 samples of surrounding sediment were randomly collected from each area. We collected background sediment from areas comprising no mud shrimp burrow as a control to be compared with the burrow sample. In total, about 300 sediment samples were collected to find out the difference in the composition and to measure the ash free dry weight (AFDW, organic matter, carbon content) for three areas. After collection, the samples were placed carefully in separate zip-lock bags and were carried to the laboratory.

In the laboratory, the samples were stored in a -20^0C refrigerator until analysis. In addition, for measuring the diameter of the burrow we collected 3 resin casts of the burrow of *A. edulis* in November 2015 from Hanbow according to Li. et al, 2008. We measured the diameter of the burrow at every 10 cm depth from the surface to the bottom in all the three resin casts.

The other objective was to study the structure of the burrow wall of *A. edulis*, which required a different technique. The objective was to acquire the complete burrow shape and to show thick patches of fine sediments accumulated by the mud shrimp during their burrow building processes. A considerable depth and a wide surface area containing at least a major portion of one burrow with two openings were necessary to collect. Wooden planks were hammered into the sediment from all four sides for taking a mud block of the following size $30\times30\times30$ cm^3 that contained a portion of the outer dimension of a burrow. After this, two shovels were inserted from two opposite sides into the bottom of the mud block. This was done at all four sides for easy removal of the mud block from the tidal flat. Then, we wrapped and tied the planks with adhesive tapes repeatedly to make the structure firmer. The wooden box was then lifted up carefully from the mud flat and was carried to the laboratory (Fig. 2.2).

Fig 2.2. Process to acquire complete shape of the burrow (up to 30 cm) to study the morphology.

There, after the removal of the wooden planks a weak water stream comparable to the tidal hydrological force was used to remove the loose sediment. Coarse sediment that was not part of the burrow was gently washed away, and the hard burrow structure got gradually exposed. This burrow structure was used to measure the traits of the outer wall and for photo documentation.

2.2.3 Sediment handling and analysis

We randomly selected 8 samples of burrow and 8 samples of background sediments in total from each sampling area in order to measure the sedimentary composition. Particle size was determined by passing each sample of sediment through a series of sieves by following Krumbien, 1934. The fraction remaining on each sieve was collected in a pre-weighed 100 ml beaker. A total of 7 mesh sizes (4, 2, 1, 0.5, 0.21, 0.105 and 0.063 mm) were used to pass the samples and gradually separate them into different size groups (Fig 2.3). After collecting the remaining fraction, the total weight was again noted for each size fraction and was expressed as a percentage of the weight of the original sample.

For measuring the volume of the burrow wall, the portion of one entire burrow collected from 30cm depth was wrapped tightly with Polyethylene wrap film to measure the total volume by using the water displacement method [Hughes et al., 2005].

For measuring the burrow lumen,

$$Volume = \pi \ r^2 \ l \qquad \text{………..} \ (1)$$

where, r = radius of the resin casting, which is 2 cm (from the resin cast collected in November 2015), length (l) is the height of the mud block, which is 30cm.

For revealing the variability of sedimentological characteristics of mud shrimp burrows, we used a total of 12 samples from three sampling areas to measure the void ratio (e).

$$e = Vv \div Vs \qquad \text{…….} \ (2)$$

where, *Vv* is volume of void (equal to volume of water), *Vs* is volume of solid sediment sample (equal to volume of burrow sample).

Millimeters (mm)		Micrometers (μm)	Phi (ϕ)	Wentworth size class	
	4096		-12.0	Boulder	Gravel
	256		-8.0	Cobble	
	64		-6.0	Pebble	
	4		-2.0	Granule	
	2.00		-1.0	Very coarse sand	Sand
	1.00		0.0	Coarse sand	
1/2	0.50	500	1.0	Medium sand	
1/4	0.25	250	2.0	Fine sand	
1/8	0.125	125	3.0	Very fine sand	
1/16	0.0625	63	4.0	Coarse silt	Silt
1/32	0.031	31	5.0	Medium silt	
1/64	0.0156	15.6	6.0	Fine silt	
1/128	0.0078	7.8	7.0	Very fine silt	
1/256	0.0039	3.9	8.0	Clay	Mud
	0.00006	0.06	14.0		

Fig 2.3 Wentworth (1922) grain size classification

2.2.4 Estimation of organic matter of the mud shrimp burrow wall

We weighed a total of 10 adult individuals of mud shrimps (5 male and 5 female, carapace length: 12.22 ± 13.97 mm, 13.39 ± 0.69 mm (mean ± standard deviation), 4 samples of background sediment and 12 samples of the inner surface of the mud shrimp burrow from three sampling areas for wet weight (WW). The dry weight (DW) of the samples was determined by drying in an electric oven at a constant temperature of 60^{0}C for 24 hours. Both WW and DW were measured by analytical microbalance (Type AG 135, Mettler Toledo, Switzerland) and recorded. Dried samples were then placed in an electric oven and combusted at a constant temperature of 500^{0}C for 16 hours to measure the ash weight (AW). For revealing the organic content of the samples, the AFDW was calculated by deducting the AW from the DW.

In order to calculate the organic matter of the mud shrimp burrow wall (MSBW), a definite volume of the burrow sample was required. For this, a volume of 10 cm^3 burrow sediment from the inner surface was used to measure the AFDW. Hence, the estimation of the AFDW of one whole mud shrimp burrow was following the equation (3):

$$AFDWt = IDB \times \pi \times LB \times AFDWbm \div Vm \quad \ldots\ldots\ldots\ldots (3)$$

where, *AFDWt* is represented by the total AFDW in one whole mud shrimp burrow, *IDB* is the inner diameter of the burrow, *LB* is the length of the burrow, *AFDWbm* is the AFDW in the burrow mud, and *Vm* is the volume of the burrow mud used to measure the AFDW. In this study, the inner diameter of the burrow was 20 mm from the resin samples collected in November 2015 (20 ± 0.12 mm). The length of the burrow was considered to be 100 cm since in previous studies the lengths of the burrows at the sampling sites near this study area were ranging between 80 ± 100 cm [Li et al., 2008].

2.2.5 Data and statistical analysis

To compare the composition of sediment samples in the mud shrimp burrow and surroundings, Student's t-test was applied to identify the differences between different sizes of sediment particles. The data for the proportion of sediment (%) and the proportion of AFDW in the sediment (%) were arcsine transformed in order to satisfy the assumptions of normality and homogeneity of variances. To identify the differences in the AFDW (carbon content) of the surroundings and mud shrimp burrow in three sampling areas, one-way analysis of variance (ANOVA) followed by post hoc Tukey's honest significant difference (HSD) test were applied. To evaluate whether the carbon content inside the burrow was sufficient to support the shrimp living inside, the ratio of AFDW in MSBW and a single individual of shrimp (*A. edulis*) was calculated.

2.3 Results

2.3.1 Morphological traits of the mud shrimp burrow wall

The sediment texture of mud shrimp burrows appeared finer than the background sediment in all the three sampling areas. We studied the outer structure, which is the burrow wall, and the inner narrow tube, referred to as the burrow lumen from the portion of the mud block collected at 30cm depth. The burrow wall of the mud shrimp burrow was very broad and appeared huge in contrast to the burrow lumen, which only represented a thin narrow hollow tube of a confined shape (Fig 2.4). From the morphology of the burrow wall, a round-shaped opening was noted on the upper part (Fig 2.4 a). Gradual thickening was significant from the top to the bottom of the burrow with the diameter being 4±5 cm at the top and 20±25 cm at the bottom (in about 30 cm depth). Distinctive portions are the opening of the burrow and a chimney-like trunk narrowing from the lower to the upper portion (Fig 2.4 b). An extremely irregular deposition of thick mud without a distinct shape was noted on the outer surface of the burrow (Fig 2.4 c). The trunk of the burrow appeared to be strong and not fragile when it got dry after 1 year (Fig 2.4 d). The volume of the burrow wall showed a huge difference with the volume of the burrow lumen (Fig 2.5). The volume of the burrow wall was about 19.1 (± 6.0) times of the burrow lumen (n = 2).

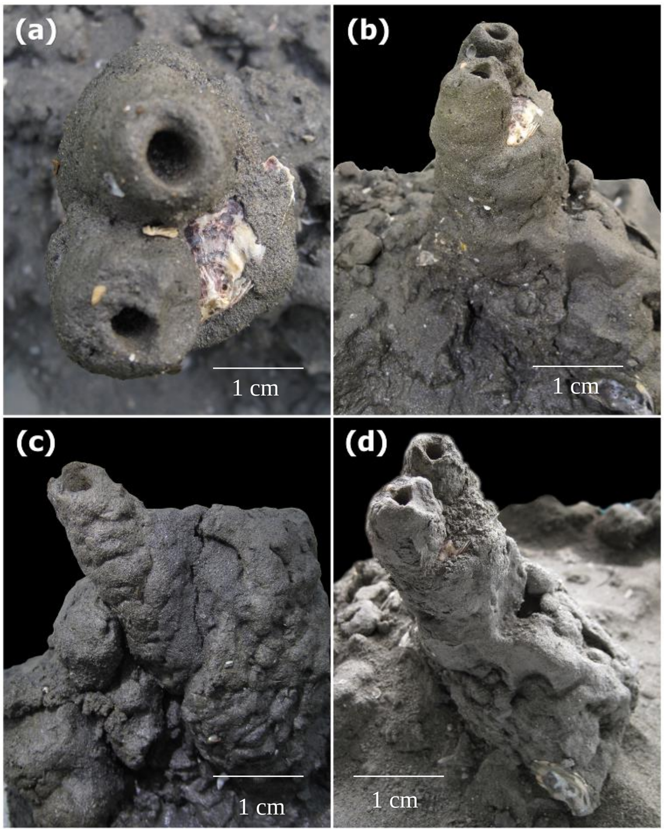

Fig 2.4 Photo of the mud shrimp burrow wall. Top view of the burrow showing the opening (a), the trunk of the burrow gradually thickening with depth (b), irregular deposition of the clay in the burrow wall (c), and intact morphology of the burrow wall 1 year after collection (d).

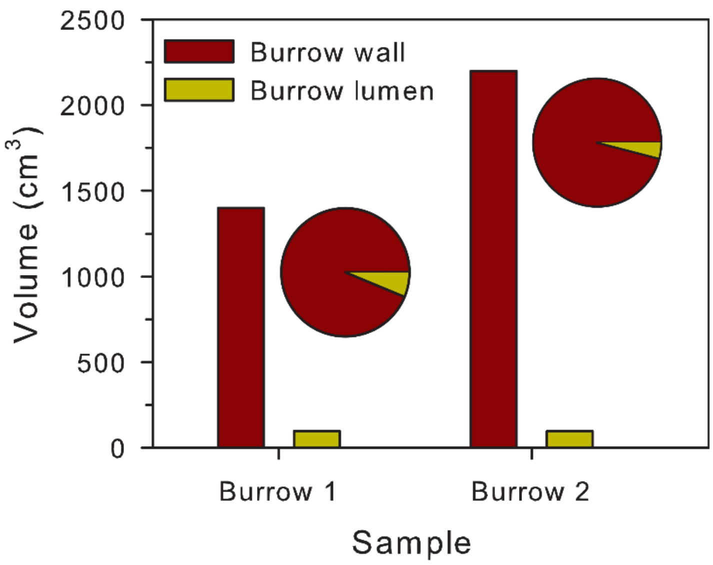

Fig 2.5 The volume of the burrow wall and the burrow lumen. Pie chart- the proportion of the burrow wall and the burrow lumen in a mud block.

2.3.2 Composition of the mud shrimp burrow wall

The composition of MSBW showed a clear variation with background sediments in all three sampling areas (Fig. 2.6). The composition of background sediments indicated that the mud shrimp used the habitat with a higher proportion of medium sand (> 70% of size Φ1.63) and clay (size Φ9). The accumulation of clay (size Φ9) in the burrow wall was noticed in three study areas when compared with the background sediment (Fig. 2.6). Most of the size categories in the MSBW were altered particularly in Wangong. When the results of all MSBW samples were combined, the Student's t-test revealed that proportions of size Φ 1.63, were significantly higher in background samples than in burrow samples (t = 6.61, p < 0.001). Nevertheless, the proportions of size Φ -0.5 (t = -2.09, p = 0.043), 3.63 (t = -5.22, p < 0.001) and 9 (t = -25.01, p < 0.001) were significantly higher in burrow samples than in background samples (Table 2.2). Taken together, the results of the composition of MSBW revealed: (1) the ability and the preference of mud shrimps to select fine sediments to build their burrows, and (2) the changes in the sediment characteristics caused by the burrowing behavior of the mud shrimps because they changed the physical distribution of sediment particles of the tidal flat by accumulating finer sediments inside their burrows.

Furthermore, the traits of MSBW showed an average value of the void ratio (*e*) 0.43 ± 0.04 (%), 0.4 ± 0.06 (%) and 0.24 ± 0.04 (%) collected from Shengang, Hanbow and Wangong, respectively (Fig. 2.6a). The void ratio found in all three areas was very low.

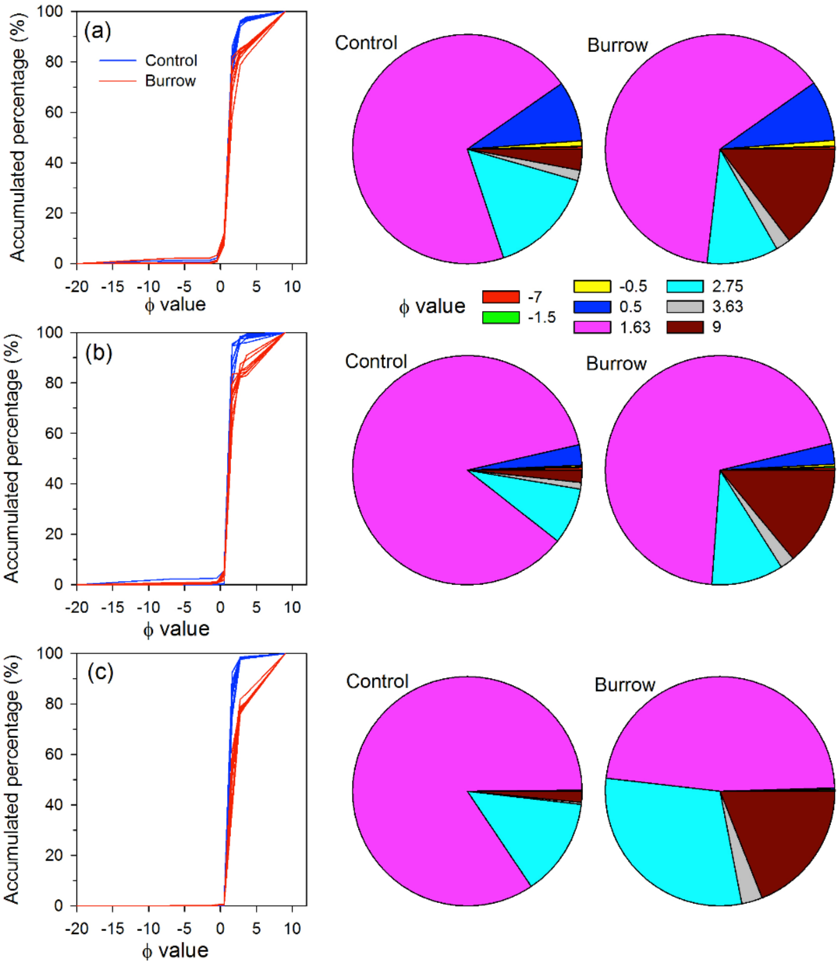

Fig 2.5 Accumulated percentage and proportion of sediments in burrow wall and surroundings from three sampling areas. Shengang (a), Hanbao (b), and Wangong (c).

Table 2.2 Results of Student's t-test comparison for proportion (%) of each size category of sediment between habitat (control) and burrow. n is number of samples. WSC is Wentworth Size Class.

Location	Sengang (n =8)			Hanbao (n =8)			Wangong (n =8)			All (n =24)		
Φ value WSC	Control	Burrow	t-value, *p*-value	Control	Burrow	t-value, *p*-value	Control	Burrow	t-value, *p*-value	Control	Burrow	t-value, *p*-value
-7 Cobble	0.32	0.32	0.70, 0.50	0.34	0.29	-0.27, 0.79	< 0.01	0.01	-0.47, 0.65	0.22	0.20	0.24, 0.81
-1.5 Granule	0.08	0.08	-0.341, 0.74	0.13	0.20	-1.69, 0.11	0.02	0.02	-0.45, 0.66	0.08	0.10	-1.05, 0.30
-0.5 Very coarse sand	0.77	0.82	-0.82, 0.43	0.19	0.34	-2.60, 0.021*	0.03	0.22	-8.85, 0.001**	0.33	0.46	-2.09, 0.043*
0.5 Coarse sand	8.51	8.55	-0.15, 0.99	2.90	2.90	-0.23, 0.82	0.08	0.19	-7.50, <0.001**	3.83	3.88	-0.23, 0.82
1.63 Medium sand	70.44	63.42	2.66, 0.02*	85.82	70.13	4.77, <0.001**	84.25	47.76	9.77, <0.001**	80.17	60.44	6.61, <0.001**
2.75 Fine sand	15.42	10.04	2.42, 0.03*	7.93	10.12	-0.59, 0.57	13.76	29.86	-4.89, <0.001**	12.37	16.67	-1.26, 0.21
3.63 Very fine sand	1.48	2.08	-1.80, 0.10	0.93	2.00	-1.67, 0.12	0.27	2.92	-19.67, <0.001**	0.90	2.33	-5.22, <0.001**
9 Clay	2.98	14.70	-24.61, <0.001**	1.74	14.02	-12.82, <0.001**	1.58	19.02	-45.22, <0.001**	2.10	15.92	-25.01, <0.001**

2.3.3 AFDW analysis

A total of 12 mud shrimp burrow samples from three areas were used to measure the content of AFDW. The content of AFDW in the three sampling area varied, ranging between 1.55 ± 0.10 (% of dry burrow sample, Wangong) and 1.74 ± 0.08 (% of dry burrow sample, Hanbow). The statistical results showed no significant difference in MSBW among the 3 sampling areas, but all AFDW values in the MSBW were higher than the background (control) samples ($p < 0.001$, one-way ANOVA, Fig 2.6 b). The average content of AFDW in MSBW was 1.23 ± 0.11 (% of dry burrow sample). By using the equation (3) the AFDW in one entire burrow (inner surface 1 cm thick) was found to be 14.17 ± 2.82 g.

The average AFDW of one adult mud shrimp was 0.586 ± 0.038 g, whereas no significant difference was found between the two sexes ($p > 0.05$, Student T-test). Further, the ratio of AFDW in a single burrow to AFDW of one shrimp was 24.2 (Fig 2.6 c).

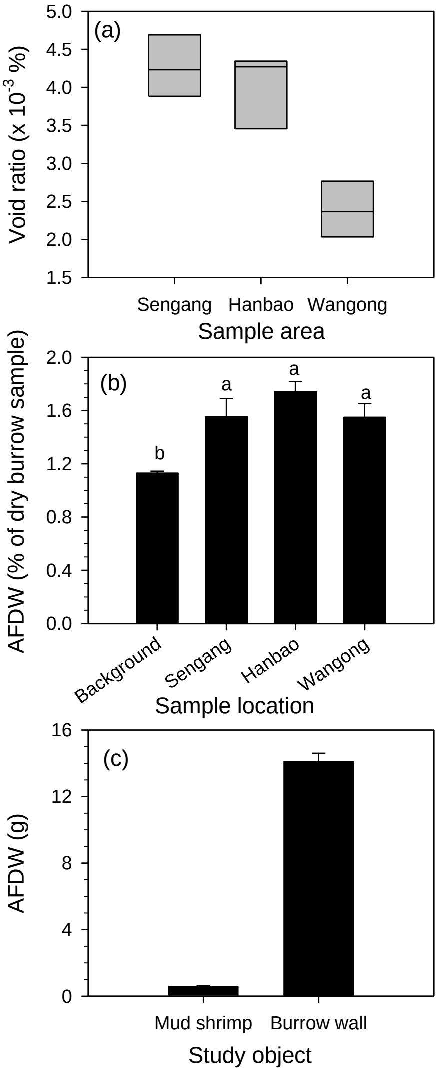

Fig 2.6 Void ratio of mud shrimp burrow wall in 3 sampling areas collected from September 2015 to November 2016 (a), comparison of ash free dry weight of background sediment and mud shrimp burrow wall using one-way analysis of variance, followed by Tukey's test (b), relative weight of ash free dry weight of one individual of mud shrimp and one whole mud shrimp burrow wall (c).

2.4 Discussion

2.4.1 Characteristics of the mud shrimp burrow wall

The burrow of *A. edulis* comprises of an upper U section and a central shaft, thereby giving an overall Y shaped appearance (Li et al., 2008). The structure of the burrow of *A. edulis* is similar to the suspension feeding upogebiid shrimps like having small and narrow circular tunnels (Kinoshita, 2002; Dworschak, 1983; Li et al., 2008; Griffis and Suchanek, 1991; Candisani, et al., 2001). The upper U section of the burrow is generally formed for the exchange of water from outside to inside of the burrow since these shrimps are mainly filter feeders (Li et al., 2008; Nickell and Atkinson, 1995; Kinoshita and Itani, 2005, 2010). The burrow of *A. edulis* showed the presence of circular chambers, which is used for turning the body inside the burrow (Li et al., 2008).

The inner structure of the burrow of *A. edulis* is a narrow tube with a definite dimension of arm width extending vertically into the sediment by building a central shaft (Li et al., 2008). Also, arm width, volume and the total depth of *A. edulis* burrows were significantly positively correlated with size of the shrimp (Li et al., 2008). The present study is the first record showing the outer morphological structure of the burrow. A lot of interspecific variations were noticed in the thalassinidean shrimp burrow morphology with respect to their structure, shape, and dimension (Li et al., 2008; Kinoshita and Itani, 2010). In the case of the deposit feeder ghost shrimp *Nihonotrypaea petalura*, the extension of the burrow was horizontally greater than vertically, having a single opening at the surface (Shimoda and Tamaki, 2004). Even though the inner burrow wall of *A. edulis* had a distinct Y shaped appearance, the outer wall of the burrow was thick with an overall irregular shape and became extended with accumulated sediment. In a previous study, *Upogebia pusilla* was observed to push significant amounts of sediment against the burrow wall in order to considerably enlarge the burrow and build the burrow lining [Dworschak, 1983; Ott et al., 1976). The result of the present study supports the above inference about the behavior of the mud shrimp (*A. edulis*), because their burrowing behavior leads to a thick deposition of clayey particles, ultimately strengthening their burrow.

2.4.2 Difference in the composition of sediment between background and burrow

Burrowing animals exhibit a strong influence on the physical characteristics of the sediment by altering the penetrability and permeability to water, and the water content of the burrow [D'Andrea and DeWitt, 2009; Botto and Iribarne, 2000; Dorgan, 2015). Studies on how the burrowing or other biological activities affect the physical characteristics of the sediment are few (Meadows and Tait, 1989). The pattern for the alteration of physical characteristics of the sediment for mud crab species like *Uca uruguayensis* and *Chasmagnathus granulata* was marked by higher penetrability and lower permeability (Botto and Iribarne, 2000). Several studies reported the same phenomena about the effects of bioturbating animals (Meadows and Tait, 1989; Rhoads and Young, 1970; Levinton, 1989; Davis, 1993). In the present study, *A. edulis* was found to accumulate finer sediments (clay) when building their burrow. This was also noticed in the crab *C. granulata* where the burrow is characterized by the accumulation of finer particles, which meant that they could selectively choose respective sediments for building their burrow wall (Botto and Iribarne, 2000). Their report revealed the trapping of clayey or finer sediments inside the burrow tunnel during high tide. The present study also confirmed the presence of clay particles in the burrow wall when compared to the sediment composition of the background.

Thalassinidean shrimps have funnel shaped burrow openings which are supposed to act as sediment traps by collecting clayey particles and accumulating them in their burrow linings (Suchanek, 1983; Nickell and Atkinson, 1995; Witbaard and Duineveld, 1989). The burrowing crabs are supposed to deposit the sediment in the form of mounds on the tidal flat over many cycles of tides, eventually covering the crab bed surface (Botto and Iribarne, 2000). Furthermore, the accumulated sediments are cohesive, dense and not easy to transport (Botto and Iribarne, 2000). This result was in accordance with the present study where the mud shrimp *A. edulis* accumulated clayey particles and deposited them in their burrow randomly, providing an overall irregular outer burrow morphology. The accumulation of fine sediments might also happen because of the breakdown of coarser sediments into finer particles by the mud shrimp while burrowing. Both possibilities reflect the alteration of the sediment characteristics by the mud shrimp, which provides a sedimentological impact on the mudflat (Rossi et al., 2013).

The ability of selecting particles according to their size was noted before in thalassinidean shrimps in some studies (Coelho et al., 2000, Scott et al. 1988). These studies were mainly associated with understanding the trophic modes of mud shrimps. For example, the trophic mode of *Upogebia omissa* was reported to have the ability to select finer particles based on size by re-suspension during deposit feeding (Coelho et al., 2000, Scott et al. 1988). This ability of *U. omissa* to separate particles by size was not well documented before. The present study showed that mud shrimps can separate particles not only when feeding, but also during the burrowing process by separating finer sediments and accumulating them in their burrow.

In this study the void ratio in the burrow wall was found to be very small (less than 0.5 %), and indicates a low permeability. A tendency of the shrimp to isolate themselves from the outer world by building strong, compact burrows with very small opening indicates that this animal does not need to access the surface (Nickell and Atkinson, 1995). A previous study on the mudflat amphipod *Corophium volutator* showed that the permeability of the sediment decreased with an increase in population density [Meadows and Tait, 1989]. The results of these authors suggest an inverse relationship between shear strength and permeability of the sediment that might be responsible for biological activity. The result of the present study confirms that the influence of some mud flat animals can change certain characteristics of the sediment, which may, therefore, affect other benthic animals living in vicinity (Lavesque et al., 2016). In fact, ecosystem engineers can create their own modified habitat by impacting the functioning and the structure of the ecosystem (Heuner et al., 2015).

The low void ratio and the compactness due to the accumulation of clayey particles increase the shear strength of the burrow [Meadows and Tait, 1989; Trask and Rolston, 1950; Yong et al., 1966; Sassa et al., 2011]. These characteristics of the burrow can protect mud shrimps from predators (Kinoshita and Itani, 2005). Several studies noticed that upogebiid shrimps reduce the diameter of their burrow opening [Dworschak, 1983, Vaugelas et al., 1990]. This phenomenon of small openings could be to maximize the generation of currents and to hide from predators (Nickell and Atkinson, 1995). Some ghost shrimps could survive in very low oxygen and have their own response mechanisms in order to thrive under hypoxic conditions (Leiva et al., 2015). The present results support previous studies and highlight the fact that mud shrimps alter the physical characteristics of the sediment in order to build strong burrows.

2.4.3 Organic material in the mud shrimp burrow wall

The content of the organic matter in the MSBW is an important parameter to understand the feeding strategies of mud shrimps. Previous studies have shown higher values of organic matter in the burrow wall than those of the background sediment in most thalassinidean shrimps (Kinoshita et al., 2008; Papaspyrou et al., 2006; Vaugelas et al., 1990; Leiva et al., 2015; Kristensen et al., 1991; Felder and Griffis, 1994; Papaspyrou et al., 2005). In the present study, the organic content in the burrow wall was also found to be higher than in background sediments. In fact a recent study reported high quality particulate organic matter (POM) as an essential component in the diet of the ghost shrimp *N. californiensis*. This POM reaches out to the bottom of the deep burrow commonly through sediment reworking or burrowing behavior of ghost shrimps (Bosley et al., 2017).

From previous studies, the Upogebiidea shrimps have been reported mainly as filter feeding animals [Nickell and Atkinson, 1995, Scott et al. 1988, MacGinitie, 1930; Schaefer, 1970; Powell, 1974; Dworschak, 1987). However, there are other studies that reflected different trophic modes and sometimes even more than a single mode (Nickell and Atkinson, 1995, Dworschak, 1987). According to the report of Coelho et al. (2000), the feeding mode of *U. omissa* had a strong tendency for deposit feeding and described this species as a generalist feeder. This dual trophic behavior has been previously reported for *U. pusilla* [Powell, 1974] and *U. stellata* (Nickell and Atkinson, 1995). Hence, a detailed study of the burrowing and feeding behavior of mud shrimps is necessary.

In the present study, the value of AFDW of one entire mud shrimp burrow was 24 times higher than that of an adult mud shrimp. According to the ten percent rule in a trophic pyramid, during the transfer of energy from the organic food via the lower trophic level to the higher trophic level, approximately 10 percent of the energy from organic sources is left in the higher trophic level (Lindeman, 1942). Shrimps belonging to the Upogebiidea are shown to have trophic plasticity (Nickell and Atkinson, 1995). A study on the thalassinidean shrimp *Callianassa subterranea* showed that ground dried algae and dried zooplankton can let these animals survive in the laboratory for more than 2 years (Rowden and Jones, 1994; Stamhuis et al., 1996). According to the report of Kinoshita et al. (2008), organic particles were more easily trapped in the burrow of *Upogebia major*, although upogebiid shrimps are mainly considered to be filter feeders. In laboratory cultivation, *A. edulis* rejected food offered into their burrow opening such as fish and shrimp meat, planktonic algae, dead copepods, and aquaculture feed of shrimp larvae (Das et al. unpublished data). From the observation, this shrimp is perhaps not a pure filter-feeding animal.

Chapter – 3: Effects of cadmium exposure on antioxidant enzymes and histological changes in the mud-shrimp *Austinogebia edulis* (Crustacea: Decapoda)

3.1 Introduction

Cadmium is a widespread trace metal of the earth's crust. It is released from industrial production as a polluting by-product mainly when lead, copper or zinc are extracted from ore (Mishra et al. 2006; Ma et al. 2013). In aquatic systems Cd is usually carried to the ground or the sediments where benthic organisms can take up this element, resulting in the entry of cadmium (Cd) into food chains and trophic webs. Increasing environmental Cd concentrations are potential threats for organisms (Folgar et al. 2009; Baki et al. 2018). Benthic animals can take up Cd through different physiological processes such as waterborne adsorption or by dietary ingestion of sediments and food items (Wu et al. 2014). This may harm or affect organisms in different organs like kidney, intestine, testes, liver, hepatopancreas, alimentary system and others (Kuriwaki et al. 2005; Wu et al. 2013, 2014). Most crustaceans are very sensitive to harmful chemicals or environmental stressors such as trace metals, organic pollutants, etc. Since these organisms have lower response amplitudes, they are particularly used for ecotoxicological studies and can act as bioindicators to notify possible toxicological risks (Hui et al. 2005; Rainbow and Black 2005; Núñez-Nogueira et al. 2012; Soegianto et al. 2013; Chiodi Boudet et al. 2015) or for the investigation of mechanisms of toxic action (Dahms et al. 2016). Previous studies have shown that high Cd concentrations can induce oxidative stress in several invertebrates such as crabs and shrimps as well as in clams (Cuypers et al. 2010; Xu 2011; Wang et al. 2011; Wu et al. 2013, 2014). Most heavy metals have the ability to induce peroxide and as a result to produce a huge amount of free radicals, which may have a negative impact on the secretion of hormones (Lin et al. 2017). In the presence of Cd or other heavy metals, reactive oxygen species (ROS) are released from cells. At increased concentrations, these induce oxidative stress by reacting with bio-macromolecules such as proteins, lipids, and nucleic acids, thereby also decreasing the cellular antioxidant capacity (Corticeiro et al. 2006). This reaction leads to various disturbances, such as cellular damage, mutation, and the peroxidation of lipids (Valko et al. 2006; Pathak et al. 2006; Oh et al. 2006; Ognjanovic´ et al. 2010; Cuypers et al.

2010; Xu 2011). Common indicators of oxidative stress are enzymes of the antioxidant defense system, which have the role of detoxification through the removal of free radicals and protecting the organism under stress (Yan et al. 2007; Zhang et al. 2009; Cao et al. 2010; Singaram et al. 2013). Such enzymes are among others, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Several studies have shown that at a higher concentration of Cd, the activities of the antioxidant enzymes can be significantly reduced which may lead to damages at cellular, tissue, or organ level (Wu et al. 2013; 2014; Chiodi Boudet et al. 2015; Lin et al. 2017; Zhou et al. 2017). The final product of the peroxidation of lipids is malondialdehyde (MDA), which gets induced by increasing amounts of free radicals inside the organ. This compound is often used as a parameter to determine the degree of damage or the level of lipid peroxidation and the aggregation of ROS species in an organism (Otitoloju and Olagoke 2011; Ma et al. 2012; Lin et al. 2017).

In this study a commercially important decapod crustacean and particularly endangered species was used to analyze the effect of Cd on its antioxidant enzymes. *Austinogebia edulis* (Ngoc-Ho & Chan 1992) is a globally rare species of mud shrimps, which is found along the western coast of Taiwan and is one of the bio-indicator candidates of the environmental protection agency of Taiwan. This animal can construct deep burrows (>1m) and the shape of the burrow is usually Y-shaped (Das et al. 2017). The area where *A. edulis* is quite abundant is close to an industrial park. Here, also several trace metals are released from the industry, which is contaminating sediments nearby (Peng et al., 2006). A report on the concentration of trace metals at the west coast of Taiwan has shown that Cd is quite abundant and readily available through sediment bioturbation by benthic organisms (Peng et al. 2006). It was even reported that Cd has one of the highest enrichment factors and its concentration got increased both in the sediment of southwestern Taiwan and at the northern coast of Taiwan (Keelung river drainage basin) over the past decades (Huang and Lin 2003; Chen et al. 2007). Therefore, studying the biochemical and histopathological changes after Cd exposure in the mud shrimp *A. edulis* is warranted. Studies of benthic organisms are particularly necessary since benthic marine animals have a high capacity to accumulate trace metals (Kargin et al. 2001; Wu et al. 2008; Liu et al. 2013). Several marine benthic invertebrates like *A. edulis* are deposit feeders and they directly ingest contaminated sediments that might have long-term harmful sublethal to even lethal effects that might lead to a reduction of population size and even to their extinction. This is the first study to show biochemical and histological changes in a thalassinidean mud shrimp when experimentally exposed to Cd.

Since the hepatopancreas of crustaceans plays crucial roles in several metabolic functions like digestion, absorption, secretion etc., it is an important organ that should be considered in the study of biochemical changes in crustaceans caused by pollutants (Bhavan and Geraldine 2000; Lin 2017). The crustacean hepatopancreas has bio-transforming, sequestering, and detoxifying functions involving several specific enzymes (Wu et al. 2008). However, among the xenobiotics certain pesticides can affect the hepatopancreas and may induce toxicity, which leads to histopathological changes and reductions of some enzymatic activities (e.g. of digestive and antioxidant enzymes) (Wu et al. 2008; Wu et al. 2013, 2014). Gills are well exposed to environmental toxins. They are more vulnerable to the absorption of toxins than other organs because of their large surface area and thin epithelia, which provides little barrier for environmental pollutants (Pandey et al. 2008).

The aims of this study were to investigate the antioxidant response upon Cd exposure and to obtain detailed knowledge about the antioxidant defense mechanism of *A. edulis*. We particularly tested the hypothesis here that Cd exposure causes impairment of different organs and the antioxidant defense systems of the mud shrimp *A. edulis*. We studied the effects of Cd on the histology, and antioxidant enzyme regulation in the tissues of three organs: hepatopancreas, gills, and musculature.

3.2 Materials and methods

3.2.1 Animal handling and treatments

The mud shrimp *Austinogebia edulis* (Ngoc-Ho & Chan 1992) was collected from the tidal flat of Shengang, which is close to an industrial park at the central western coast of Taiwan where *A. edulis* is commonly found. After collection the animals were immediately transferred to the laboratory. In the laboratory, the mud shrimps were maintained in sediment-filled small transparent boxes (15cm×8cm×6cm) with one individual in each box. These boxes were then immersed in aerated seawater with a filtering system and 24 hours continuous aeration. The temperature of the water and salinity was maintained at the conditions of the sampling site, i.e. 20^0C and a salinity of 33, respectively. Prior to the experiments the shrimps were acclimated to the above laboratory conditions for 1 month.

3.2.2 Exposure experiment

After this acclimation, similar sized, healthy shrimps (24-29g) were exposed to different cadmium concentrations, like control (0 mg/kg), 0.5 mg/kg, and 5 mg/kg. In order to maintain the cadmium concentration, the treatment water was changed every 24 hours at static exposure. During exposure, all other parameters were the same to above acclimation conditions.

3.2.3 Biochemical analysis

After a period of 96 hours, the shrimps were sacrificed and opened on ice by cutting the carapace dorsally. The hepatopancreas, gills, and a portion of the abdominal musculature were removed from the body. The individual organs were weighed at a ratio of 1:9 (w/v) and phosphate buffer solution (pH 7.2) was added. An electric homogenizer was used to homogenize the samples that were then centrifuged for 10 min (9300 g). The supernatant was used to measure antioxidant enzyme concentrations, namely SOD, CAT, and GPX along with measurements of MDA content by following the protocol described by the kits used for the experiment (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Xanthine/ Xanthine oxidase induced the generation of superoxide anion radicals during the measurement of SOD (Nishikimi 1975; Sun et al. 1988; Assady et al. 2011). This superoxide radical will then be converted to oxygen and hence the amount of enzyme required to inhibit this oxidation reaction by 50 % is defined as one unit of SOD (Wang et al. 2011; Assady et al. 2011). Absorbance was measured at 550 nm. Catalase activity (CAT) was expressed as 1 µmol H_2O_2 consumed sec^{-1} and mg protein and the absorbance was measured at 405 nm, following the ammonium molybdate colorimetric method (Góth 1991; Ahmad et al. 2000). MDA content analysis (nanomoles per milligram of protein) was based on the reaction with thiobarbituric acid, and lipid peroxidation was measured at an absorbance of 532 nm at 95 ^{0}C (Ohkawa et al. 1979). For analyzing the GPx activity, absorbance of 412 nm was used and the reaction was mainly based on the interaction of glutathione, which was left after the action of GPx and 5,59-dithio bis-(2-nitrobenzoic acid)(Rotruck et al. 1973). The protein content of aliquots was measured by using bovine serum albumin as a standard (Bradford 1976). All of the above mentioned measurements were carried out by using a microplate reader (Spectramax M5, Molecular Devices, USA) in triplicate.

3.2.4 Histological examinations

For revealing the effects on the hepatopancreas of *A. edulis,* histological examinations were performed for finding abnormalities using a light microscope (Olympus BX51). After dissection, all shrimp samples were preserved in seawater containing 10 % buffered formaldehyde for 24 h. The specimens were dehydrated through a graded series of ethanol and cleaned with xylene solutions, following routine histological procedures (Gurr 1962). Sections were obtained using a microtome (Thermo Scientific HM 430 Sliding Microtome, Wilmington, US) and the thickness of each section was 4-6-µm. The sections were stained with hematoxylin and eosin (HE) for observing the cell structure of the hepatopancreatic tissues. For functional examination, periodic Acid-Schiff (PAS) stain was used to test the secretion of mucus (Hagemans et al. 2010).

3.2.5 Data and statistical analysis

To compare the enzyme activity of 4 different enzymes at different concentrations of Cd, a Student's T-test was applied. In the case the data met the requirements of a parametric test, one-way analysis of variance (ANOVA) was used to estimate the differences between the activity of different enzymes in groups treated with Cd and the control group with the number of treated days. The cross effects of the concentrations of the treatment and periods of exposure on each tested enzyme activity among distinct Cd solutions in *A. edulis* was analyzed using a two-way ANOVA. In addition, a post hoc Tukey's honest significance difference (HSD) test was applied to identify the differences of concentrations and exposure periods. The statistical software SPSS.24 was used for all analyses. Principal component analysis (PCA) was performed to evaluate the relationships among activities of four enzymes and three tissues obtained from 240 tested samples (4 enzymes × 20 individuals × 3 tissues) at both high and low concentrations by using the Paleontological Statistics Software Package (Hammer et al. 2001).

3.3 Results

3.3.1 Biochemical analysis

The activity of enzymes in the different tissues, gill, hepatopancreas, and muscle varied (Fig 3.1, 3.2, 3.3). In gills, the activity of SOD (Fig 3.1a) and GPx (Fig 3.1c) was significantly lower than in the control group. The activity of CAT (Fig 3.1b) decreased significantly on the 4th day of exposure from the control group. In contrast, the MDA activity in both the treatment groups showed an increasing trend and finally was significantly higher than in the control group. The activity at 5 mg/kg concentration was higher than at the 0.5 mg/kg concentration group during the whole experimental period (Fig 3.1d).

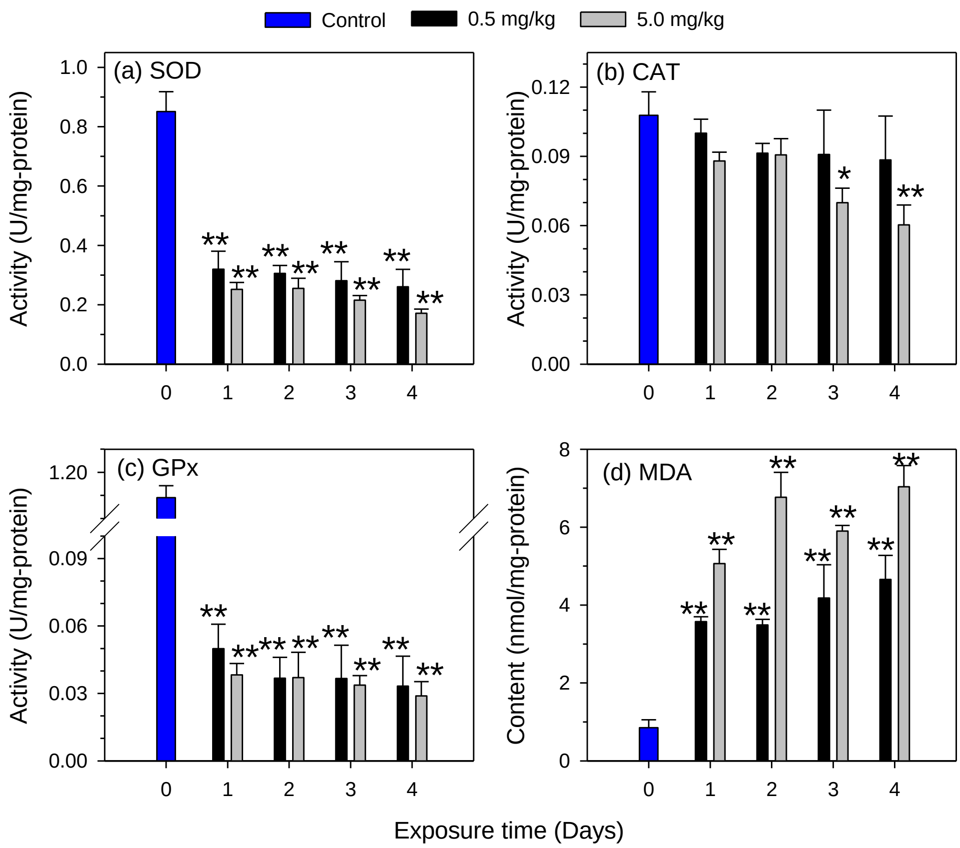

Fig 3.1 Activities of antioxidant enzymes [(a) SOD activity; (b) CAT; (c) GPx; (d) MDA] and the membrane lipid peroxidation in the gill of *A. edulis.* Values that are

used to calculate are means ± SD from five individual samples. Significance is indicated by * p <0.05 and ** p <0.01

In the hepatopancreas, the activity of SOD at 5 mg/kg concentration was significantly lower than in the control group on the 2nd and 4th day of exposure (Fig 3.2a). The activity of GPx was significantly lower in the treatment groups than in the control during the whole experimental period. The activity at 5 mg/kg concentration showed a decreasing trend from the 1st day to the 4th day of exposure (Fig 3.2c). The activity of CAT (Fig 3.2b) and MDA (Fig 3.2d) was not significantly different to the control group in both treatment groups.

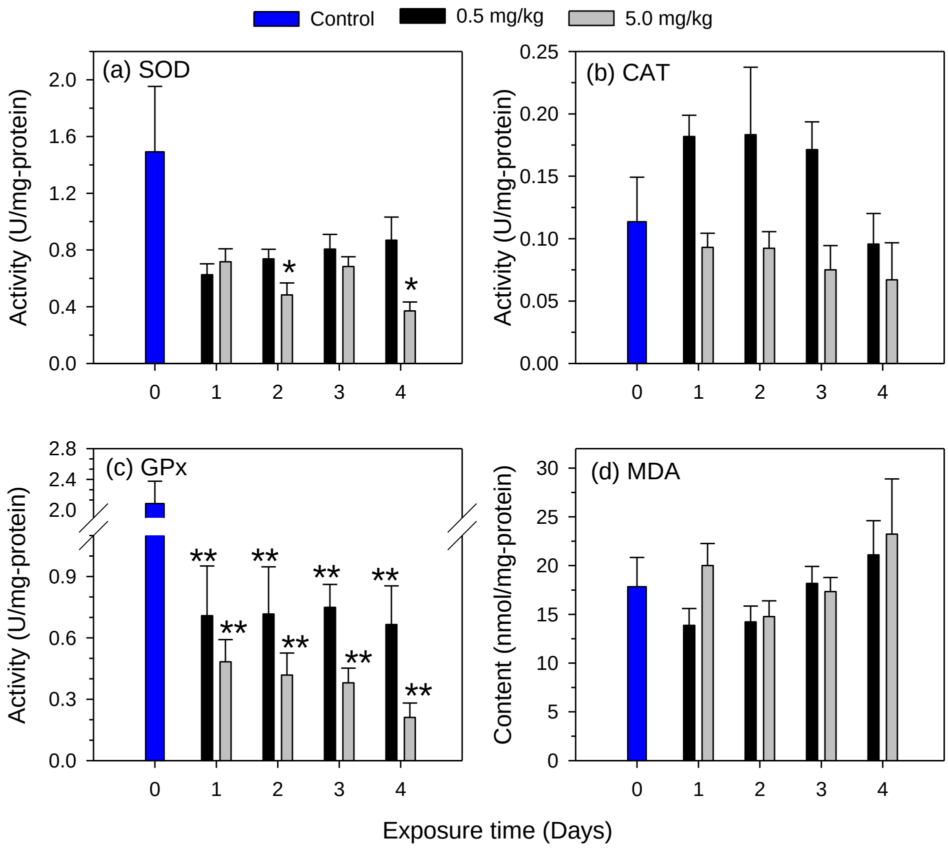

Fig 3.2 The activities of antioxidant enzymes [(a) SOD activity; (b) CAT; (c) GPx; (d) MDA] and membrane lipid peroxidation in the hepatopancreas of *A. edulis*. The values are means ± SD from five individual samples. Significance is indicated by * p <0.05 and ** p <0.01

In the musculature, most of the enzymes did not show much sensitivity to Cd exposure (Fig 3.3). However, the activity in both treatment groups was different, such as in SOD (Fig 3.3a), CAT (Fig 3.3b), and GPx (Fig 3.3c) the activity at 0.5 mg/kg concentration was higher than in the 5 mg/kg concentration group. In contrast, the activity of MDA (Fig 3.3d) at 0.5 mg/kg concentration was mostly lower than in the 5 mg/kg concentration group. As for CAT at 0.5 mg/kg the activity was significantly higher only on the 1st day than in the control group, then it decreased to a concentration similar to the control (Fig 3.3b).

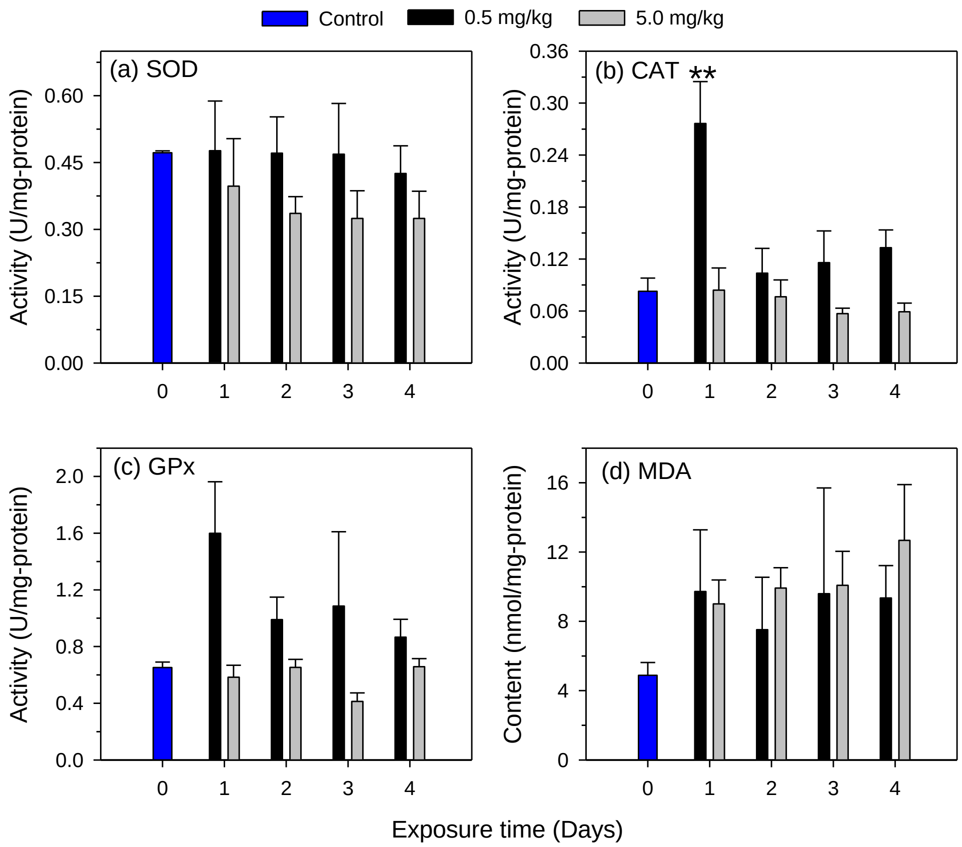

Fig 3.3 The activities of antioxidant enzymes [(a) SOD activity; (b) CAT; (c) GPx; (d) MDA] and membrane lipid peroxidation in the muscle of *A. edulis.* The values are means ± SD from five individual samples. Significance is indicated by * p <0.05 and ** p <0.01.

By using Student's t-test, the average value of each enzyme activity was used to compare the strength of the two concentrations in different tissues (Table 3.1). In gills, the activity of MDA at 5 mg/kg concentration was significantly higher than in the 0.5 mg/kg group (t=-2.99, p=0.004**). In the hepatopancreas, the activity of CAT at 0.5 mg/kg concentration was significantly higher than in the 5 mg/kg concentration group (t=3.35, p=0.002**).

In muscles, the activity of SOD (t=2.04, *p*=0.05*), CAT (t=3.38, *p*=0.002**) and GPx (t=3.14, *p*=0.004**) was significantly higher in the 0.5 mg/kg concentration than in the 5 mg/kg concentration group.

Table 3.1 Results of Student's t-test comparison of the antioxidant enzyme activity and MDA concentration with different concentrations in different tissues, number of samples = 5

Test tissue	Gill			Hepatopancreas			Muscle		
Tested enzymes	0.5 mg/kg	5mg/kg	t-value, *p*-value	0.5 mg/kg	5mg/kg	t-value, *p*-value	0.5 mg/kg	5mg/kg	t-value, *p*-value
SOD	0.40	0.35	0.73, 0.47	0.91	0.75	0.96, 0.34	0.46	0.37	2.04, 0.05*
CAT	0.10	0.08	1.65, 0.11	0.15	0.09	3.35, 0.002**	0.14	0.07	3.38, 0.002**
MDA	3.35	5.13	−2.99, 0.004**	17.04	18.63	−0.87, 0.39	8.21	9.31	−0.62, 0.54
GPX	0.25	0.25	0.03, 0.98	0.99	0.72	1.28, 0.21	1.04	0.59	3.14, 0.004**

Table 3.2 Statistical results of two-way ANOVA of the activity of enzymes in three experimental tissues with different time of exposure and treatment concentrations. The value of degree of freedom is 3, 1 and 3 for Day, Concentration and Day × Concentration, respectively. SS is Sum of Squares. *Significant at the $p < 0.05$ level (2-tailed); ** significant at the $p < 0.01$ level (2-tailed)

	Gill			Hepatopancreas			Muscle		
Enzyme	SS	F	*p*-value	SS	F	*p*-value	SS	F	*p*-value
SOD									
Day	0.03	0.94	0.43	0.11	.15	0.92	0.02	0.24	0.86
Concentration	0.05	4.08	0.05*	0.39	1.59	0.21	0.13	4.81	0.03*
Day × Concentration	0.00	0.06	0.98	0.46	0.63	0.60	0.01	0.07	0.97
CAT									
Day	0.0021	1.22	0.31	0.02	1.88	0.14	0.06	6.22	<0.001**
Concentration	0.0022	3.87	0.56	0.06	16.08	< 0.001**	0.08	23.41	<0.001**
Day × Concentration	0.0012	0.67	0.57	0.01	0.74	0.53	0.04	4.04	0.013**
MDA									
Day	11.71	3.85	0.016*	306.84	2.59	0.06	28.11	0.23	0.87

Concentration	49.13	48.45	< 0.001**	39.56	1.05	0.32	18.89	0.47	0.49
Day × Concentration	4.82	1.58	0.208	68.11	0.57	0.63	25.01	0.21	0.88
GPX									
Day	0.0011	0.12	0.94	0.15	0.28	0.83	0.77	1.12	0.35
Concentration	0.0002	0.05	0.82	1.13	6.44	0.015*	3.10	13.58	<0.001**
Day × Concentration	0.0002	0.02	0.99	0.07	0.13	0.93	0.99	1.44	0.24

Furthermore, results of two-way ANOVA revealed that the concentration and time of exposure were important factors and had various influence on each enzyme in different tissues (Table 3.2). The exposure time influenced the activities of MDA in gill ($p = 0.016$) and CAT in muscle ($p < 0.001$). The concentration of Cd altered the activity of SOD in gill ($p = 0.05$) and muscle ($p = 0.03$); activity of CAT in hepatopancreas ($p < 0.001$) and muscle ($p < 0.001$); activity of MDA in gill ($p < 0.001$), and activity of GPX in hepatopancreas ($p = 0.015$) and muscle ($p < 0.001$). In addition, the cross interaction of concentration and time of exposure significantly affected the activity of CAT in muscle ($p = 0.013$), indicating that the change in concentration and time of exposure caused the activities of CAT to shift over time.

The result of PCA for the enzyme activity of 240 tested samples collected from different sets of experiments is shown in figure 3.4. At 0.5 mg/kg Cd concentration (Fig 3.4a) the pattern of distribution of different enzymes in gill is not much as varied as in other tissues like hepatopancreas and muscles. The difference between the tissues of hepatopancreas and muscle reflects a huge variation of MDA during the different exposure periods. At 5.0 mg/kg Cd concentration (Fig 3.4b), the variations in hepatopancreas were higher than in other tissues. A certain enzyme MDA was distributed in a large area indicating high fluctuations during the experimental period. Based on the PCA results, the alteration of enzyme activities in gills was lower at both concentrations, whereas changes of enzyme activities in muscle were higher than at the 0.5 mg/kg concentration or the 5.0 mg/kg concentration. In the hepatopancreas changes in enzyme activity were higher than in the tissues of gill and muscle (Fig 3.4).

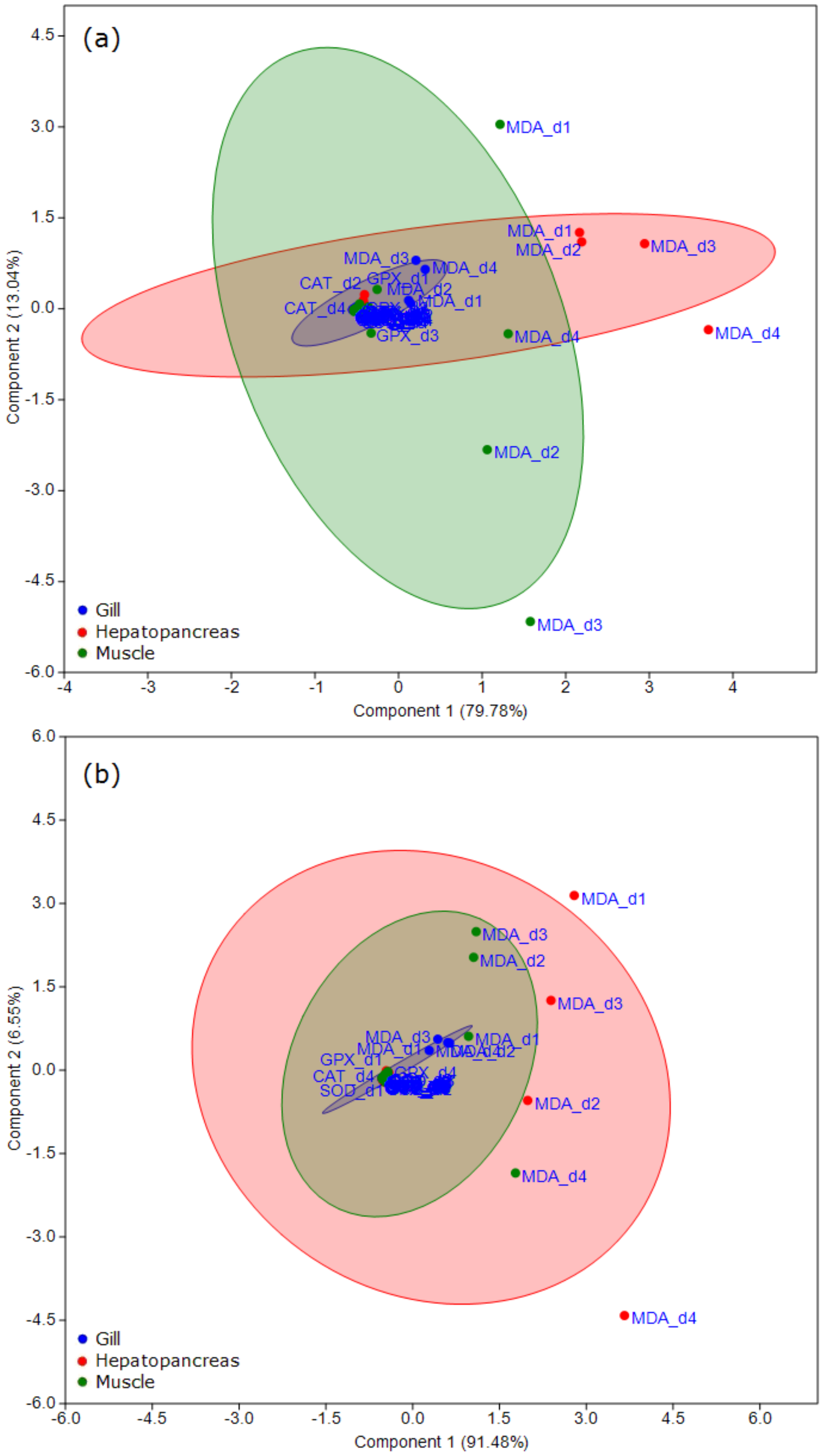

Fig 3.4 The PCA results show the activity of antioxidant enzymes in different tissues for the two different concentrations of Cd, which are (a) 0.05 mg/kg and (b) 5 mg/kg

3.3.2 Histological examination

Sections of H.E. staining showed that the hepatopancreatic cells were circular in shape in the control, providing a cell-specific typical pattern. The epithelial cells were tightly packed. Various kinds of cells were arranged having a distinct margin. On the inner surface; a border containing striations was noticed (Fig 3.5 a, b). At 5 mg/kg concentration, damage became evident in the disappearance of the epithelial cell boundaries. Large areas of vacuolization became apparent. Several epithelial cells were detached and the hepatopancreas appeared to be necrotic and swollen. The arrangements of the nuclei were irregular which indicated that the tissue structure was altered by the exposure to Cd (Fig 3.5 c, d). Pictures of the PAS stain in the control group demonstrated that the deep purple and pink color in the tissue of hepatopancreatic cells secreted large amounts of mucus (Fig 3.5 e, f). In contrast, at 5 mg/kg concentration the tissue of the hepatopancreas was lightly stained, resulting in grey or light purple color. This implied that the secretion of mucus was much less than in the control group (Fig 3.5g, h).

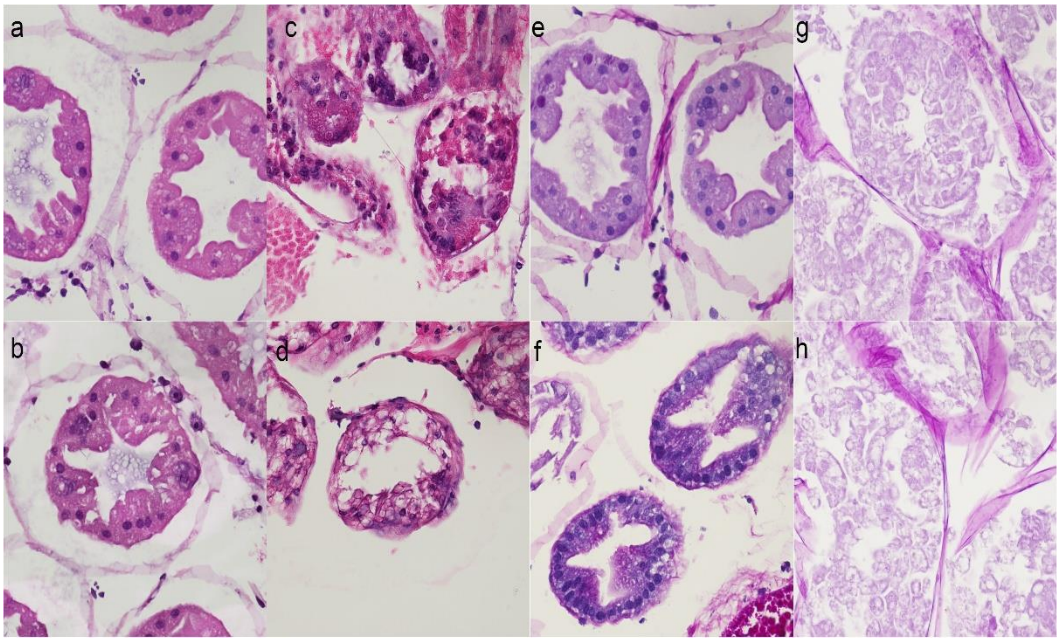

Fig 3.5 The structure of hepatopancreas after treatment with cadmium (5 mg/kg) under light microscopy (H.E. staining). a, b × 400, control group showing a normal structure of hepatopancreas with tightly packed columnar epithelial cells. c, d × 400, groups treated with 5 mg/kg Cd concentration showing the disappearance of the epithelial cell boundaries and the loss of the star-shaped lumen with swelling and necrosis of cells. The structure of hepatopancreas after treatment with cadmium (5 mg/kg) under light microscopy (PAS stain).

e, f × 400, control group showing hepatopancreatic cells with deeply stained (purple) mucus and cell specific patterns of the organ. g, h × 400, groups treated with 5 mg/kg Cd concentration showing lightly stained cells with a lack of mucus or glycogen

3.4 Discussion

3.4.1 Biochemical changes after Cd exposure

Several studies reported that Cd induces oxidative stress in most organisms that were studied, including crustaceans (Liu et al. 2009; Patra et al. 2011; Chiodi Boudet et al. 2015; Lin et al. 2017). Reactive oxygen species (ROS) are commonly produced when organisms encounter oxidative stress due to high concentrations of certain toxins, like trace metals, xenobiotics etc. (Ahmad et al. 2000). The production of ROS and free radicals get higher with increasing concentrations of metals or toxins. As a result they cause severe damages by exerting direct impacts on lipids, DNA, and proteins (Wu et al. 2014). The present study observed a decrease in the activities of all antioxidant enzymes in three tissues (hepatopancreas, gills, and muscles) at higher concentrations of Cd. The effect of inhibition and induction is dynamic and varies with different biochemical parameters and concentrations of metals, as it was investigated for Cd (Lei et al. 2011; Wu et al. 2013). Increased ROS has been previously reported to affect cardinal cellular components through apoptosis (Lin et al. 2017). The ability of ROS to bind or attack polyunsaturated fatty acids causes peroxidation of lipids, which is a key process that primarily identifies or denotes oxidative cell damage (Anane and Creppy 2001). Peroxidation of lipids produces the secondary product malondialdehyde (MDA), which indicates oxidative cell damage by binding to free amino acids from a protein and thereafter crosslinking within and between protein molecules (Papadimitriou and Loumbourdis 2002; Badisa et al. 2007). Therefore, an increase in the activity of MDA is a primary mechanism for oxidative damage of cells. The present study reports a significant increase in the MDA activity in all three tissues studied (gills, hepatopancreas and muscles) after acute intoxication of the mud shrimp *A. edulis* by Cd. Several other studies reported an increase in MDA activity and considered the accumulation of MDA as a marker for the risk of oxidative cell damage (Parikh et al. 2003; Liu et al. 2013; Soegianto et al. 2013; Lin et al. 2017; Zhou, et al. 2017).

Antioxidant enzymes such as SOD, CAT and GPx play a crucial role in the cellular defense against xenobiotic exposure (Sevcikova et al. 2011). SOD is known to catalyze the search of superoxide anion radicals and they can quickly convert oxygen-free radicals to hydrogen peroxide molecules, thereby balancing the metabolism of free radicals and imparting protection from cellular damage (Filho 2007; Cao et al. 2010). Both CAT and GPx are known to have the ability to eliminate and transform H_2O_2 into H_2O and O_2, thereby, helping in the elimination of H_2O_2 and as a result reducing tissue injuries (Yao et al. 2006). All mentioned enzymes provide a first line of defense of organisms encountering stress due to exposure to toxins. Their proper functioning and activity is necessary to prevent any cellular damage or death (Pandey et al. 2008).

An increase in the activity of SOD, CAT and GPx in the present study was observed at a lower concentration of Cd (0.5 mg/kg Cd). This can be explained as following. When the organism experiences a lower concentration of Cd, it starts combating the harmful effect of the toxin by activating antioxidant enzymes, which are lining up as a defense mechanism. This phenomenon is more likely to be a defensive mechanism or strategy against an increase of ROS species. An increase or stimulation in SOD activity increases the concentration of H_2O_2, which in turn gets eliminated by CAT. Therefore, both enzymes got activated at 0.5 mg/kg Cd (Chen et al. 1994). At higher concentrations of Cd (5 mg/kg Cd) all enzymes were inhibited due to an increase of ROS concentration and eventually losing their defensive functions due to increasing toxicity in all three tissues. An exceeding range of Cd concentrations could eventually impair the functions of antioxidant enzymes and result in the reduction of SOD activity. This causes an accumulation of O^{2-} in the organs of *A. edulis* which leads to oxidative cellular damage or even cellular death. Also, our results show that transforming or eliminating properties of CAT enzymes was reduced due to increased Cd concentrations. Several studies inferred the same phenomenon about biochemical alterations upon exposure to Cd toxicity (Pandey et al. 2008; Messaoudi et al. 2010; Wang et al. 2011; Lin et al. 2017; Bao et al. 2018).

For the activity of GPx, selenium is an essential element. The active site of the GPx enzyme is selenocysteine (Se-Cys), which after exposure to metal such as Cd assists to reduce the toxicity of the trace metal. An increasing trace metal concentration (Cd) can lead to a change in the active site of the enzyme and as a result GPx tends to lose its activity (Iszard et al. 1995; Wu et al. 2013). Therefore, an increasing accumulation of H_2O_2 and OH leads to oxidative cellular damage and GPx synthesis reaction gets inhibited with increasing Cd concentration (Hultberg et al. 1998; Wu et al. 2014). According to a previous study, the increasing accumulation of H_2O_2 can lead to an induction of peroxidation of lipids, thereby increasing the activity of MDA (Wang et al. 2013). Collectively, such results reveal that Cd exposure to aquatic animals can lead to the accumulation of MDA and ROS species that actually reduce the activity of antioxidant enzymes and further increase the level of oxidative damages.

3.4.2 Histological alterations in the hepatopancreas after Cd exposure

Common pathways for the uptake of xenobiotics or any metal ion of an aquatic organism is from the water (waterborne) or through the uptake of food (diet-borne). Both cause harmful damage in the alimentary system of an organism (Rainbow and White 1989). The hepatopancreas being an important digestive organ plays crucial roles such as nutrient absorption, secretion, and production of digestive enzymes (Wu et al. 2014). In crustaceans, the hepatopancreas performs almost similar functions than the liver of higher organisms and is considered to be analogous to it (Chiodi-Boudet et al. 2015). Hence, any damage of the cellular structure or changes in the structure of epithelial cells can potentially affect the function of the hepatopancreas. The hepatopancreas of crustaceans is considered to be the main depository organ for toxic metals and is, therefore, widely studied as a marker to diagnose harmful effects of pollutants or other toxic chemicals (Soegianto et al. 2013; Chiodi Boudet et al. 2015; Lin et al. 2017; Zhou et al. 2017). In the present study a comparison of the cellular structures of the hepatopancreas of healthy individuals (control group) with those treated with cadmium (5 mg/kg) showed substantial changes in the histological structure of the mud shrimp *A. edulis*. The boundary of epithelial cells disappeared with the exposure to Cd at 5 mg/kg concentration and also a separation of the cells from the basal lamina was observed in our study. A change of epithelial cells was a common finding in several other studies that examined the effects of exposure to noxious metals or toxic chemicals on the alteration of the structure of the hepatopancreas of several crustaceans (Bhavan and Geraldine 2000; Wu et al. 2008; Wu et al. 2014; Chiodi Boudet et al. 2015). Other studies related to heavy metal exposure or xenobiotics (aflatoxin, fungicide, pesticide etc.) reported typical responses such as infiltration of hemocytes, necrosis of the branched tubules, separation of cells from the basal lamina and the thickening of the basal lamina in other shrimp or prawn species in the hepatopancreas (Lightner et al. 1996; Wu et al. 2008; Lin et al. 2017). Studies have reported heavy vacuolization in the cytoplasm when exposed to heavy metals and this result was in accordance with our findings of normal to heavy vacuolization in epithelial cells lining the branched tubules of the hepatopancreas (Vogt 1990; Wu et al. 2008). Several studies have reported sensitivity of the hepatopancreas to environmental pollutants, like heavy metals, pesticides, fungicides, and also environmental changes indicated by histological and molecular biomarkers (Vasanthi et al. 2017; Zhao et al. 2017). The present study also reflected some significant structural damages in the hepatopancreas of *A. edulis*, which inevitably affected its secretion, absorption, and digestion abilities that in turn caused a negative impact on the physiology of the mud shrimp. It is quite evident that cellular damage of tissues is a common phenomenon in animals that were exposed to high concentrations of Cd (Liu et al. 2013; Soegianto et al. 2013; Lin et al. 2017). Our study also showed a reduction in glycogen and mucus in the hepatopancreatic tissues after exposure to Cd when stained by PAS. PAS staining is generally used for the detection of glycogen or polysaccharides and mucus, like glycoproteins, mucins, and glycolipids that are present in different tissues of an organ (Hagemans et al. 2010). Our results showed a greyish appearance of the tissue with no glycoproteins or mucus, which is in contrast to the deep magenta color of the control group, indicating a healthy tissue full of mucosubstances. All these observations clearly indicated that Cd was readily accumulated in the hepatopancreas of *A. edulis* and caused considerable morphological damages even though the exposure time was short (96 hours). This observation was similar to a recent study that showed increasing folds of Cd accumulation and considerable histological damage in the hepatopancreas of *Eriocheir sinensis* with increasing exposure time (6 days) (Lin et al. 2017). Such results may encourage ecotoxicologists to determine areas, which are threatening aquatic life due to increased concentrations of xenobiotics such as heavy metals.

Chapter 4 – Spatial and temporal distribution of persistent organic pollutants in sediment near Changhua Industrial park, Taiwan

4.1 Introduction

Persistent organic pollutants (POPs) are the pervasive contaminant found in different domains of the environment. The sources of these compounds are usually anthropogenic and they originate through multiple routes. POPs are compounds that mostly accumulate from the run offs in urban areas, discharges of industries, domestic regions, stack emissions, oil spilling, incomplete combustion, etc. (Ramdine et al., 2012; Ahmed et al., 2017; Nascimento et al., 2017; Ramzi et al., 2017). A rise in industrialization along the coastal regions has proved to be highly threatening to the surrounding ecosystem. The increasing anthropogenic activities in the industries have significantly elevated the pollutant loading like discharge of pesticides, fertilizers, petrochemicals, etc., to the underlying sediment matrix. The sediment has often been considered as the ultimate sink for numerous chemical pollutants (Denis et al., 2012; Bayen, 2012; Cao et al., 2015; Fusi et al., 2016; Tongo et al., 2017; Yuan et al., 2017; Huang et al., 2017). Intertidal zone is an area considered to be most stressful because of the fluctuation of different physical parameters and hence are more susceptible to anthropogenic activities (Chapman and Reed 2006; Wright et al. 2004). Thus, contamination of such tidal flats is a common scenario and measures should be taken to protect such highly productive areas.

Organic pollutants are known to include endocrine disrupting compounds (EDCs) and micro-pollutants. These have been studied as regards to their origin, distribution and destination in the environment during the last few decades (Ponce-Velez et al. 2006; Kucklick et al. 1997; Magi et al. 2002; Yunker et al. 1999, 2001 and 2002; Lipiatou and Saliot 1991 and reference therein) and counted among the major groups of environmental pollutants due to their bio-accumulative potential, carcinogenicity and high persistence. They are composed by pesticides, polycyclic aromatic hydrocarbons, polychlorinated compounds, personal and hygiene care products, pharmaceuticals, dioxins, etc. In particular EDCs are known to be persistent and considered to be extremely toxic to human beings and the environment. The presence of such compounds can potentially affect the basic functions of hormones in human body like secreting, synthesis, binding, transporting and eliminating, and as consequence generate cancerous tumors, abnormal patterns of growth, delays for neurodevelopment in children, hypo/ hyperthyroid, etc. (Fowler et al., 2012; Bergman et al., 2013; Sifakis et al., 2017). Several studies have showed that EDCs can be accumulated in the benthic or aquatic organisms from the surrounding contaminated sediments. Therefore consuming such organisms or even drinking contaminated water and agro-based supplements by the human population can cause serious diseases, which in long term might prove to be fatal (Salgueiro-González et al. 2015; de Castro-Català et al. 2016; Casatta et al. 2016; Liu et al. 2017, Keshavarzifard et al. 2017b). Moreover, EDCs have been reported to cause intersexual changes in fish (Aris et al., 2014; Zheng et al., 2015; Adeogun et al., 2016). It has been reported that aquatic animals are more vulnerable and at a higher risk to bio-accumulate POPs since their metabolic functions are not well developed as compared to terrestrial animals (Martí et al., 2010, Chang, 2017).

On one side, PAHs are known to be carcinogenic and mutagenic toxic compounds that can pose serious threats or can be hazardous to the environment. Due to this, the European Union (EU) and the United States Environmental Protection Agency (USEPA) has listed PAHs as a contaminant with priority and since then PAHs monitoring has been performed in different areas of the world (Arias et al., 2009; Maioli et al., 2010; Lakhani, 2012; Nguyen et al., 2014; Li et al., 2014; Tongo et al., 2017 and references therein). Similarly, Polybrominated diphenyl ethers (PBDEs) are brominated flame retardants extensively used to reduce the flammability of many combustible products, including textiles, plastics, electronic components and finishing materials (Nelson et al., 2015). The rising concern due to their widespread distribution particularly in the marine environment has resulted in their worldwide monitoring (Chen et al., 2018; Zanaroli et al., 2015; Bodin et al., 2011 and reference therein). On the other side, although the organochlorine compounds were widely used in agriculture and disease control programs from the 1940s to 1960s with dramatic benefits, they fell into disfavor because of their persistence in the environment, wildlife, and humans. However, the relatively low cost of these pesticides and unavailability of complete substitutes (particularly for DDT), ensured a continuous use in several developing countries (Caldas et al. 1999, Senthilkumar et al. 2001, Jergentz et al. 2004, Miglioranza et al. 2004, Peruzzo et al. 2008). Pesticides have numerous effects in organisms, causing general stimulation of the nervous system (Carvalho et al. 2002), endocrine disruption (Colborn 2004) and food chain biomagnification (Galassi et al. 1994), because of their lipophilicity and environmental persistence. Finally, Polychlorinated biphenyls (PCBs) are a family of 209 hydrophobic chlorinated compounds characterized by high persistence, bio-accumulative potential and toxic properties, reflecting the lipophilicity and widespread distribution of these compounds in the environment (Sbriz *et al*., 1998; Konat *et al*., 2001; Frignani *et al*., 2004; Samara *et al*., 2006; Dercova *et al*., 2009). PCBs were first synthesized in 1929, as various (commercial) technical mixtures, including “Aroclor” (USA), “Clophen” (Germany), “Phenoclor” (France), “Fenclor” (Italy) and “Kanechlor” (Japan). PCBs were extensively used for different industrial applications (e.g., dielectric fluids, insulators for transformers and capacitors, hydraulic fluids, casting wax, carbonless carbon paper, compressors, heat transfer systems, plasticizers, pigments, adhesives, liquid cooled electric motors and others) until the late 1970’s when the production, processing and distribution of these substances were banned in most countries. All these compounds tend to strongly adsorb on the surface of the organic compounds due to a considerably higher partition coefficient and its low water solubility (Hong et al. 2016; Keshavarzifard et al. 2017a). Once in the marine environment, they enter to the trophic web and tend to accumulate in organisms and bio-magnify, rapidly reaching the long-lived apex predators (Clark, 2001)

Although the application or usage of organic pollutants (POPs) have been prohibited in several countries, few developing nations indulge in their production and usage due to the cost effectiveness (Chang, 2017) and hence such toxic chemicals continue to prevail in different compartments indicating a global threat to the ecosystem (Cleemann et al., 2000; Feng et al., 2003; Verweij et al., 2004; Wurl and Obbard, 2005; Chau, 2006; Katsoyiannis, 2006, Doong et al., 2008). In 1988, after several years of extensive usage and application, the Environmental Protection Administration of Taiwan listed 10 POPs (out of 12) as toxic substances (Liou et al., 2006). Despite this, there are precedents demonstrating extensive levels of POPs in Taiwan, including PAHs, PBDEs, OCPs mostly in rivers and coastal areas; for instance, the Gao-ping River, Kenting coral reef waters, Danshui River and Keelung River (Hung et al.2007, Jiang et al., 2011, Hung et al., 2010, Fang et al., 2008, Cheng and Ko, 2018, Doong et al. 2004, Cheng et al., 2010). Considering the western coast of Taiwan, a highly anthropized area which includes prolific industries for electroplating and dyeing (Yeh et al., 2009), precedents on POPs concentration are scarce. Particularly, the Changhua Coastal Industrial park, also known as the Changbin Industrial park, is basically a conglomeration of industries, which includes a variety of chemical industries, processors for metals, spinning and also food production. In simultaneous this area is also distinguished for artisanal collection of clams, oysters and fishing for native inhabitants. Precedents for the area have demonstrated poor air quality plus elevated levels of trace metals and PAHs (Chen, et al., 2016; Hsu et al., 2016). In fact, according to Yuan and Chen et al., an increase in the level of urinary 1-hydroxypyrene in adult residents have been noticed due to the emissions from the nearby petrochemical industry at Yunlin County (Yuan et al., 2015, Chen et al., 2016). Considering this scenario, the main aim of this study is to investigate the spatial and temporal distribution of several POPs in sediments and benthic organisms near Changhua Industrial park and identify the possible sources of POPs and assess the potential ecological risks to benthic organisms (mud shrimp, *A. edulis*) at the area.

4.2 Material and methods

4.2.1 Sampling area and sample collection

Changhua County, Taiwan, represents a complex environmental and ecological system with numerous sources of pollution, a vast intertidal flat with a huge biodiversity and climatic variation (Chen, et al., 2016). This tidal flat is the habitat of a rich biodiversity of crabs, shrimps, fish, clams, etc. Out of these *Austinogebia edulis,* is a mud shrimp that builds deep burrows (>1m) and is largely consumed as seafood (Das et al., 2017). It is an uncommon species of mud shrimp found in the tidal flat of western coast of Taiwan, Hong Kong and northern Vietnam. This mud shrimp presents a high occurrence at the Changhua County and is considered to be ecologically and commercially essential; hence, it is frequently monitored through different governmental programs. Previous study also showed the sensitivity of *A. edulis* to trace metals and how they alter the enzymatic activities and physiological damage based on higher concentrations of Cadmium (Das et al., 2018). During the last 10 years, *A. edulis* population has extensively declined. Some areas where the abundance of this shrimp was much high, has lowered down to almost zero in recent times; however, the rationale for this is still unknown. Benthic organisms are often more likely to get exposed to POPs contaminated sediment when comparing to air and water (Hussain and Hoque, 2015) leading the sediment matrix as a good index for recording the contamination levels (Soukarieh et al. 2018). A rising hypothesis is a higher pollution pressure over *A. edulis* since their burrows are commonly high in organic matter contents when compared to the surrounding sediment and therefore a higher possibility of accumulation of POPs in these sediments is possible (Das et al. 2017).

In order to assess the persistent pollutant occurrence at the area we collected sediment samples from around the industrial area in the western coast of Taiwan. Total 5 stations were chosen parallel to the coastline namely, Station A, B, C, D and E (Figure 4.1). The sampling sites experience a wet season mostly from late June until October and a dry season from December until April. The seasonal monsoon affects the ocean currents and the climate of Taiwan. The air temperature is usually 12°C during winter and in summer it can get more than 30°C (Das et al., 2017), with an average salinity of 33. Sampling was conducted once in the wet season (September, 2017, avg. temp – 28°C) and once in the dry season (January, 2018, avg. temp- 15^{0} C) (Table 4.1). We used a coring device to get the sediment samples from the deepest layer at 50cm, middle layer at 25cm and the surface at 0 cm. Some sediment samples were kept for the analysis of Total Organic Carbon (TOC) and grain size. The mud shrimps *A. edulis* were collected only from Station A and Station E because in the other stations population density of *A. edulis* was found to be zero. All the samples were put into the icebox after wrapping in aluminum foils and immediately transported to the laboratory. Once there, samples were initially homogenized on a clean tray made of stainless steel and then transferred to pre-cleaned acetone and hexane rinsed glass bottles and stored in -20^0 C until further analysis.

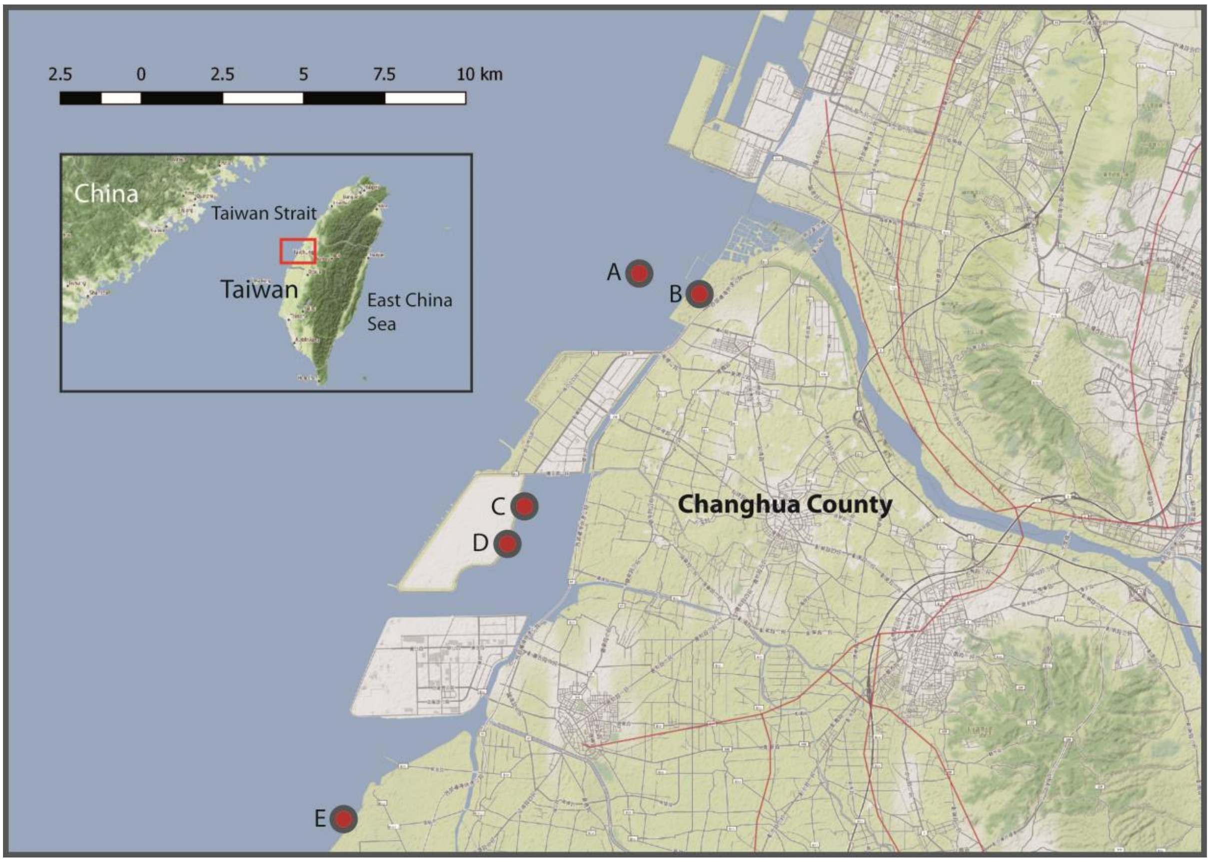

Fig 4.1 Map showing the sampling stations (A, B, C, D and E) at the western coast of Taiwan. The station C and D is situated near the northern and southern part of the Changhua Industrial Park.

Table 4.1 Sampling stations around the Changhua Industrial Park along the western coast of Taiwan with coordinates

Stations	Latitude (N^0)	Longitude (E^0)	Description
A	24.173	120.456	Located in the northern side of the Industrial Park. Also the mud shrimp conservation area.
B	24.164	120.458	Similar location as Station A, but outside the mud shrimp conservation area.
C	24.124	120.418	Located in the northern side within the Industrial park.
D	24.119	120.417	Located in the southern side within the Industrial park.
E	24.015	120.349	Located in the southern part of the western coast of Taiwan

4.2.2 Preparation and extraction of sediment samples

For the preparation and extraction of sediment samples and mud shrimp, *A. edulis* we followed the procedure described by Ko and Baker (1995, 2004). About 5g of dried sediment and organs from each sample were taken and grinded finely with anhydrous Na_2SO_4 in a mortar pestle before extraction. The organs that were used for the bioaccumulation of POPs were namely exoskeleton, muscle, gill and hepatopancreas. This was followed by extraction of the samples in a Soxhlet apparatus for 24 h with hexane and acetone as extraction solvents mixed in 1:1 (v/v) ratio. In the extraction tubes, every sample was spiked with surrogate standards (d8-Napthalene, d10-Fluorene, d10-Fluoranthene and d12-perlene for PAHs and PCB 14, PCB65 and 166 for PCBs, OCPs and PBDEs) prior to extraction in order to calculate the percentage of procedural recovery. We used small-activated copper wires or granules in order to remove the elemental Sulfur from all the samples. For concentrating the extract a rotary vacuum evaporator was used at several steps in the whole procedure of PAHs analysis. Fractionation and further cleaning of the extract to remove polar interference was performed by using 8g of 6% deactivated alumina packed in a column which was followed by concentrating the extract first with rotary evaporator and then further concentrating or drying up to 1 ml by using a gentle purified N_2 stream. Perdeuterated internal standards of PAH were added to each sample before analyzing the PAHs concentration in the gas chromatography-mass spectrometer (GC–MS) (Varian-320). After PAH analysis, the sample extracts were passed through a glass column packed with 8g of 2.5% deactivated florisil and covered with Na_2SO_4 (~1cm) on top. The column was conditioned with 35 ml petroleum ether (Pet. Ether) and dichloromethane (DCM) mixture (1:1) solvent with further addition of 35 ml Pet. Ether. The PCBs, OCPs and PBDEs were eluted with 35 ml Pet. Ether. Then solvent was transferred to hexane and was concentrated by rotary evaporation. The volume of elution was further reduced to less than 0.1 ml under a gentle stream of nitrogen. For quantification, the internal standards (PCB 30 and PCB 204 for PCBs, PCB 204 for OCPs and PCB 204 and MBDE 209 for PBDEs) were added to each sample before analyzing the PCBs, OCPs and PBDEs concentration in GC-triple-quadruple mass spectrometry system (TQ-8050, Shimadzu).

4.2.3 Persistent Organic Pollutants analysis

The PAHs was determined using a Varian 320 GC-MS, while PCB, DDTs, HCB and PBDEs analysis were conducted via GC-MS/MS (TQ-8050, Shimadzu). Separation was performed with a VF-5ms column (30 m, 0.25 mm i.d., 0.25 μm film thicknesses) for PAHs, and OCPs, VF5ht (15 m, 0.25 mm i.d., 0.1 μm film thickness) for all PBDE congeners and DB-5 (60m, 0.25 mm i.d., 0.25 μm film thickness) for PCBs. The oven temperature programs for POPs of the instrumental analysis were described in the supplementary data. The compound name, method detection limits (MDLs) and recovery of POPs analyzed in this study was listed in Table 4.2 and 4.3.

Table 4.2. Method detection limits (MDLs) and recovery (%) of PAHs analyzed in sediment samples in this study.

No.	Compounds	Initial	MDL (ng)	Recovery (%)
1	Naphthalene	Nap	19.2	65.3±8.9
2	2-Methylnaphthalene	2-MNap	14.3	71.3±8.7
3	1-Methylnaphthalene	1-MNap	1.9	62.8±8.3
4	2,6-Dimethylnaphthalene	2,6-MNap	0.4	66.7±9.4
5	1,3-Dimethylnaphthalene	1,3-MNap	0.8	66.8±8.7
6	1,6-Dimethylnaphthalene	1,6-MNap	1.1	68.7±6.3
7	1,4-Dimethylnaphthalene	1,4-MNap	0.4	66.6±8.4
8	1,5-Dimethylnaphthalene	1,5MNap	0.4	67.5±8.9
9	Acenaphthylene	Acy	0.3	67.9±8.9
10	1,2-Dimethylnaphthalene	1,2-MNap	0.7	69.0±8.7
11	Acenaphthene	Ace	0.8	67.9±8.8
12	Fluorene	Flu	1.0	73.5±9.1
13	1-Methylfluorene	1MFlu	0.6	75.9±6.0
14	Dibenzothiophene	DBT	0.5	79.1±6.4
15	Phenanthrene	Phe	4.9	82.8±7.8
16	Anthracene	Ant	0.7	77.3±5.2
17	2-Methylphenanthrene	2MPhe	4.5	82.8±9.2
18	2-Methylanthracene	2MAnt	0.5	74.5±5.1
19	4,5-Methylenephenanthrene	4,5-MPhe	1.4	80.1±6.4
20	1-Methylanthracene	1-MAnt	0.9	70.7±4.2
21	1-Methylphenanthrene	1MPhe	1.0	82.6±6.0
22	4,6-Dimethyldibenzothiophene	4,6-MDBT	1.6	70.8±2.5
23	Fluoranthene	Flo	4.3	83.5±8.0
24	Pyrene	Pyr	2.5	83.6±9.7
25	Retene	Ret	1.9	82.7±8.0
26	Benzo[a]fluorene	BaFu	0.8	80.7±7.1
27	Benzo[b]fluorene	BbFu	0.9	73.7±7.4

28	1-Methylpyrene	1MPye	0.7	81.9±9.1
29	Benz[a]anthracene	BaA	0.6	83.1±9.7
30/31	Chrysene+Triphenylene	Chr+TPh	0.6	85.4±9.6
32/33	4/6-Methylchrysene	4/6-NChr	0.9	92.7±9.5
34	Benzo[b]fluoranthene	BbF	0.5	94.3±9.5

Table 4.2 (Continued) Method detection limits (MDLs) and recovery (%) of PAHs analyzed in sediment samples in this study.

No.	Compounds	Initial	MDL (ng)	Recovery (%)
35	Benzo[k]fluoranthene	BkF	0.5	96.5±7.7
36	Benzo[e]pyrene	BeP	0.7	95.5±9.4
37	Benzo[a]pyrene	BaP	0.6	81.2±7.6
38	Perylene	Per	0.4	76.0±8.8
39	Indeno[1,2,3-c,d]pyrene	IP	0.5	77.4±7.9
40	Dibenz[a,h]anthracene	DA	0.4	84.5±9.9
41	Benzo[g,h,i]perylene	BP	0.4	84.7±10.5
42	Coronene	Cor	0.6	100.6±13.9

Table 4.3. Method detection limits (MDLs) and recovery (%) of PCBs, OCPs and PBDEs analyzed in sediment samples in this study.

Name	Cl-No.	MDL(pg)	Recovery%	Name	Cl-No.	MDL(pg)	Recovery%
#1	Cl-1	6.8	60.1±12.4	#70+76	Cl-4	162.5	83.7±15.8
#3	Cl-1	7.4	63.9±11.7	#74	Cl-4	41.1	79.8±17.9
#4+10	Cl-2	2.0	63.7±11.0	#77	Cl-4	31.0	74.5±18.7
#5+8	Cl-2	18.5	71.9±10.6	#82	Cl-5	24.0	76.9±14.0
#6	Cl-2	4.0	68.8±10.3	#84+92	Cl-5	115.7	64.3±12.2
#7+9	Cl-2	2.5	68.7±11.9	#85	Cl-5	41.5	76.1±16.5
#16+32	Cl-3	14.2	77.3±11.3	#87	Cl-5	111.5	81.6±8.1
#17	Cl-3	13.6	73.9±11.9	#89	Cl-5	57.7	82.6±12.6
#18	Cl-3	8.4	74.4±11.4	#91	Cl-5	25.3	72.1±19.4
#21+33	Cl-3	9.2	80.9±11.9	#95	Cl-5	51.4	84.5±11.6
#22	Cl-3	16.1	83.1±14.4	#97	Cl-5	19.6	90.6±10.9
#24	Cl-3	3.9	70.5±9.6	#99	Cl-5	14.4	83.1±18.4
#25	Cl-3	9.8	75±16.1	#101	Cl-5	14.3	82.4±20.1
#26	Cl-3	8.3	71.8±13.5	#110	Cl-5	9.9	82.4±11.0
#28	Cl-3	9.2	76.4±16.2	#118	Cl-5	12.3	77.9±18.8
#31	Cl-3	10.2	75.7±14.8	#132+153	Cl-6	31.5	83.7±13.0
#37	Cl-3	9.9	87.4±12.2	#136	Cl-6	12.2	76.3±10.3
#40	Cl-4	22.9	84.2±15.6	#138+163	Cl-6	25.2	79.4±15.0
#41+64+71	Cl-4	101.8	86.8±8.3	#141	Cl-6	38.1	83.1±11.5
#42*	Cl-4	15.7	82.9±13.5	#149	Cl-6	19.3	78.2±15.9
#44	Cl-4	17.3	84.7±9.5	#151	Cl-6	21.8	80.7±13.9
#45	Cl-4	14.9	79.1±11.5	#170	Cl-7	30.3	85.3±18.6
#46	Cl-4	21.0	75.2±12.9	#171	Cl-8	36.1	84±12.2
#47	Cl-4	152.7	82.8±18.6	#174	Cl-7	26.5	80.4±16.5
#48	Cl-4	15.3	80.2±10.0	#176	Cl-7	25.4	75.7±17.5
#49	Cl-4	11.7	77.6±14.6	#177	Cl-7	32.3	78.5±21.1
#51	Cl-4	37.7	79.3±9.4	#178	Cl-7	39.5	83±18.0

#52	Cl-4	12.1	78.1±13.3	#180	Cl-7	26.2	85.7±14.9
#53	Cl-4	29.7	78±12.4	#182+187	Cl-7	32.2	84.7±15.3
#56+60	Cl-4	18.4	89.7±13.3	#183	Cl-7	33.6	85.9±15.5
#63	Cl-4	16.8	87.4±13.7	#185	Cl-7	34.9	80.6±8.9
#66	Cl-4	11.5	82.8±14.5	#190	Cl-8	108.6	86±9.5

Table 4.3 (Continued) Method detection limits (MDLs) and recovery (%) of PCBs, OCPs and PBDEs analyzed in sediment samples in this study.

Name	Cl-No.	MDL(pg)	Recovery%	Name	Br-No.	MDL(ng)	Recovery%
#193	Cl-8	31.0	79.1±16.2	BDE02	Br-1	0.02	881.±23.4
#194	Cl-8	25.0	79.1±11.6	BDE15	Br-2	0.06	92.8±13.1
#195	Cl-8	50.1	78.7±20.1	BDE17	Br-3	0.46	106.5±9.2
#196+203	Cl-8	52.3	85.2±16.4	BDE28	Br-3	0.50	103.6±9.7
#201	Cl-8	47.9	87±16.9	BDE47	Br-4	0.26	107.0±7.4
#202	Cl-8	22.1	84.4±20.1	BDE66	Br-4	0.26	108.8±5.1
#205	Cl-8	42.4	87.3±3.6	BDE71	Br-4	0.35	105.1±9.7
#206	Cl-9	66.3	82.3±15.7	BDE85	Br-5	0.92	117.6±16.3
Name		MDL(ng)	Recovery%	BDE99	Br-5	0.34	107.7±7.1
Hexachlorobenzene (HCB)		0.5	72.3±11.2	BDE100	Br-5	0.54	105.5±10.7
o,p'-DDE		0.1	86.3±11.6	BDE138	Br-6	0.81	106.7±27.6
p,p'-DDE		0.1	85.6±10.1	BDE153	Br-6	0.53	104.0±17.5
o,p'-DDD		0.1	86.1±10.1	BDE154	Br-6	0.63	98.9±11.4
p,p'-DDD		0.1	83.4±12.0	BDE183	Br-7	0.47	105.9±27.6
o,p'-DDT		0.1	73.4±12.9	BDE190	Br-7	0.39	88.0±24.0
p,p'-DDT		0.1	73.5±12.8	BDE203	Br-8	0.27	86.3±33.3
Coelution				BDE205	Br-8	0.83	74.0±17.1
				BDE206	Br-9	1.0	67.3±0.7
				BDE209	Br-10	3.53	97.4±2.2

4.2.4 Particle size and total organic carbon determinations

The measurement of the total organic carbon (TOC) in the sediment from different station and depths was accomplished by the instrument Elementar Vario EL III. (Cheng et al., 2012; Jiang et al., 2011). For each sample three replicates was used to determine both the grain size and the TOC of the sediment from each station.

4.2.5 Quality assurance and quality control

The analytical procedure was carefully conducted by maintaining the standard quality assurance protocols. The glassware and apparatus that were used was washed initially with detergent and double distilled water followed by a rinse with acetone and hexane at each step of the analysis. In all the batches of analysis, procedural blanks, samples (in duplicate) and spiked samples were analyzed to assure the quality of the extraction and to detect any transmission of contaminants that might have occurred during analysis. In order to find out the recovery %, four PAHs surrogates, d8-napthalene, d10-fluorene, d10-fluoranthene, and d12-perylene, and three PCB surrogates, PCB14, PCB65 and PCB166, were added prior to extraction in all the tubes including blank. The average recoveries of spike were 62.8±13.5%, 84.2±10.7%, and 89.9±12.4%, 82.9±18.3% for PAHs and 73.9±14.5%, 83.3±16.8%, 84.0±17.3% for PCBs, respectively. The concentrations of POPs were not corrected for surrogate recoveries. The ranges of recovery of individual POPs from spike were 65.3% - 100.6% for PAHs, 57.2%-90.6% for PCBs, 64.4%-80.3% for HCB, 64.4%-94.6% for DDTs and 66.6%-118.8% for PBDEs, respectively. The method detection limits (MDLs) of POPs were defined as the average mass of each compound in the blanks plus three times the standard deviation. The mass of compounds below the MDLs were computed as zero. Quantification of POPs was done by the internal standard method. The relative standard deviation of relative response factor (RRF) was below 10%.

4.3 Results and Discussion

4.3.1 Spatial and temporal distribution of POPs

Fig 4.2a shows the concentration of different POPs in the sediments of 5 sampling stations around Changhua County that were collected in two seasons, namely the wet (August) and the dry season (January). Out of the 5 POPs series that were analyzed, PAHs, PCBs, DDTs and PBDEs were detected in all the samples except HCBs in Station A and E. The station C showed the highest concentration of t-PAHs (332.95 ng/g dw), t-PCBs (4.34 ng/g dw), t-HCBs (0.18 ng/g dw) and t-PBDEs (58.36 ng/g dw) and in Station D concentration of t-DDTs was the highest (2.63 ng/g dw). In comparison to Station C and D, Station A and E had much lower concentration of all the measured t-POPs. This indicated site-specific pollution levels at stations C and D, probably due to their proximity to the industrial park of Changhua. In spite of the fact that previous studies reported higher concentration of POPs in dry season and lower concentration in wet season -probably due to the dilution effect of the rain during the monsoon (Doong et al., 2002b, Adedayo O. Adeleye et al., 2016)- the concentration of POPs in the present survey did not show statistically significant differences between seasons.

Fig 4.2b shows the total concentration of all the POPs that were estimated in three depths of sediment cores (0cm-surface, 25cm-middle, 50cm-deep) considering the 5 sampling stations at the Changhua County. Results showed no significant differences between the concentrations of POPs at different depths. The spatial distribution for the individual concentration of all the POPs was site specific and influenced by the land use/cover (industrial).

Amongst all studied POPs, PAH showed the highest levels ranging from 19.96 to 332.95 ng/g dw, followed by PBDEs, PCBs and HCBs, and these levels were statistically higher at stations C and D for all the compound series, thus indicating an industrial hotspot area.

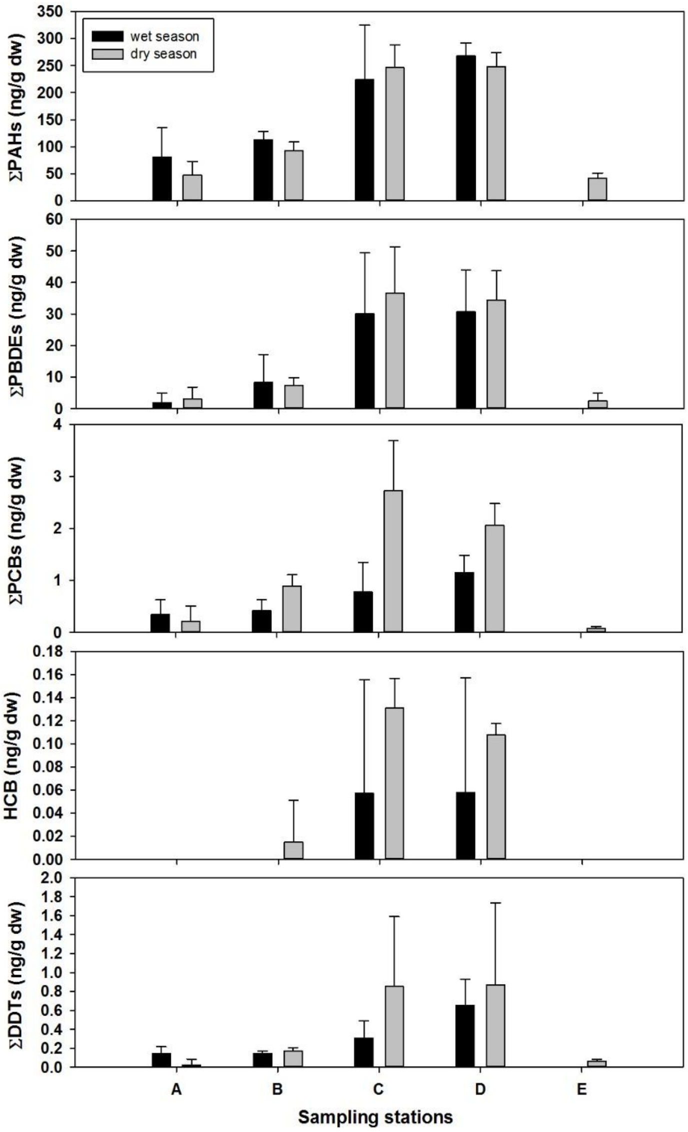

Figure 4.2a. Seasonal variation of POPs in different stations (A, B, C, D, E).

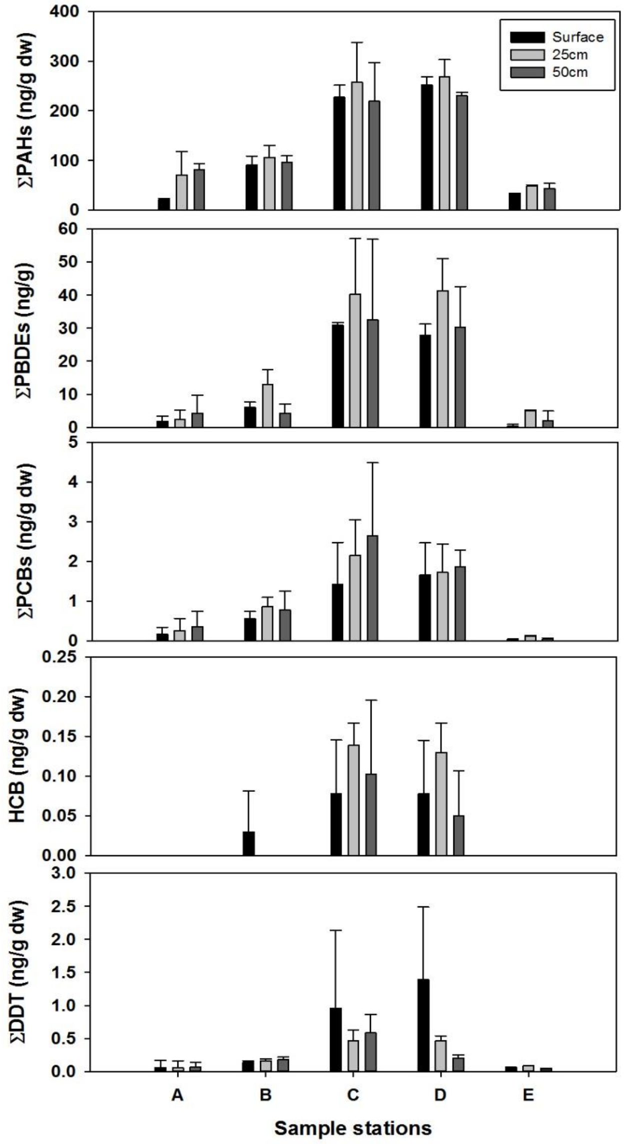

Figure 4.2b Total concentration of POPs in different stations (A, B, C, D, E) and depth (0cm, 25cm, 50cm).

4.3.2 Comparison of POPs concentrations with other coastal regions and estuaries in the world.

The concentration of PAHs in the present study was in the similar range of Gao-ping estuary of South Taiwan (Doong et al. 2008, Doong and Lin 2004) (Table 4.4). Gao-ping River is considered to be highly contaminated with several tons of wastewater from the nearby petrochemical industries and from the adjacent developed cities in the south of Taiwan. The level of t-PAH in sediments in southeast Asian countries have been reported to be similar like the Xiamen Harbor, China, East China Sea, coastal regions of Singapore, etc. (Table 4.4). However, extremely high level of t-PAH has been reported in the sediments of the Haihe estuary, China, followed by the Pearl River Estuary, China and coastal regions of Korea (Table 4.4). In the other parts of the world, the t-PAH concentration that was recorded is considerably higher than in Taiwan. Previous studies in the bay of San Francisco have documented abnormally high concentrations of t- PAH (2944-29590 ng/g dw) in the sediments and also extremely high concentration of DDT (11-23330 ng/g dw) long time ago (Table 4.4). In the Bay of Bengal, t-PAH concentration is also higher than many Asian coastal areas but PCBs or DDTs was in the similar range of estuaries in Taiwan including the present study. The PCBs concentration recorded in Puerto Rico, USA is much higher than many parts of the world like estuaries and coastal regions of Spain, China, Singapore, Korea, etc. The t-PCB concentration recorded in this study is in the similar range of South Taiwan, which is substantially lower than the other coastal areas of the world like Singapore, Korea, USA, China, Hong Kong etc. (Table 4.4). Apart from the outstandingly high concentration of DDTs in San Francisco Bay, Xiamen Harbor in China, Coast of Korea, Singapore and the Western Coast of India has also shown higher concentration of DDTs in a decreasing order. In this study, the DDTs concentration near the Industrial Park was much higher (almost 10 times) than the other parts of Changhua County. The concentration of DDTs in the industrial park was slightly higher than the Gao-ping estuary in South Taiwan, but conversely it is much lower when compared to the ranges of the DDTs concentration across the globe. In this study we also analyzed the concentration of PBDE, which was detected in all the stations and in both the seasons. The t- PBDE concentration ranged from 0-58.36 ng/g dw. This range of t- PBDE was similar, yet higher than the Macao coast in China where the reported range was from 0.6-41.3 ng/g dw (Mai et al. 2005). In this study we found that the discharge of PBDE in the industrial park was much higher than the other parts of Changhua County including the southern part of the western coast (station E) where the t-PBDE concentration was below the detection limit. Previous studies in Taiwan reported similar range of t-PBDE in Danshui River (1.4-52.7 ng/g dw) which is considered to be polluted due to its heavy drainage from the capital city of Taiwan, Taipei and the adjacent industrial areas from the other metropolitan cities (J.-O. Cheng, F.-C. Ko 2018). Keelung River being one of the tributaries of Danshui River, is also highly polluted and has been addressed as the habitat where the aquatic species are heavily impaired by certain toxic compounds (J.-O. Cheng, F.-C. Ko, 2018). Recent study shows that the highest recorded t-PBDE concentration in Taiwan is in

Keelung River with the range (3.9- 96.1 ng/g dw), which is much higher than that of the Changhua Industrial Park (J.-O. Cheng, F.-C. Ko, 2018). Also the above study shows that the Wenzichuan Stream of Taiwan has an extremely high concentration of particularly BDE209 with the range being 814.9-10,464.1 ng/g dw. Collectively, the concentration of t-PBDE has been found to be much higher in the industrial parks and rivers beside the mega cities when compared to other coastal areas and rivers of Taiwan where the range was in between 0.58-12.15 ng/g dw (J.-O. Cheng, F.-C. Ko, 2018). This study integrates the information of t-PBDE concentration of the western coast of Taiwan and marks the concern for the pollution control. Worldwide, the concentration of t-PBDE was found to be extremely high in Osaka, Japan (8-352 ng/g dw), in the rivers and estuaries of United Kingdom (UK) especially near a manufacturing factory for flame retardants in Tees Estuary (1.3- 1270 ng/g dw) and specifically in the San Francisco Bay of USA (ND-212 ng/g dw) (Ohta et al. 2002, Allchin et al. 1999, Oros et al. 2005). In the other coastal areas of the World including China, South Korea, Japan (Tokyo Bay), Spain, Portugal, USA (Great Lake), the concentration of PBDE in sediments is found to be lower than 50 ng/g dw (Mai et al. 2005, Pan et al. 2007, Moon et al. 2007, Minh et al. 2007, Song et al. 2004, 2005a, b, Eljarrat et al. 2005, Lacorte et al. 2003). However, our study shows higher concentration of t- PBDE in the sediment of Changhua Industrial Park when compared with the coastal areas and rivers worldwide.

Table 4.4 Comparison of POPs reported in other studies with the present study

Locations	∑ **PAH** (ng/g dw)	∑**PCB** (ng/g dw)	∑**DDT** (ng/g dw)	**Reference**
Xiamen Harbor, China	247–480	0.05–7.2	4.5–311	Hong et al. (1995) ; Zhou et al. 2000
Haihe Estuary, China	775–255372	ND–36.10	ND–0.34	Jiang Bin et al. (2007); Zhao et al. (2010)
Pearl River Estuary, China	138–1100	-	1.38–25.4	Chen et al. (2006) ; Hong et al. (1995)
ECS and its estuaries	32.10–71.10	ND–63	ND–5.10	A.O. Adeleye et al. (2016)
Victoria Harbor, Hong Kong		3.2–27	1.4–30	Connell et al. (1998)
Coastal region, Singapore	12.65-93.85	1.40–330	3.40–46.10	Wurl and Obbard (2005); Basheer et al. (2003)
Coast of Korea	9.1-1400	0.17–371	0.01–135	Hong et al. (2006); Kim et al. (1999)
Gao-ping Estuaries, Taiwan	1.43–356	0.38–5.90	0.44–1.88	Doong et al. (2008), Doong and Lin (2004)
Western Coast of Taiwan	**19.96-129.66**	**0.04-1.11**	**0.03-0.21**	**This study**
Changhua Industrial Park	**135.15-332.95**	**0.24-4.34**	**0.106- 2.63**	**This study**
Northeastern coast of India	-	0.18–2.33	0.18–1.93	L. Guzzella et al., 2005
Bay of Bengal, India	20.35-2615.38	0.02–6.57	0.04–4.79	Rajendran et al. (2005); Binelli et al. (2008)
West coast of India	-	-	1.47–25.17	Sarkar et al. (1997)
Bay of Biscay, France	0.7–300 (mg/kg, dw)	ND–375	-	Bartolomé et al. (2006)
The Baltic Sea	9.5-1900	0.01–6.20	0.13–0.50	Witt (1995); Pikkarainen (2007)
Cantabrian Sea, Spain	19–2123	ND–160	4.2–25	Sánchez-Avila et al. (2013)
San Francisco Bay, CA	2944–29590	-	11-23330	Pereira et al. 1996
Guánica Bay, Puerto Rico, USA	0.64–4663	14 0.11–3059.90	0.00–69.25	Whitall et al. (2014)
Bahia Blanca Estuary, Argentina	15-10260	0.61-17.6	ND-2.3	Arias et al, 2009, Tombesi et al, 2016; Arias et al., 2010

4.3.3. Compositional profiles

4.3.3.1. PAHs

PAHs ratios are traditionally used to determine PAHs sources classify samples by location and estimate the importance of combustion and petroleum derived PAHs (Lipiatou & Saliot 1991, Yunker et al. 1999 and 2001, Budzinsky et al. 1997). The usual index of combustion and/or anthropogenic input is an increase in the proportion of the less stable and/or kinetically produced parent PAH isomers relative to the thermodynamically stable isomers; e.g., fluoranthene relative to pyrene, or to the molecular mass totals (Yunker et al. 2001). Index calculations are traditionally restricted to PAHs within a given molecular mass to minimize factors such as differences in volatility, water/carbon partition coefficients, adsorption (Mc Veety & Hites 1988) and in most cases appear to closely reflect the source characteristics of PAHs (Yunker et al. 2002).

Primarily, the proportions of fluoranthene to fluoranthene plus pyrene (Fl/202) and indeno-[1, 2, 3-cd]pyrene (IP) to IP plus benzo[ghi]perylene (IP/276) (Figure 4.3) were used. Fl/202 ratios less than about 0.40 usually indicate petroleum (oil, diesel, and coal), between 0.40 and 0.50 indicate liquid fossil fuel (vehicle and crude oil) combustion, while ratios over 0.50 are attributable to grass, wood or coal combustion. Similarly, IP/276 ratios less than approximately 0.20 imply petroleum, between 0.20 and 0.50 liquid fossil fuel (vehicle and crude oil) combustion while ratios over 0.50 are attributable to grass, wood or coal combustion (Yunker et al. 2001). Further, these two parent PAHs ratios are supplemented by anthracene (An) to An plus phenanthrene (An/178) (Figure 4.3). An/178 ratios < 0.10 are usually taken as an indication of petroleum, while ratios > 0.10 indicate combustion (Budzinsky et al. 1997; Soclo et al. 2000; Yunker et al. 2001).

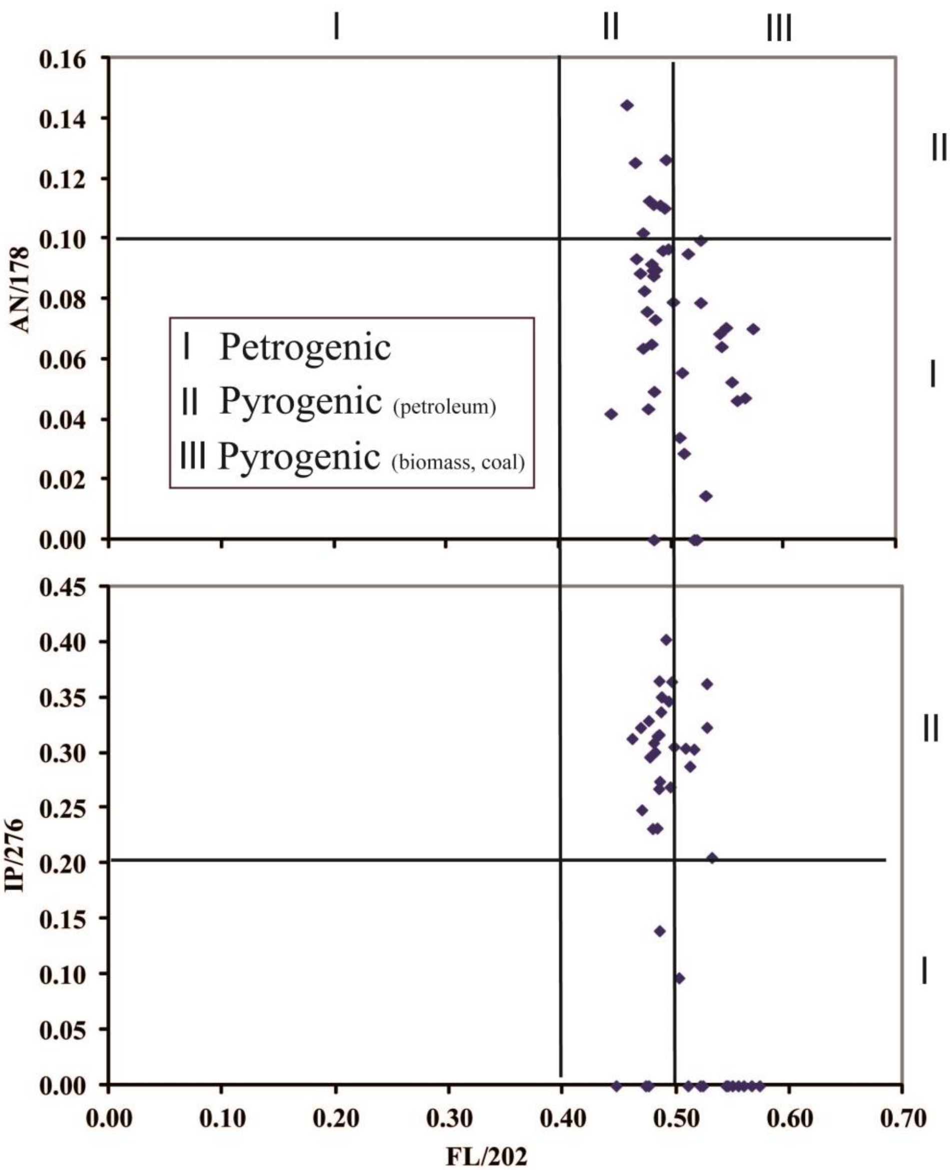

Figure 4.3 Comparison of selected PAHs ratios for 42 sediment samples along the area of study. Abbreviations refer to the ratios of fluoranthene plus pyrene (Fl/202), indeno-[1, 2, 3-*cd*]pyrene (IP) to IP plus benzo[*ghi*]perylene (IP/276) and anthracene (An) to An plus phenanthrene (An/178)

Results showed mixed origins for the 42 samples analysed; while sediment samples presented a mean Fl/202 ratio of 0.50 ± 0.03 (n=42) and an IP/276 mean ratio of 0.25 ± 0.12 (n=35) indicating a pyrogenic source impacting the area, the mean An/128 ratio was 0.07± 0.03 (n=42), pointing to petrogenic inputs. In order to deepen this analysis a PCA was performed.

Principal Components Analysis (PCA) allowed to extract underlying common factors (principal components, PCs) for analyzing relationships among the observed variables. As a result of an effective extraction process, PC1 accounted for the major proportion of the total data variance while the second and following PCs progressively explained smaller amounts of data variation. Prior to analysis, values under the Limit of Detection or Minimum Detection Limit (MDL) in the data set were replaced with random values under the MDL value. Concentrations of 40 PAHs as active variables and 42 samples as cases were used. The number of factors extracted from the variables was determined according to Kaiser´s rule, which retains only factors with eigenvalues that exceed one. As performed in other studies; e.g., Golobocanin et al. 2004, a way of factor rotation to get as many positive loadings as possible to achieve a more meaningful and interpretable solution was preferred (Varimax normalized).

The majority of the variance (88.27%) was explained by three principal components vectors. PC1 explained 73.13 % of the total variance and PC2 accounted for 6.16%, while PC3 explained 3.9% of the variance. Figure 4.4a shows the loadings for the individual PAHs at the principal components plot. Along the PC1 axis, almost all the compounds were found to have positive coordinate; indeed, PC1 had dominant correlations (> 0.7) with alkylated derivatives of PAHs which are markers of petrogenic origin. This component also included some pyrogenic markers such as Fluoranthene, Pyrene, benz[x]anthracene, and chrysene (coal combustion) and even retene, a marker of wood combustion (Simcik et al., 1990). In addition, PC1 compounds gave strong correlation with the Total PAHs concentration ($r > 0.90$; mean $r^2 = 0.91$) indicating PC1 as a mixed origin with over-imposition of petrogenic origin plus a quantitative correlation component.

Secondly, PC2 presented significant positive loadings for two 3-ringed PAHs compounds: Fluorene and Dibenzotiophene. Fluorene has been reported as a dominant PAH in the coke oven signature (Khalili et al. 1995) while Dibenzotiophene –tiophenes in general-has been signed as marker of diesel-powered vehicles (Duval and Friedlander, 1981). Such PAHs are the result of combustion/pyrolitic processes and are absent in crude oil or refined products. Consequently, PC2 was defined as a pyrogenic component including coke combustion and diesel motors exhaust.

Thirdly, PC3 presented a significant correlation with Indeno [1, 2, 3-c,d]pyrene a six fused ring compound which is a common marker of pyrolysis (gasoline exhausts, coal tar and soot). An overview of the principal components plot (Figure 4.4a) shows different PAHs clustering; PAHs in the three main clusters may be originated from different origin sources. As previously stated, the (2+3+4) ring cluster including all the complete alkylated PAHs series, with low loadings on PC2, is primarily a petrogenic cluster; however, it could either be named “mixed”. The other two clusters were defined as pyrogenic with diverse origin. Following these approaches, a portion of the total variance of PAHs concentrations is explained by source contribution, resulting in the petrogenic origin as the prevalent contribution over the sampled area. These results are consistent with our previous molecular ratios findings. Figure 4.4b shows the 2 D score plot of PC1 and PC2 axis. This allowed the characterization of the sampling stations according to the first and the second component. The plot revealed how the samples are related to each other given the measurements that have been made. In this context, we identified three major groups of samples distributed along the three axes. PC1 positive coordinates include 19 samples dominated by stations C and D. These samples are located in a petro-chemical industrial park wastewater discharge zone, an area defined above as a hotspot because of its total PAHs levels. The remaining samples can be divided into different PC2 and PC3 contributions achieving higher pyrolitic/combustion: while sites B and E mainly coordinated with PC2 (coke + diesel combustion), A and B did it with PC3 (gasoline, coal combustion)

In brief, PCA allowed us to separate PAHs compounds enabling the assessment of different PAH sources at the area; accordingly, it also allowed the classification of sampling sites. These facts are in excellent agreement with molecular ratios analysis seen on section “ratios”.

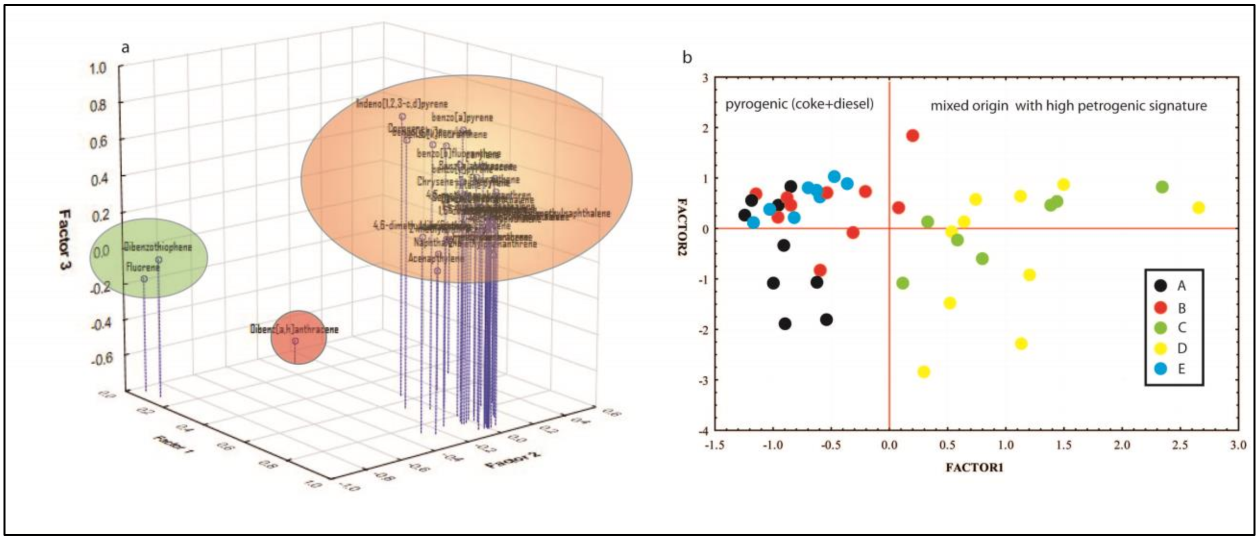

Figure 4.4a The PCA loading plot of sedimentary PAHs; b. Score plot illustrating the distribution of PAHs compounds in the sampled areas along PC1 and PC2 axis

Finally, as shown in Figure 4.4b, stations C and D represented marked petrogenic PAHs inputs. This outlines that PAHs inputs at the most impacted locations in Changhua County, consisted mainly petrogenic inputs, probably not due to combusted oil and petroleum derivatives.

4.3.3.2. Organochlorines

Commercial grade DDT generally contains 75% p, p'-DDT, 15%, p'-DDT, 5% p, p'-DDE, <0.5% p, p'-DDD, <0.5%, p'-DDD, <0.5%, p'-DDE and <0.5% unidentified compounds (WHO, 1979). DDT-isomers have a long persistence in the environment, gradually degrading to DDE and DDD under both aerobic and anaerobic conditions. In general, the pattern of DDT and its metabolites for sediment samples was in the order of DDT >DDD/DDE indicating quite recent inputs of commercial DDT to the environment. Despite this, different spatial trends were identified: for the north (catchment of city sewage outlet and harbours) there were medium DDT levels (0.17 ng/g dw)) and the concentration pattern for its derivatives was DDT >DDD> DDE. On the opposite, for the southern zone (reserve), DDXs levels were the minimum recorded (from

0.02 to 0.06 ng/g dw), while the industrial stations C and D showed the maximum levels (>0.8 ng/g dw). The dominance of DDTs in C and D sediments, plus the maximum concentration achieved in top layer, indicates slow degradation of DDTs or recent inputs of fresh DDT at these locations (Tavares et al., 1999; Yuan et al., 2001).

4.3.3.3. PCBs

Concentrations of PCBs in worldwide comparison were relatively lower (< 4 ng/g dw). PCBs levels were maximum in C and D, lower in A and B and the lowest in E, in agreement with each land use/cover. The major compounds found in the area were congeners 132/153, 31, 28 and 5+8. Comparing the pattern of % of chlorinated compounds with the average known Aroclor mixes (UNEP), considering the five sampling sites as one, a mixed pattern involving Aroclor 1016 and 1260 was found.

4.3.3.4. PBDEs

The concentration of PBDE (Σ10) in Changhua County ranged from 0-57.6 ng/g dw with BDE-209 being the most abundant congener in all the stations and in both the season (dry and wet). The highest concentration of PBDE was found in Station C (57.60 ng/g dw) in the deepest layer (50cm) of sediment in the dry season (January). This result was in accordance with the studies in the other part of world which accounted for greater concentration of POPs in the dry season than in the wet season and also higher PBDE concentration near various chemical industries (Doong et al., 2002b, Moon et al., 2007; Pan et al., 2011; Adedayo O. Adeleye et al., 2016; Cheng and Ko; 2018). The PBDE concentration was higher in the stations within the industrial parks (Station C and Station D) having closer proximity to the source point of the contaminants. Similar to the other POPs, the concentration of PBDE in Station A, B and E was low, although the top layer of Station A showed the presence of various PBDE congeners like BDE-02, BDE-15, BDE-71, BDE-66, and BDE-99 in small quantities only in the dry season. In Station A, the concentration of BDE-209 was highest in the deepest layer (50 cm, 5.53 ng/g dw) in the dry season (January) and in middle layer (25cm, 5.38 ng/g dw) in the wet season (September). However, the concentration of BDE-209 congener was higher in the middle layer of station B (10.23 ng/g dw) and E (4.99 ng/g dw) in the dry season. BDE-209 being the most abundant congener composed of almost 85-90% of the total PBDE concentration in all the stations and was dominant in both the season. The highest BDE-209 concentration in both the season was recorded in Station C with the values being 57.60 ng/g dw at 50cm in dry season and 47.89 ng/g dw at 25 cm in wet season. Similar results have been published by previous studies, where BDE 209 was found to be the most abundant congener and accounting for almost 90-100% of the composition of entire PBDE undoubtedly showing highly variable concentrations throughout the world (Voorspoels et al., 2004; Chen et al., 2006 **;** Moon et al. 2007; Pan et al., 2011; Pozo et al., 2015 ; Cheng and Ko; 2018). Recent study demonstrated the high commercial usage of Br-10 mixtures in Northern Taiwan, which is in accordance with the data of the present study showing higher deca-BDE mixtures also in the west coast of Taiwan, which lies close to the industrial park. The low brominated BDE found in the top layer of Station A might be the process of debromination of higher brominated congeners of PBDE (Moon et al., 2007; Cheng and Ko; 2018).

4.3.4 The concentrations of POPs related with sediment characteristics

The TOC of the sediment is often considered as a prime factor for the distribution of POPs in the a particular area and are widely compared in the studies related to organic contaminants (Hung et al., 2006; Hung et al., 2007; Hung et al., 2010; Yang et al., 2011; Gao et al., 2013; Cheng and Ko; 2018) Persistence of POPs in aquatic sediments is due to their low rate of degradation and vaporization, low water solubility, and partitioning to particles and organic carbon (Kennish, 1992). To test this in the present study, a correlation between each POP level and % TOC was assessed (Figure 4.5)

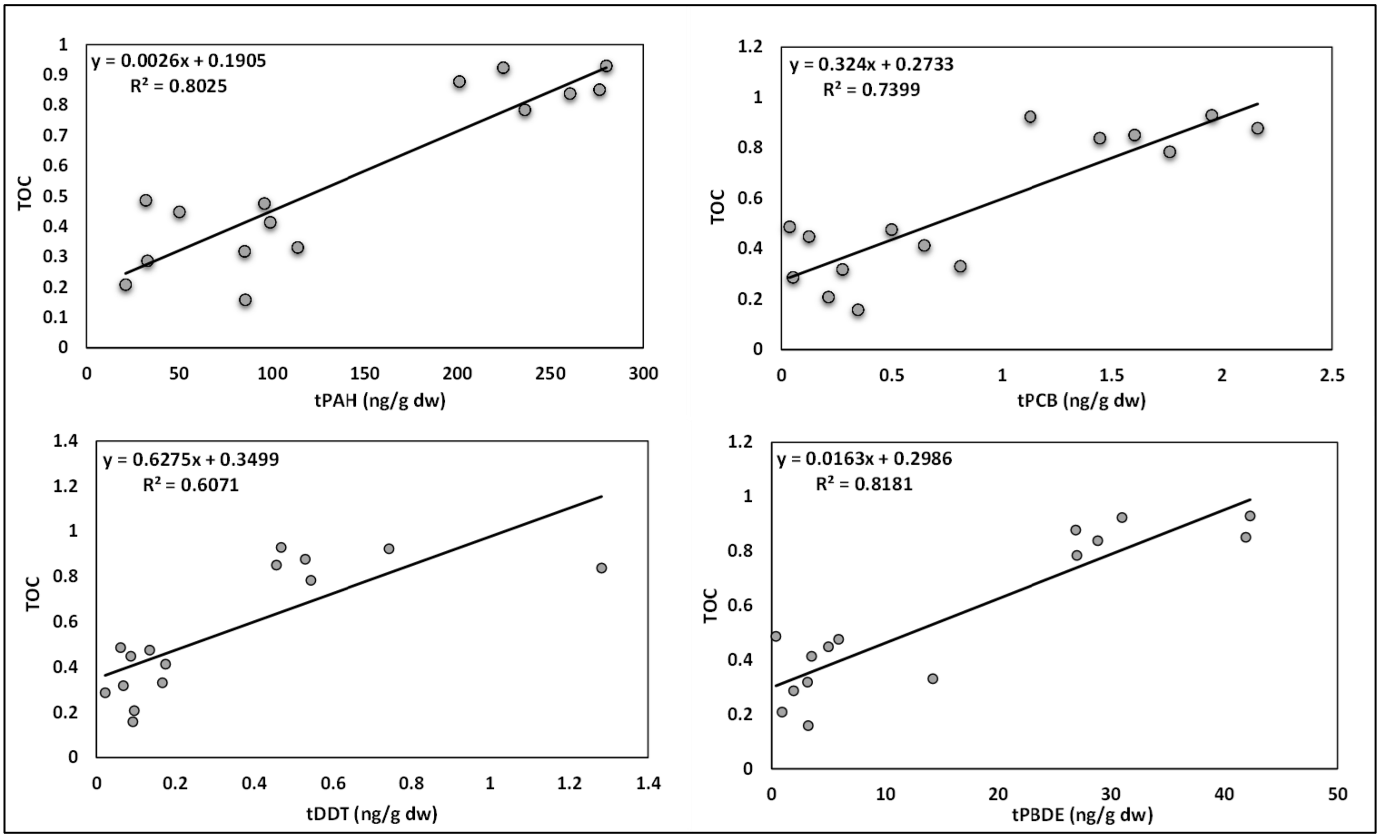

Figure 4.5 The relationship between TOC (%) and t-PAH, t-PCB, t-DDT, t-PBDE in ng/g dw.

In the present study the range for the contents of TOC was from 0.158- 0.929. The lowest TOC content was recorded in Station A and the highest in Station C both in the middle layer (25cm) of the sediment column. A strong and significant correlation between each POP (t-PAH, t-PCB, t-DDT and t-PBDE) with TOC % was noticed in this study. Previous studies have reported similar findings when TOC and POPs concentration were compared (Doong et al., 2008; Lee et al., 2014; X.T. Wang et al., 2015; Zhao et al., 2012, Cheng and Ko; 2018). As shown in figure 5, the relationship between TOC and t-PAH, t-PCB, t-DDT and t-PBDE showed a significant correlation with r^2 = 0.8, p value <0.01; r^2 = 0.73, p value <0.01; r^2 = 0.6, p value <0.01 and r^2 = 0.81, p value <0.01respectively. Hence, TOC content can be considered as a useful tool to assess or surveil the concentration of diverse organic contaminants in sediments (Doong et al., 2008; Lee et al., 2014; Cheng and Ko; 2018).

4.3.5 Ecological risk assessments of POPs in sediments

A standard for the concentration of individual POPs in seafood and sediment is still lacking in Taiwan. However, previous studies have used the yardstick or the general rule presented by Long et al., 1995 from the Canadian councils of Ministers of the Environment (CCME, 2002). In the above-mentioned report the Threshold Effect Level (TEL) and the Probable Effect Level (PEL) was acquired to estimate the risk of POPs in sediments and benthic organisms. This standard criterion represents the limit above which (with 50% frequency, “Effects Range-Median”) the concentration will be considered as toxic and below the 10% frequency (“Effects Range-Low”), the concentration will cause rare detrimental effects. In comparison with the sediment quality guidelines (SQG) set by Long et al., 1995, it is evident that none of stations exceeded the POPs concentration set by the standard guidelines (Table 4.5).

Table 4.5 Comparison of standard values set for toxicity (SQG and CCME values ng/g, dw) with the concentration of POPs in sediment from this study.

SQG (Long et al., 1995) and CCME (2002)					This study				
	ERM	**ERL**	**PEL**	**TEL**	**Station A**	**Station B**	**Station C**	**Station D**	**Station E**
t-PAHs	44,792	4022	6676	655	58.42	99.20	238.93	254.38	41.80
t-PCBs	180	22.7	189	22	0.25	0.73	2.07	1.75	0.07
t-DDTs	46.1	1.58	4.77	3.89	0.06	0.16	0.67	0.79	0.06

However, the value of t-DDTs concentration in station C and station D is closer to the standardized ERL values. This can be explained by the use of pesticides for the nearby agricultural use and also the closer proximity of these two stations with the industrial park. In fact, the concentration of t-PAHs and t-PCBs are also higher in Station C and D from all the other areas sampled but much lower than the standardized guidelines. Therefore, the concentration of POPs in the sediment of the western coast of Taiwan including the Changhua Industrial Park can be assumed to have rudimentary or marginal effects on the benthic organisms.

4.3.6 Bioaccumulation of POPs in the mud shrimp

Austinogebia edulis is a common seafood consumed by the locals of the western coast of Taiwan and it has high economic importance. Although it is consumed all over Taiwan but this shrimp can only be found in the western coast. The egg-bearing females are in demand with higher market values during the season because it is a delicacy in the Central part of Taiwan. The concentration of each measured POPs in *A. edulis* has been listed in Table 4.6. Out of the five stations (A, B, C, D and E) only two stations (A and E) showed the presence of mud shrimps. The concentration of t-PAH was higher when compared to the other POPs concentration and was predominant. Hexachlorobenzene (HCB) was not detected in any of the samples that were tested. The concentration of all the POPs tested was found to be much higher in Station E than Station A. The results can be explained as, since Station A is a restricted mud shrimp conservation area under the Government of Taiwan, so the level of contamination by organic pollutants are comparatively lower than in surrounding unrestricted and open areas.

Table 4.6 The total concentrations of POPs in Shrimp from station A and E.

Station	**t-PAHs**	**t-PCBs**	**t-PBDEs**	**t-DDTs**	**HCB**
A	61.9	0.5	1.2	0.4	n.d
E	94.1	3.9	2.2	1.1	n.d

n.d: lower than MDLs

The individual bioaccumulation factor (BAFs) for each pollutant in Station A and E was calculated by using the equation (1) and listed in Table 4.5.

$$\text{BAFs} = \frac{\text{Concentration of POPs in mudshrimp}}{\text{Concentration of POPs in sediment}} \qquad \text{…………. (1)}$$

Table 4.7 The bioaccumulation factor of mud shrimps from station A and E.

Station	**BAF (t-PAH)**	**BAF (t-PCB)**	**BAF (t-PBDE)**	**BAF (t-DDT)**
A	1.060	1.936	0.459	6.190
E	2.250	54.59	0.888	17.325

The BAFs in the mud shrimp *A. edulis* was found to be much higher in Station E than in Station A. Higher values of BAFs was seen for t-PCB and t-DDT in Station E (Table 4.7). In order to implement a tool for the regulation of contaminated habitats or areas, and for the assessment of potential risk to benthic animals, the calculation of BAFs have been proved a useful approach (Parkerton, et al., 1993; Nakata et al., 2003). *Austinogebia edulis* are burrowers of muddy substratum and they get exposed to the sediment column actively throughout their entire life span. We emphasize this point, since previous literatures documented that BAFs values are highly variable and dependent of various parameters, like direct contact or exposure of the organisms with the sediment column, amount of the residue present in the sediment etc. (Hawker, et al., 1985; Froese et al., 1998; Nakata et al., 2003; Iannuzzi et al., 2011; Chang, 2017). Hence, a higher BAF value even for a hydrophobic compound like PCB is quite a possible circumstance in the body of a burrowing mud shrimp. Previous literature noted ranges of BAF for PAH in certain crabs and lugworms to be higher than in oysters and clams (Nakata et al., 2003). Several studies have accounted for varying BAFs for PCB and PBDE in fishes, crabs, mussels, shrimps etc. In fact, precisely the Chinese Mitten Crab showed the highest value of 2900 of Biota-Sediment Accumulation Factor (BSAF) for PCB which was followed by the shore crab 2330 (BSAF PCB) in the Scheldt estuary of Netherlands–Belgium (Van Ael et al., 2012). In the same site the value of BSAF PCB in the brown shrimp (606), blue mussels (1090), and worms (1180) were found to be comparatively lower (E. Van Ael et al., 2012). However, the BSAF PCB for the Blue crab (0.22-1.7) and the white perch fish (0.34-1.5) in the Passaic River, USA accounted for minute values of bioaccumulation factor (Iannuzzi et al., 2011). The BSAF for PBDE in the Scheldt estuary was also higher in Chinese mitten crab (2520) and shore crab (598) but in comparison was much lower in brown shrimp (162) and Blue mussels (304). The BSAF PBDE found in the northern horse mussel of the Vancouver Island, Canada varied from 0.95- 527 (deBruyn et al., 2009). Clearly, the results of the present study showed much lower values of BAFs for each measured POPs when compared to other global studies. The variations in the results can be accounted for the different trophic mode or the modes of ingestion amongst the benthic biota. For the lower molecular weight isomers in PAH like the PHE and AN has been reported to absorb compounds from the interstitial water directly, whereas heavy compounds adsorb on particulate matter (Baumard et al., 1998; Nakata et al., 2003). Therefore the several bioaccumulation pathways for different aquatic and intertidal organisms differs greatly depending on the size of the compounds, modes of ingestion, absorption through skin and as well as the ability of the organisms to bio-transform or bio-degrade the compounds that they often get exposed from their surrounding sediment or water.

Section-2 Micro crustacean copepods

Chapter 5 – Effects of sediment in re-suspension and mixture of heavy metals on the calanoid copepod *Eurytemora affinis*- a multi generation approach.

5.1 Introduction

The term ecotoxicology is often defined as the stream, which analyses the effect of toxic substances on the ecosystem with the help of the standard techniques developed from several domain of ecology (Zakrewski, 1991; Camargo et al., 2015). These toxic substances can belong or arise from a huge spectrum of sources with multiple origins. In short, they arise and they affect the organisms in the ecosystem sometimes leading to severe consequences of pollution, bioaccumulation, bio-integration to tertiary organisms, reduction in population density of some organisms, serious contamination of sediments, water column, air column, etc. Generally, such issues are dealt with regular monitoring of the sediments, water and air quality in heavily industrialized area where the loading of contaminants are much higher and can pose a significant threat to the nearby organisms. Seine estuary is one such natural habitat, situated at the interface between land and sea, which is heavily contaminated by different loads of toxic substances every year (Cailleaud, et al., 2007a and b). Apart from major shipping routes, Seine estuary also holds on to several industrial parks and enterprises, human intervention and other commercial activities for almost 5 decades (Dauvin, 2008; Zidour, et al., 2019). Being the largest mega tidal estuary along the English Channel, it has been one of the major concerns of many research groups and environmental programs, which primarily led to a decrease in fishing activities along the estuary (Minier et al., 2006). Therefore monitoring this area by implementing several ecotoxicological techniques and using various organisms, as bioindicator species is a necessary step towards conservation. A commonly used bioindicator organism is copepod, which is often used in the toxicological studies due to their crucial and important relations with the other taxonomic groups. It is known to be one of the dominant groups in the zooplankton community and often acts as a fundamental link between phytoplankton or primary producers, pico or bacterioplanktons thereby also combining the secondary producers of higher trophic levels (Sei et al., 1996; Buttino et al., 2018). The copepod *Eurytemora affinis* is a dominant zooplankton species encountered in the Seine estuary (mainly salinity gradient zone) covering up to 90% of the zooplankton abundance and has been established as an appropriate indicator species in several toxicological studies (Devreker et al., 2010 Souissi et al., 2015; Lesueur et al., 2015; Souissi et al., 2016; Kadiene et al., 2017; Zidour et al., 2019). However, in such intertidal flats or estuarine coasts, sediment plays an important role in the transfer of pollutants and as well as degeneration of many heavy toxic compounds with the contaminants often getting precipitated by physical or chemical processes in nature (Pereira and Soares-Gomes, 2002; Seriani et al., 2006). In fact sediments are often considered as a pool of contaminants, which when in re-suspension can release the pollutants into the water column thereby indirectly affecting several aquatic organisms and accumulating continuously via the food chain (Hack et al., 2008; Leuseur et al., 2015). Previous studies showed that *E. affinis* can bioaccumulate organic contaminants like PCBs, PAHs and even trace metals like Cadmium, Copper etc (Cailleaud et al., 2007a, b; Leuseur et al., 2015; Zidour et al., 2019). Studies comparing the effect of whole sediment in re-suspension and sediment elutriates on *E. affinis* are however few. Therefore, on this context the primary objective of this study was to comprehend the effect of sediment in re-suspension and also to compare it with the effect of sediment elutriate (extract) when exposed to the copepod *E. affinis* over multiple generations by following Souissi et al., (2016) multigenerational protocol. To go deeper, the presence of contaminants around us is not just one contaminant, but an amalgamation of toxic compounds mixed in diverse ratios and distributed in patches over an area (Amiard-Triquet et al., 2008; Thevenot et al., 2009; Zidour et al. 2019). Trace metals like Cadmium (Cd), Copper (Cu), Nickel (Ni), Lead (Pb), Zinc (Zn), Iron (Fe), etc are all present in the sediments and water column, sometimes in huge quantities often when released from nearby factories and industries (Rollin and Quiot, 2006). There are studies reflecting the toxicity of a single compound and its effect on the nearby organisms but studies on the combined effect of heavy metals mixed in different concentration is not well documented (Kadiene et al., 2017; Biandolino et al., 2018; Zidour et al., 2019). Along with our understanding of the effects of sediments on *E. affinis*, a tangential goal of this study was to analyze the effect of the combination of heavy metals when exposed to *E. affinis* over multiple generations. Some of these metals like Cu and Ni are essential elements and has role in the metabolism in several organisms when present in low concentrations (Simkiss and Taylor, 1995; Zidour et al., 2019). Although if the exposure exceeds a certain concentration along with loads of other contaminants which are non-essential elements can produce considerable changes in physiological, morphological and reproductive characteristics and even threaten the overall population size of the nearby organisms (Verschoor et al., 2012; Jaishankar et al., 2014; Berasategui et al., 2018). According to recent studies, such elements irrespective of being essential or non-essential can potentially bioaccumulate in the organisms and can further biomagnify to higher trophic levels (Mohammed et al., 2011; Pavlaki et al., 2017; Zidour et al., 2019). Assessment of sediment quality or the effect of different metallic concentration on bioindicator species like *E. affinis*, can give a comprehensive idea of the overall stress encountered by the nearby organisms of the Seine estuary.

5.2 Material and methods

5.2.1 Culture of copepods

The culture of *E. affinis* was maintained in the laboratory that was previously collected from the Seine estuary. Laboratory conditions were maintained according to Souissi et al., 2016, under a photoperiod of 12:12 h light/dark cycle, with a salinity of 15 and the temperature of water being (18-19) °C. Adult copepods of *E. affinis* in their late developmental stage was filtered by using 200µm mesh and cultured in an acrylic tank (300 L) with filtered seawater (1 µm) for nearly 2 months before the experiment in order to acclimatize them in the laboratory conditions and omit the impression from the past generations. The copepod culture was fed with the microalga *Rhodomonas marina* ([(P.A. Dangeard) Lemmermann, 1899) in every alternate days (Arias et al., 2016).

5.2.2 Experimental conditions

Collectively, there were three experimental conditions used to accomplish the experiment, which are described below in detail. The three different exposure conditions were the analysis of the effect of a mixture of heavy metals, the effect of the use of whole sediment in re-suspension and the effect of extracted sediment or commonly termed as elutriate on the several life traits of the copepod *E. affinis.*

5.2.2.1 Mix of heavy metals

A mixture of four heavy metals, namely, Cadmium (Cd), Copper (Cu), Nickel (Ni) and lead (Pb) was used to expose the copepods. The lethal concentration (LC) for each metal was noted (described below) and 10% of the LC50 concentration for each metal was used in the experiment. All the chemicals were obtained from LASIR Laboratory, University of Lille (France). The final volume of each stock solution was adjusted according to the 2L beakers (1800ml seawater, salinity 15) that were used for the exposure of copepods following the multigenerational approach.

5.2.2.2 Whole sediment

Sediment from the surface was sampled from the Seine estuary and prior to the exposure of copepods, basic characterization of sediment was done. The sediment was made homogenous by the elimination of very few large shells, cobbles and pebbles that were present. The grain size of the exposed sediment was <149 microns (0.149 mm) which corresponds to the phi scale >3 (very fine sand to silt) according to the Wentworth grade scale. The entire process of exposing the whole sediment to copepods directly was followed by Camargo et al., 2015. In the beaker, 0.45g of sediment was added along with 1800ml of autoclaved seawater. After testing a range of different concentration of sediment on *E. affinis*, we chose 0.25g/l concentration, which showed an overall average (male and female) mortality of 30%. The sediment was re-suspended by using moderate aeration in the experimental tank for nearly 4 hours before introducing the copepods into the beaker. In all the replicates and in each generation thereafter, the sediment re-suspension was continuous, which allowed direct contact of the whole sediment and the copepods thereby increasing the chances of ingesting or absorbing dissolved chemicals through skin from the contaminated sediment (Anderson et al., 2001, Camargo et al., 2015).

5.2.2.3 Sediment elutriate

Elutriate from the sediment was prepared by collectively using the protocols described by Harkey et al., 1994; USEPA, 2001; Buttino 2018. The sediment was mixed with filtered seawater in the ratio of 1:4 (weight: volume, adjusted to salinity 15) and then the mixture was shaken for

1h using an orbital shaker. For the particles to settle down, the mixture was allowed to stand for an additional hour. The unsettled fraction was further centrifuged for the clear separation of the two phases at 5100 x g for 20 min. The elutriate was finally decanted and stored for no longer 10 hours at 4°C before analysis.

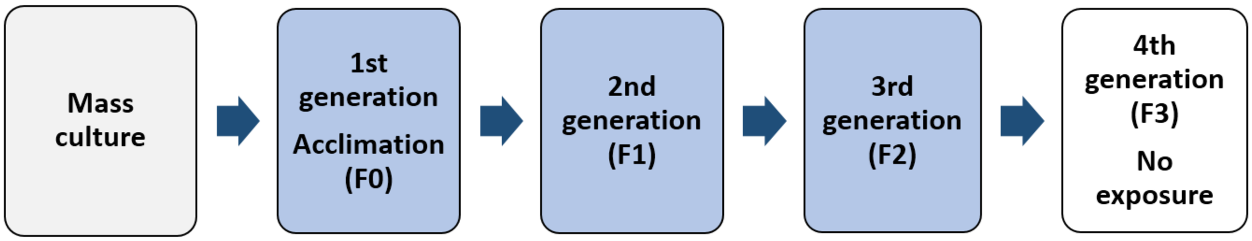

Fig 5.1 Flow-chart showing the experimental design for multi-generation.

5.2.3 Lethal concentrations

The lethal concentration for Cd, Ni and Cu for *E. affinis* has been taken from Zidour et al., 2019. In order to find out the lethal concentration for Pb the toxicity test was performed according to the method laid by Tlili et al., 2016 and Kadiene et al., 2017. A range of different concentration of Pb was prepared in 140 ml beakers (in 80ml autoclaved seawater, salinity 15) in triplicates separately for male and female *E. affinis* (25 individuals in each group). The concentrations tested were 0 µg/l, 300 µg/l, 350 µg/l, 400 µg/l, 450 µg/l, 600 µg/l. After the copepods were added in respective beakers, they were placed in a programmed incubator with the temperature of 18^0 C and a photoperiod of 12h: 12h (light: dark) for 96h. Mortality was monitored under the stereomicroscope (SZX9; Olympus, Tokyo, Japan) every day after 24h, 48h, 72h and the end point 96h and thereafter LC50 was calculated by using the Probit analysis (Tlili et al., 2016).

5.2.4 Quality assurance and quality control

All the analytical procedure was carefully conducted by maintaining the standard quality assurance protocols. The glassware, filters and apparatus that were used were washed initially with vinegar and double distilled water followed by a rinse with ethanol and again with double distilled water at each step of the analysis. In all the batches of analysis, procedural blanks, control and different exposure condition were prepared in triplicates for minimizing errors. High grades of metal salts (Cd, Cu, Ni, and Pb) were dissolved in ultrapure water in order to prepare the four stock solutions with 99.9% purity (Merck, Darmstadt, Germany). Multiple elemental standards (for ICPMS) were used as a reference to measure the targeted trace metals used in this experiment. Standard certified reference materials were used to assess the quality of the data. For the multigenerational approach, copepod, water and a well as sediment samples were collected at the starting time (t = 0) for the metal analysis to get the baseline before exposure to contaminants. Regular monitoring of salinity, pH and residual metal concentrations were conducted.

5.2.5 Multigenerational approach

In this study, the protocol described by Souissi et al, 2010, 2016 was followed to execute the multigenerational approach with slight modifications. The experimental tanks used were 2 L glass beakers filled with autoclaved filtered seawater of salinity 15 and pH 8.3. The experiment was carried out in a controlled ambience with temperature 18^0C and a photoperiod of 12h: 12h (light: dark) cycle. The first generation for acclimation (F0) started by adding 30 ovigerous female which were sorted from the large tank of copepod culture (300 L) to the respective beakers. After a period of incubation, the ovigerous females released the egg and the eggs hatched to nauplii. Immediately after the first hatch the adult females were removed to avoid the formation and hatching of the second egg sac, which might have led to additional number of nauplii thereby increasing the total population. The nauplii were then allowed to grow into adult copepods, uninterruptedly, in their respective beakers with the water of the treatment being changed after the appearance of the copepodite stage for each generation. In each treatments, generation and stages of copepod, the time of the development was noted carefully. All the experimental tanks were fed once in every two days with *R. marina* (10ml) and for the overall maintenance of algal culture and feeding technique Souissi et al. (2010, 2016) was followed.

After the appearance of the female ovigerous copepods, 30 ovigerous females were sorted carefully to start the next generation, 10 ovigerous females were sorted and put in individual Eppendorf tubes with alcohol for the morphological traits and clutch size. The rest of the population was concentrated to 1500 ml and then divided into two equal halves (750 ml in each beaker, by volume). A glass rod was used to stir the seawater inside the beaker continuously in order to generate homogenous population and immediately the separation was made in order to get equal or near to equal number of copepods in each beaker (Zidour et al., 2019). One of this half was fixed with alcohol for counting the density and stages of copepod in each generation and treatment. The other half was used for analyzing the bioaccumulation of heavy metals.

5.2.6 Bioaccumulation in copepod, water and sediment

The bioaccumulation was monitored after each generation in all the experimental tanks. The remaining copepods in 750ml (described above) were then filtered through pre-weighed cellulose nitrate filter with a porosity of 0.22mm (Sartorius Stedim Biotech, Goettingen, Germany). The filters comprising the copepods were then dried in the oven at 60 ^{0}C for 72h and weighed again. For the analysis of the residual concentration of metals in the sediment, similar technique was used as for the copepods. In the sediment treatment, where whole sediment was used in re-suspension, the residual sediment was filtered through a pre-weighed cellulose nitrate filter of porosity 0.22mm, thereafter dried in the oven at 60 ^{0}C for 72h and weighed again for each generation. For analyzing the dissolved fraction of heavy metals in the residual seawater, 12 ml of filtrate was collected in a falcon tube where 15µl of ultra-pure nitric acid (HNO_3) was added to fix it immediately.

5.2.7 Trace metals analysis

For analyzing the heavy metal concentration bio-accumulated in the whole body of *E. affinis*, the dried filters comprising the copepods were first subjected to digestion by adding nitric acid and hydrochloric acid in the ratio of 1:3 (1ml HNO_3: 3ml of HCL) in the respective Teflon tubes, and then diluted to a final volume of 5ml with double distilled water (ultrapure). Similarly, for analyzing the residual concentration in the sediment, the dried filters were digested by adding the double volume of nitric acid and hydrochloric acid used for copepods in the ratio of 1:3 (2 ml HNO_3: 6 ml of HCL). In each Teflon tube, the filters were then diluted with ultrapure water up to a final volume of 5ml. The sediment filters need to be centrifuged to get rid of the particulate and suspended matters and the final volume of the filtrate was noted. The analysis of the residual heavy metal concentration in water for each preserved sample was directly analyzed without any pre-treatment. The entire process of analyzing the heavy metal concentration was followed from the protocol described by Ouddane et al., 1990 and Zidour et al., 2019. The concentration of each element was then analyzed by using an inductive coupled plasma atomic emission spectrometry (ICP-AES; Vista Pro, axial view, Varian, Australia).

5.3 Results and discussions

5.3.1 Lethal concentration of Lead (Pb)

The lethal concentration for Cd, Ni and Cu for *E. affinis* is listed in table 5.1 taken from Zidour et al., 2019. The result for the different concentration of Pb and the corresponding mortalities that was analyzed is listed in table 5.2. This table shows the concentrations of standard Pb solution that were used to expose *E. affinis*, and the corresponding mortalities observed in male and female individuals separately after 96h.

Table 5.1 The individual acute LC 50% of Cd, Cu and Ni when exposed to *E. affinis* after 48h, 72h and 96 h (- represent slow response) as per Zidour et al., 2019

	LC50 (µg/l)								
	48h			**72h**			**96h**		
	Cd	Cu	Ni	Cd	Cu	Ni	Cd	Cu	Ni
Female	239	-	-	121	224	-	90	39	161
Male	-	145.2	-	67	51	88	127.8	28	90

From this table (Table 5.1), we can depict that the exposure time was a major determining factor for analyzing the sensitivity of the calanoid copepod *E. affinis* to heavy metals (Cd, Cu and Ni) in both the sexes, even though a huge variation between the sexes was noted. Both the sexes of *E. affinis* showed highest sensitivity to Cu after 96 h (25 µg/l for male; 38 µg/l for female). However sensitivity to Ni was higher for males by 1.8 times than in females but sensitivity to Cd was higher in females by 1.5 times thereby supporting the findings of Kadiene et al., 2017 (Zidour et al., 2019).

Table 5.2 Mortalities after 96h of Pb exposure *in E. affinis*

Concentration of Pb (µg/l)	Mortality % (Male)	Mortality % (Female)
0	16.88	15.55
300	29.33	34.33
350	41.33	46.66
400	54.66	62.66
450	81.33	80
600	84	82.6

Furthermore, we wanted to test the LC 50% concentration of Pb in *E. affinis* and from the above table (Table 5.2) we can conclude in definite that higher concentration of Pb led to higher mortalities thereby making the concentration of the metal a major determining factor for the sensitivity of *E. affinis* to metals. The different exposure times did not show much responses and so we highlighted only the mortalities after 96h in the both the sexes of *E. affinis*. Figure 5.2 is a graphical representation of the values of mortality of *E. affinis* when exposed to Pb.

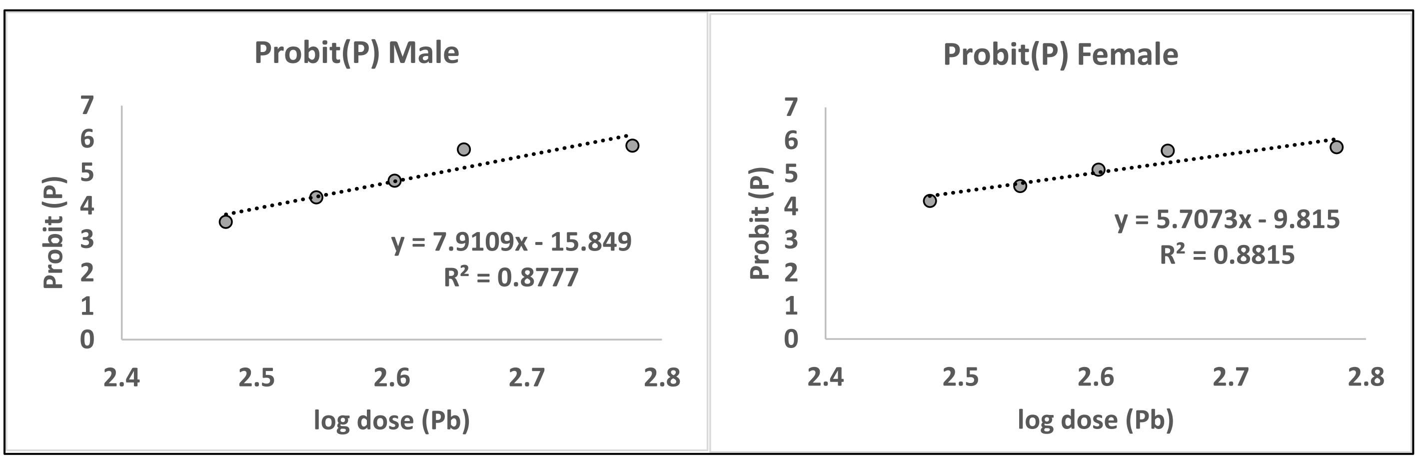

Fig 5.2 Graphical representation of the values of mortality (Probit) of *E. affinis* when exposed to Pb after 96h.

After transforming the results to Probit in order to have a linear curve of the LC50 concentration, we found the following (Table 5.3) in different hours of the acute toxicity in the individual log doses of Pb.

Table. 5.3 The individual acute LC 50% of Pb when exposed to *E. affinis* after 48h, 72h and 96 h

LC50 Pb (µg/l)	96h	72h	48h
Male	431.99	473.35	596.85
Female	394.27	473.05	607.62

From the above results, we noted that the LC 50% for the exposure of Pb to *E. affinis* was 431.99 µg/l for males showing lower sensitivity than females with 394.27 µg/l.

5.3.2 Population in each generation

In the multi generation approach, we found varied results in each generation for the total population and the stages of copepods in each experimental beaker and in each generation. We analyzed total 4 generation with exposing the copepods continuously for 3 generations in each treatment (treatments described in material and methods), F0 referring to the acclimation phase and in the 4th (F3) generation we used control seawater (autoclaved seawater, salinity 15).

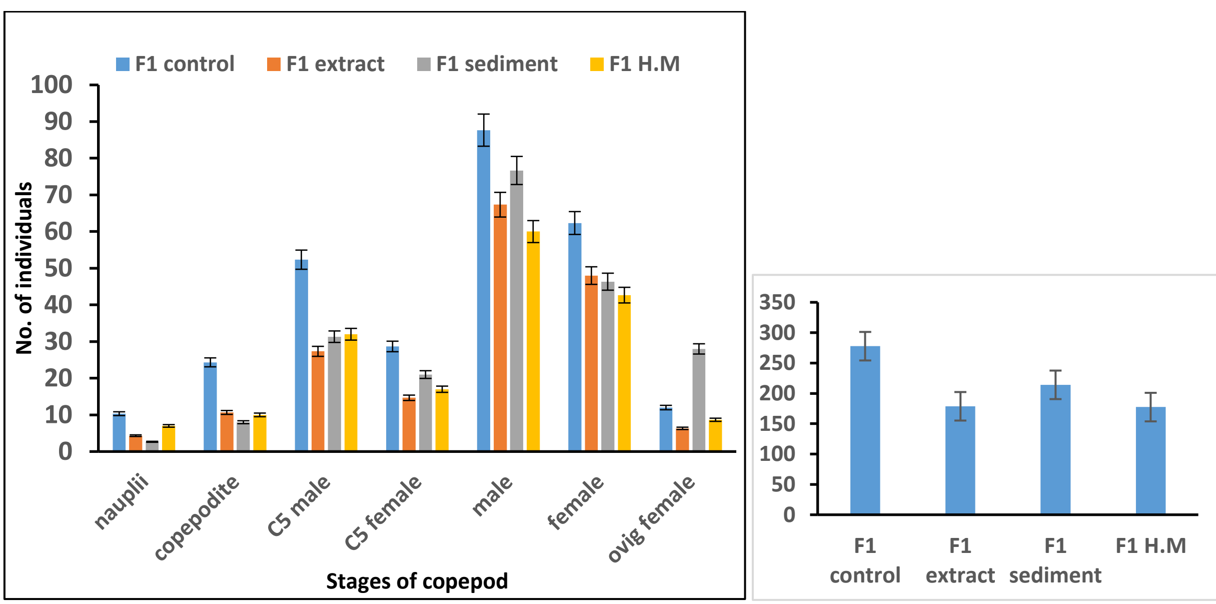

Fig 5.3 a (Left) Figure showing the variation in population of different stages of copepod in the 2nd generation (F1) for all the treatments (control - blue, extract - red, sediment - grey, H.M-

yellow) . (Right) Figure showing the total population (including nauplii) in each treatment of the 2nd generation (F1).

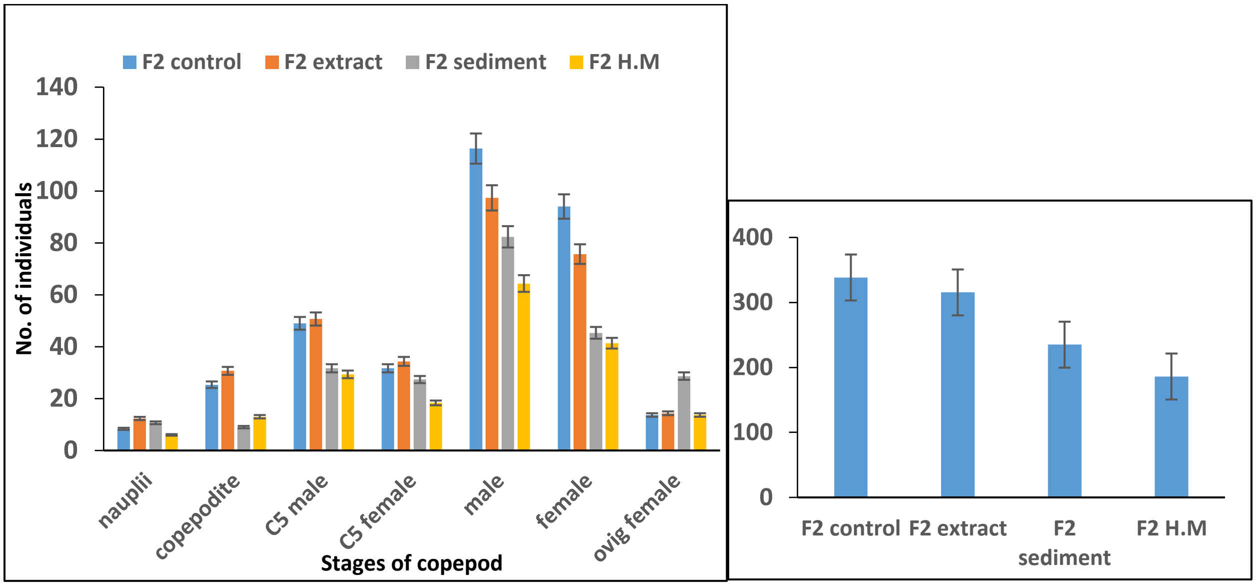

Fig 5.3 b (Left) Figure showing the variation in population of different stages of copepod in the 3rd generation (F2) for all the treatments (control - blue, extract - red, sediment - grey, H.M- yellow). (Right) Figure showing the total population (including nauplii) in each treatment of the 3rd generation (F2).

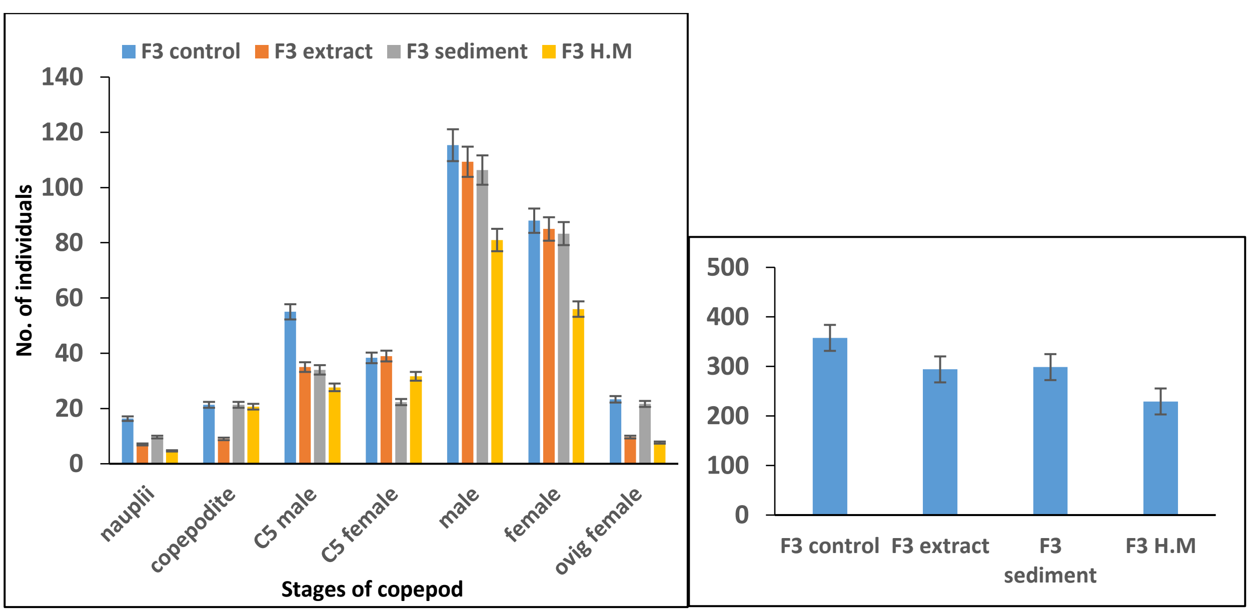

Fig 5.3 c (Left) Figure showing the variation in population of different stages of copepod in the 4^{th} generation (F3) for all the treatments (control - blue, extract - red, sediment - grey, H.M- yellow). (Right) Figure showing the total population (including nauplii) in each treatment of the 4^{th} generation (F3).

In the above figures Fig 5.2 (a, b, c), we noted that the population of adult male in all the 3 generations were higher than the other stages in each generation and treatment. The abundance of nauplii was however the least in all the generation and in each treatment and out of them heavy metal treatment showed least values of nauplii abundance in most of the cases. This result supports the findings of previous studies where low abundance of nauplii was found when exposed to heavy metals in mixture or alone compared to control treatment (Jiang et al., 2007; Mohammed et al., 2011; Kadiene et al., 2017, Zidour, 2019). However overall the population abundance of the adult stages like C5 male, C5 female, adult male, adult female was much lower in the exposed treatments than in control. The smaller stages of copepod like nauplii or copepodite recruitment in that respect was not affected at the extent of the adult stages. Inspite of the fact that such studies are still scarce, out of the few reports, our preliminary result was similar to the recent finding of Zidour et al., 2019.

However, with higher stability the population density in the control treatment increased over time and consequently having the highest population in the 4^{th} (F3) generation. On contrary the population of copepods in the heavy metal treatment was least when compared to the other exposed conditions in all the generations. The total population of copepods in the heavy metal treatment however increased slightly in the 4^{th} generation (F2=186; F3=229) where no metals were added perhaps explaining the minute recovery due to clean seawater devoid of any metals. In fact similar observations were noted down for the sediment treatment in which the population density increased in the 4^{th} generation (F2=235; F3=272) perhaps due to the use of clean seawater. In general, the population density of copepods in the extract treatment was the least in F1 without any significant pattern over the four generations. However very interestingly, all the other treatments had the lowest population of copepods in the second generation (F1). Fig 5.4 clearly represents the variation of total population in four conditions (Control, extract, sediment and heavy metal) in each generation. It further reflects the higher population in control compared to other treatments in every generation.

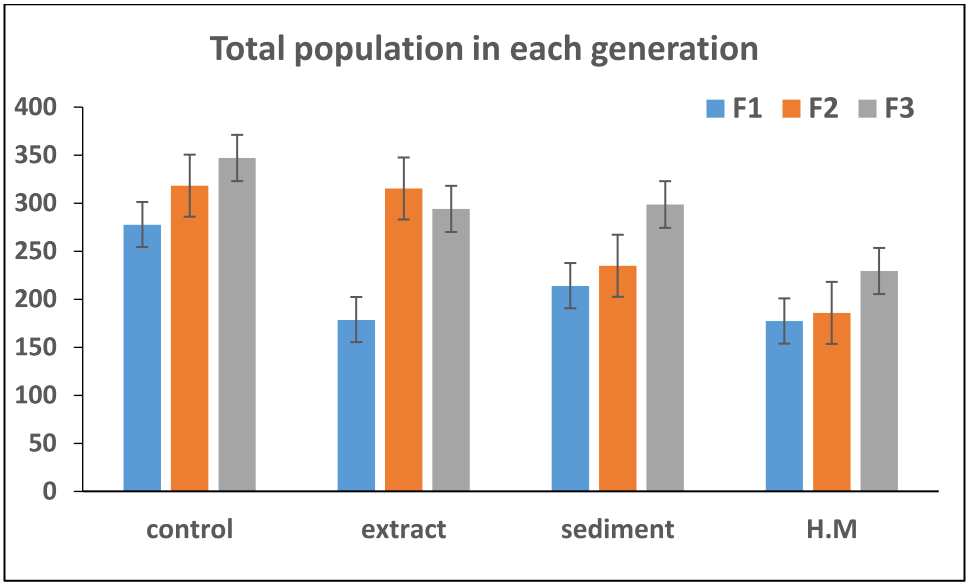

Fig 5.4 The graphical representation of the total population in each generation and treatment (F1-blue, F2- red, F3- grey).

5.3.3 Sex specific responses and mortality

From the figure below, (Fig 5.5) we can comprehend that the sex ratio was affected mostly in the sediment treatment where abundance of males in each generation decreased hence affecting the overall distribution of the stages of copepods in general. Our results show that sediment in re-suspension can possibly affect the survival of males and reduce its abundance over the time, thus making time a determining factor for the sensitivity of copepods to different exposure. In fact, over the time the percentage of males decreased in each treatment including control. However, the exposure of the mixture of metals (Cd, Cu, Ni and Pb) did not affect the availability of male copepods since the abundance of males in the heavy metal treatment was similar and comparable to that of the control.

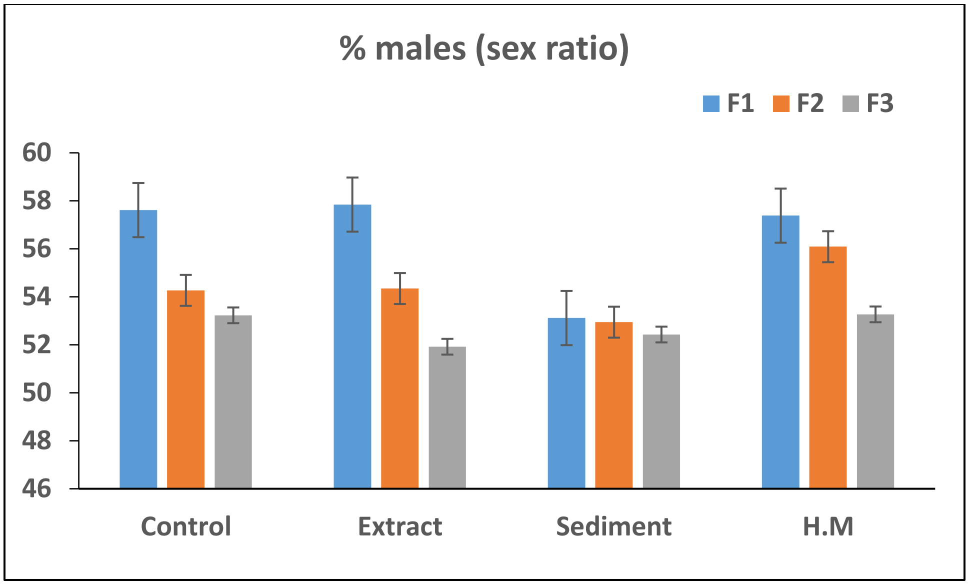

Fig 5.5 The graphical representation of the sex ratio in each generation and treatment (F1- blue, F2- red, F3- grey).

The mortality of copepods however gave interesting insights about all the exposure conditions and here effect for over multiple generations including the 4th generation (F3) where a detoxifying approach was used by providing just natural autoclaved seawater (salinity 15). Fig 5.6 shows the results for the mortality of each treatment including control in each generation. Our results show lowest mortality in the control treatment in each generation when compared to the other treatments. Nonetheless, as interestingly pointed out that after the acclimation phase (1st generation, F0), the total population in F1 was greatly affected in all the treatments showing the lowest values. On the other hand the bioaccumulation of the trace metals were also found to be higher in the second generation (F1) in all the treatments showing further highest mortality in the same generation (F1). To overall summarize we can assume that higher concentration of metals in the different exposure beakers including the influence of sediment and its toxicity affected the total population and in turn the mortality in each generation.

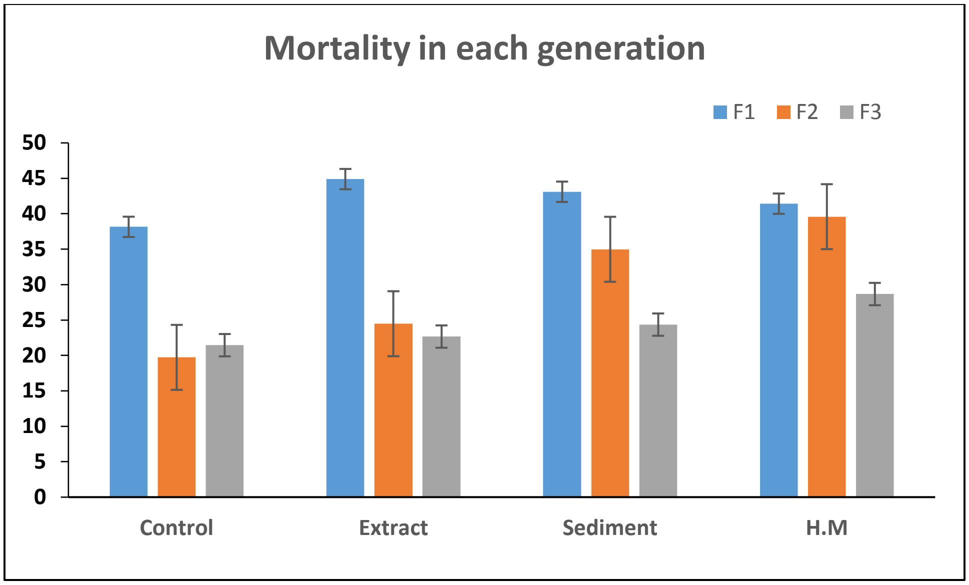

Fig 5.6 The graphical representation of the mortality % in each generation and treatment (F1-blue, F2- red, F3- grey).

5.3.4 Clutch size and prosome length

Fig 5.7 a shows the variation in the clutch size of *E. affnis* for different treatment and generations. In F0, namely the acclimation phase the copepods in the control beaker showed the highest values of all the clutch sizes and in the subsequent generation it showed similar values unlike the 2nd generation (F1). The sediment treatment however showed a decrease in the clutch size in 2nd generation similar to the control and then further increasing in the 4th generation (F3) when clean seawater was used thereby following similar trend like that of the total population and mortality. Similar observation in case of the heavy metal treatment was seen where the clutch size increased in the 4th generation (F3) but no such decrease in F1 was noticed. A sharp increase in clutch size of the extract treatment was observed in F1 thereby not giving any consistent pattern with previous findings.

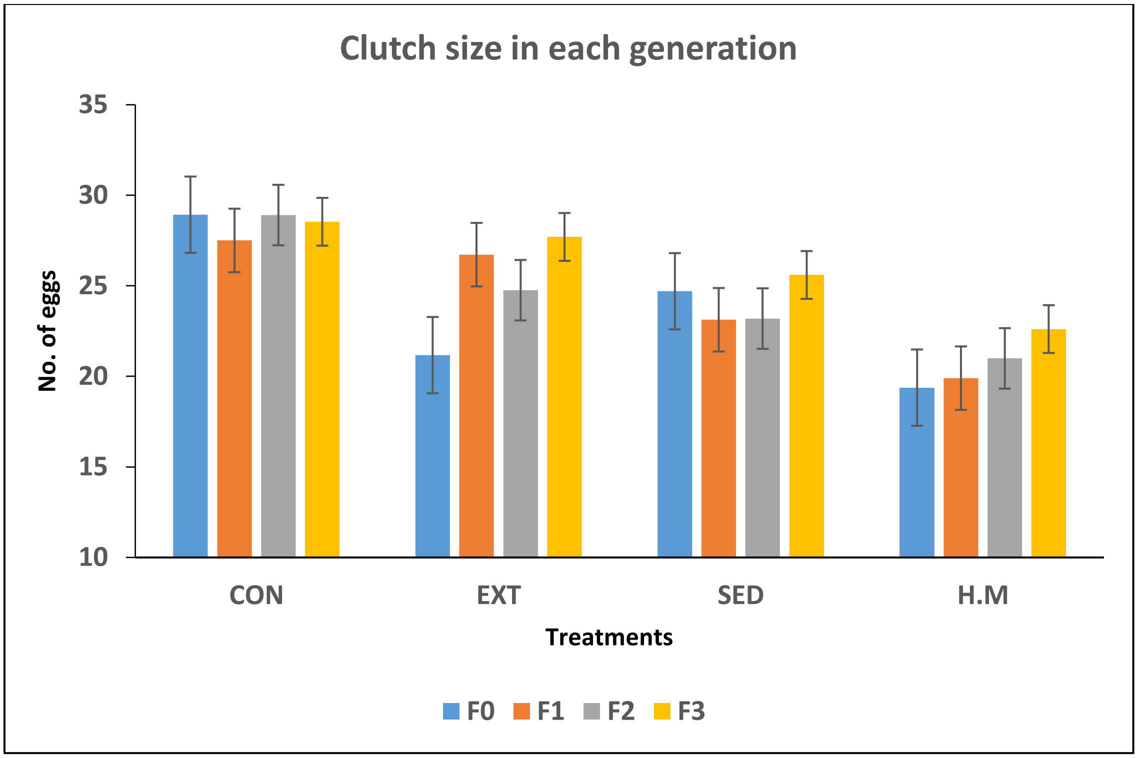

Fig 5.7 a The graph showing different clutch size in each generation and treatment (F0- blue, F1- red, F2- grey, F3- yellow).

The Prosome length of copepods in each treatment including the control in each generation was measured. Fig 5.7 b shows the values of the prosome length of the copepods in all the treatments from the acclimation phase where the control showed the highest value of prosome length in the 3rd generation (F2). Similar observation with that of the clutch size was observed for all the treatments with F1 showing lower prosome length in control and sediment treatments. The prosome length universally increased in the 4th generation for all the treatments when clean seawater was used without any exposure apart from control. A sharp increase in the prosome length of the copepods in the extract treatment was observed in F1 similar to the clutch size thus drawing a positive correlation between the both. In fact the comparison between the prosome length and clutch size for all the treatments drew a correlative relationship from our results. In fig 5.7 c the comparison between the prosome length and the clutch of the copepod *E. affnis* is shown and the results reflect a positive correlation between these two parameters in all the treatments. Statistically, significant correlations was established between the prosome length and the clutch size of *E. affinis* in all the four treatments namely extracts (p value 0.05), sediment (p value <0.05), heavy metal (p value <0.05) and control (p value <0.05). Furthermore when we compared all the values of prosome length and clutch sizes of the copepod *E. affinis* irrespective of the exposure condition in order to depict a general trend, a highly significant correlation (p value <0.01) was observed between prosome length and clutch size (Fig 5.7d).

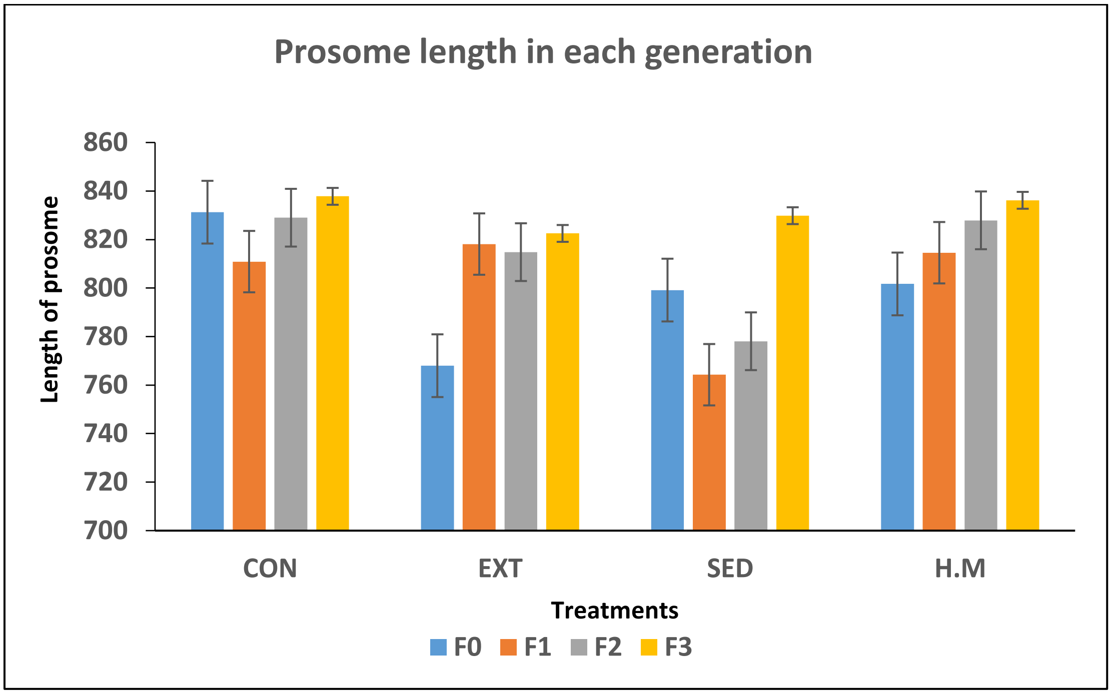

Fig 5.7 b The graph showing different prosome lengths of ovigerous female in each treatment and generation showing (F0- blue, F1- red, F2- grey, F3- yellow).

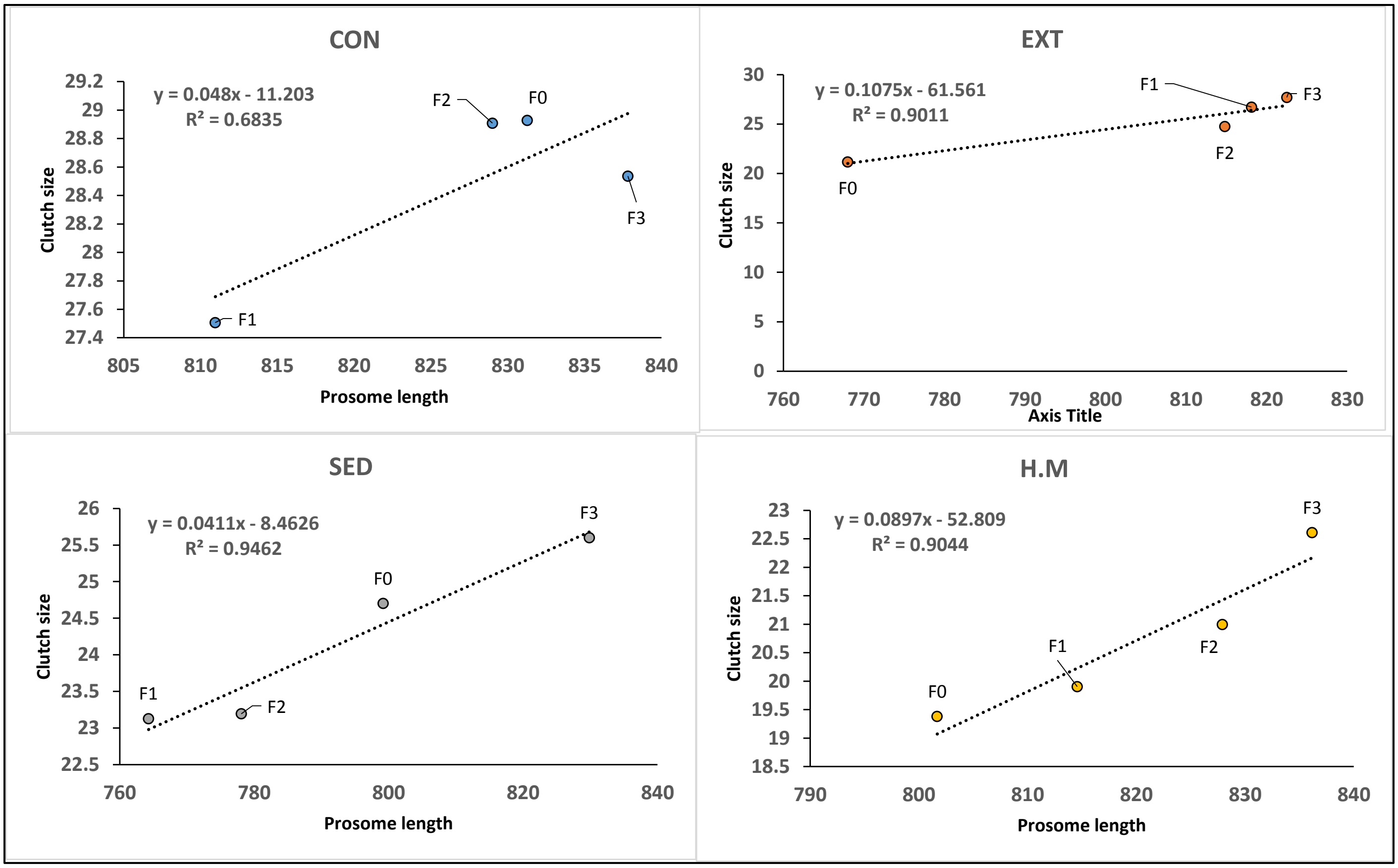

Fig 5.7 c Relationship between prosome length and the clutch size of copepods in each treatment. Linear trend was obtained through simple regression. The control is represented by blue, extract is orange, sediment is grey and heavy metal is yellow.

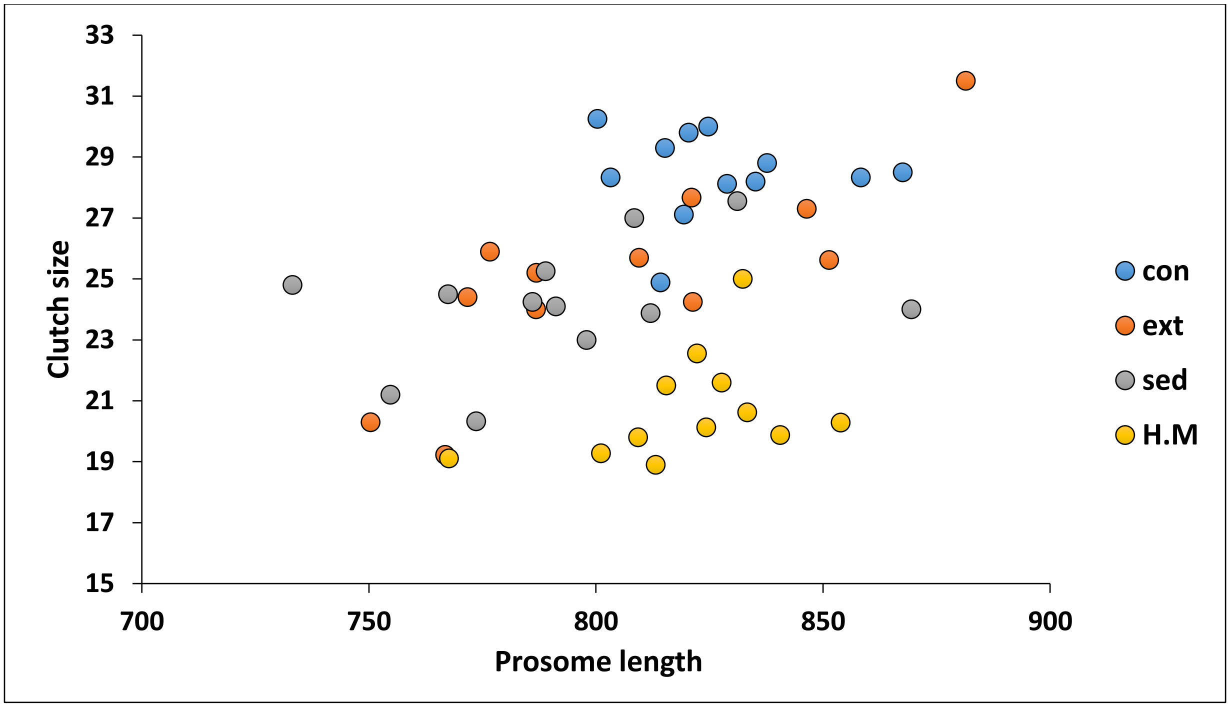

Fig 5.7 d The relationship between all the prosome length and clutch sizes of copepods in each generation. The control is represented by blue, extract is orange, sediment is grey and heavy metal is yellow.

5.3.5 Bioaccumulation effect of the heavy metals on the mortality

The bioaccumulation in *E. affinis* of each metal when mixed together was analyzed and compared with the mortality in each generation. Fig 5.8a-1 shows the relationship between the mortality and the bioaccumulation of the 4 metals namely, Cd, Cu, Ni and Pb in *E. affinis* for the sediment treatment. Exposure to sediment in re-suspension led to the bioaccumulation of the above metals with all the metals showing highest bioaccumulation in F1 (2nd generation). However, Cu showed the highest range of bioaccumulation (48.81 µg/g) followed by Ni (27.57 µg/g), Pb (20.32 µg/g) and lastly Cd (1.77 µg/g). However when all the metal concentration that was bioaccumulated in *E. affinis* was added (summation), similar to individual metals, a correlation between summation of metals together and mortality was observed (Fig 5.8 a-2).

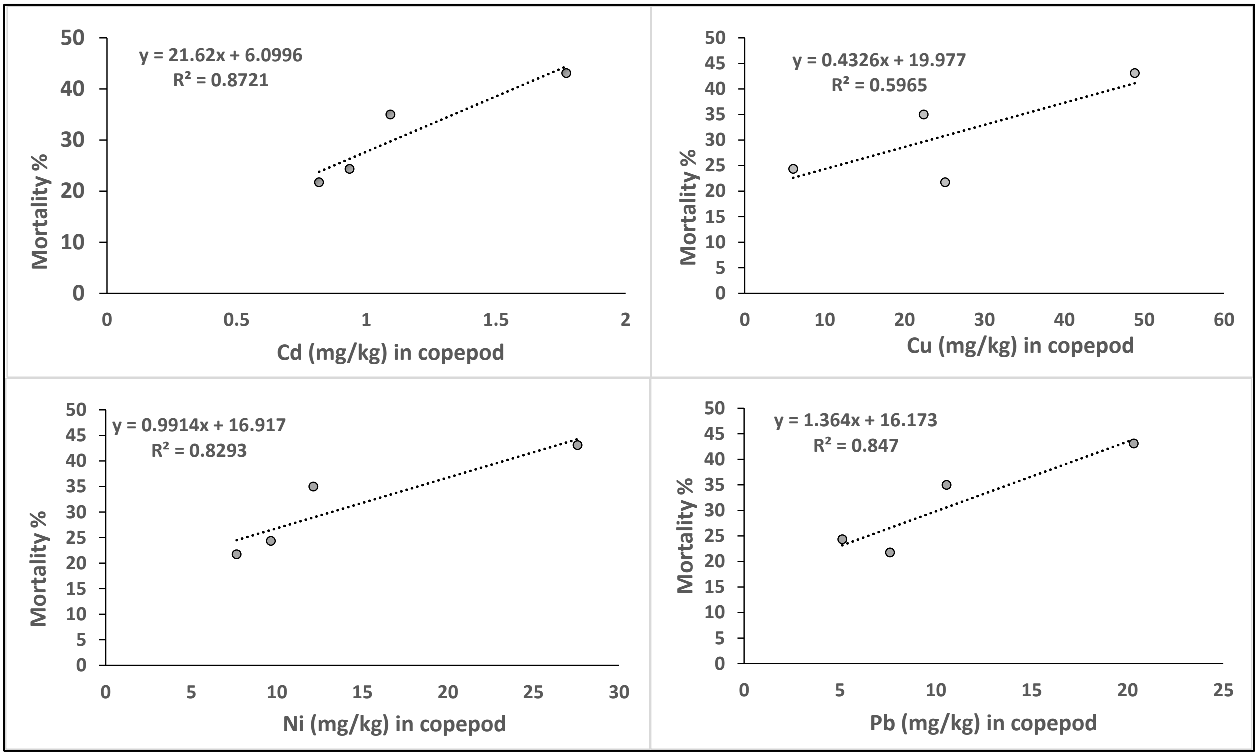

Fig 5.8 a-1 Relationship between metals bioaccumulated in copepods and mortality in sediment treatment. All the heavy metals showed a linear trend was observed through simple regression.

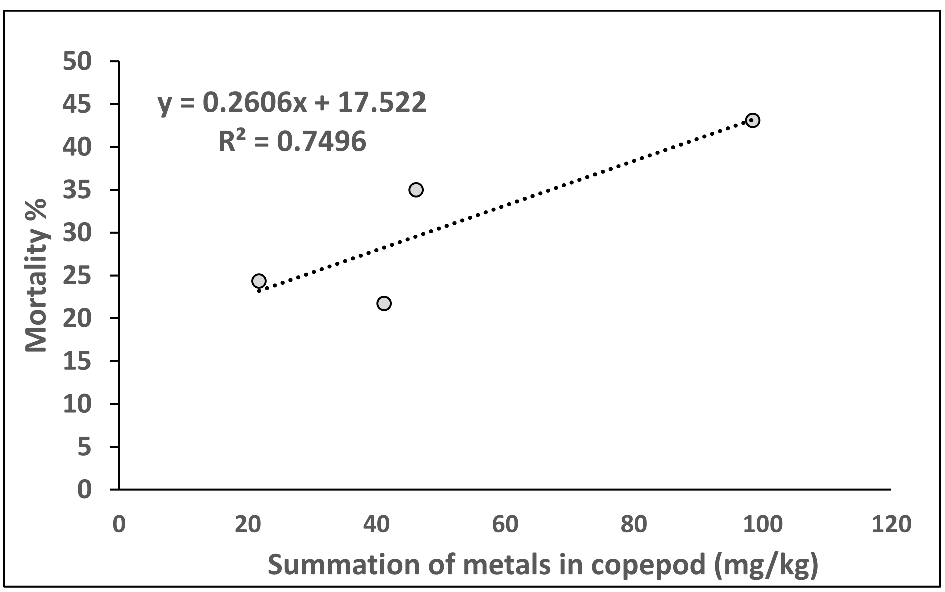

Fig 5.8 a-2 The comparison between mortality and the summation of the concentration of metals in *E. affinis* in sediment treatment. A linear trend was observed through simple regression.

In Fig 5.8 b-1 the relationship between the mortality and the bioaccumulation of the metals in *E. affinis* for the heavy metal treatment is shown. Similar observation was noticed in the heavy metal treatment with that of the sediment treatment when mix of metals led to the bioaccumulation, with all metals showing highest bioaccumulation in F1 (2nd generation). Cu again showed the highest range of bioaccumulation (127.56 µg/g) followed by Pb (56.32 µg/g), Ni (48.61 µg/g) and lastly Cd (29.05 µg/g). A correlation was depicted (from the figure below) between the mortality and the accumulated metals both being the highest in the 2nd generation (F1). In Fig 5.8 b-2 however the summation of all the metal concentrations in *E. affinis* was plotted against the mortality in each generation of the heavy metal treatment.

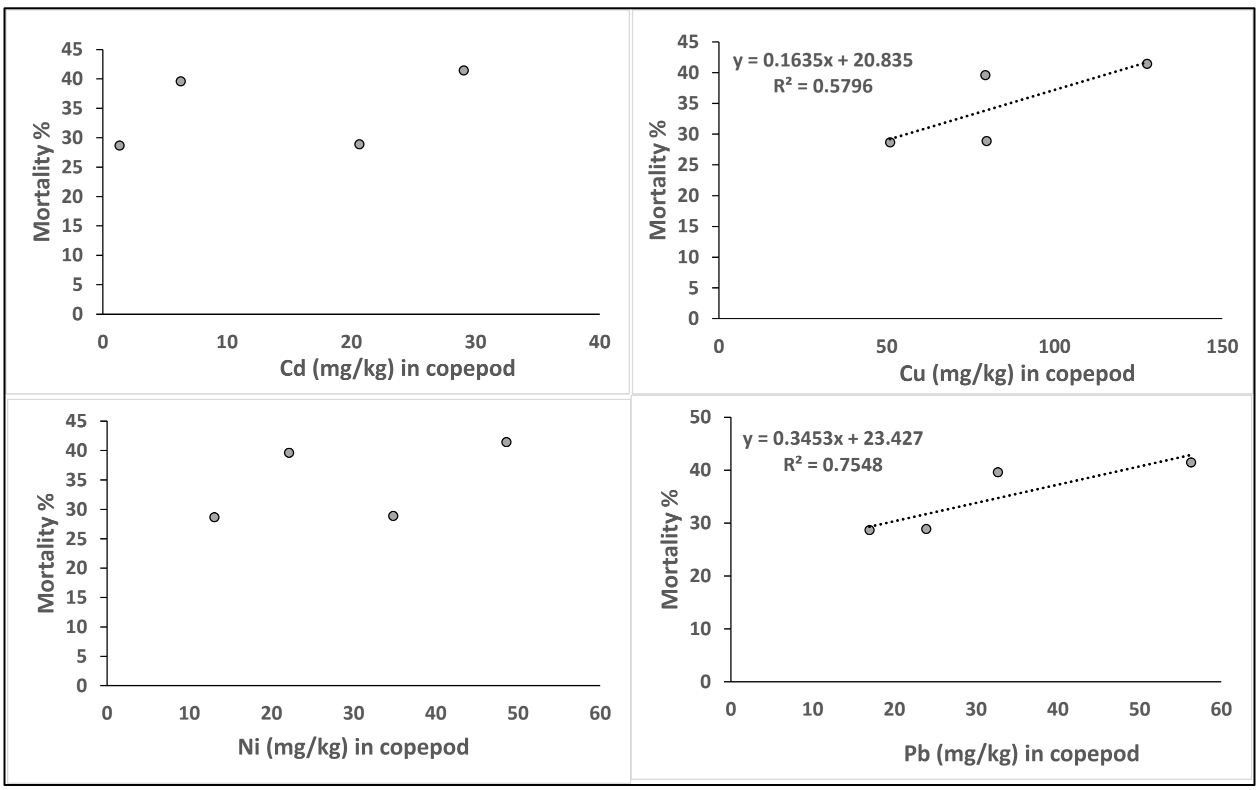

Fig 5.8 b-1 Relationship between metals bioaccumulated in copepods and mortality in heavy metal treatment. A linear trend is observed in case of Copper (Cu) and Lead (Pb) through simple regression.

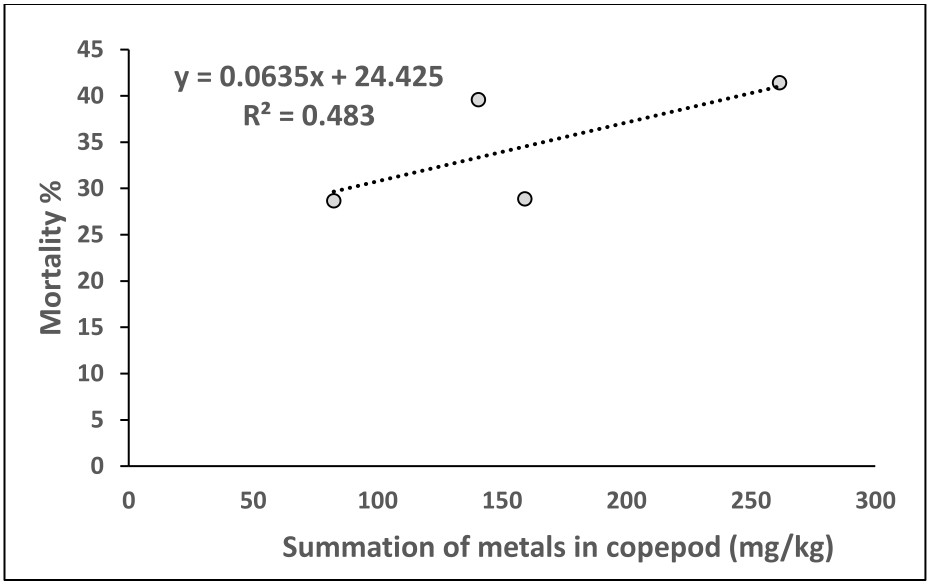

Fig 5.8 b-2 The comparison between mortality and the summation of the concentration of metals in *E. affinis* in heavy metal treatment. Linear trend is obtained by simple regression.

Fig 5.8 c-1 shows the relationship between the mortality of *E. affinis* along with the accumulated metals inside the copepod for the extract treatment. The correlation between the mortality and the accumulated metals showed a correlation between Cd bioaccumulation and mortality, but any such relationship was absent in case of Cu and Ni. However, a significant correlation between bioaccumulation of Pb and mortality in each generation (p value 0.035) was noticed. Not a strong correlation between summation of all the metal concentration and mortality in extract treatment was observed (Fig 5.8 c-2).

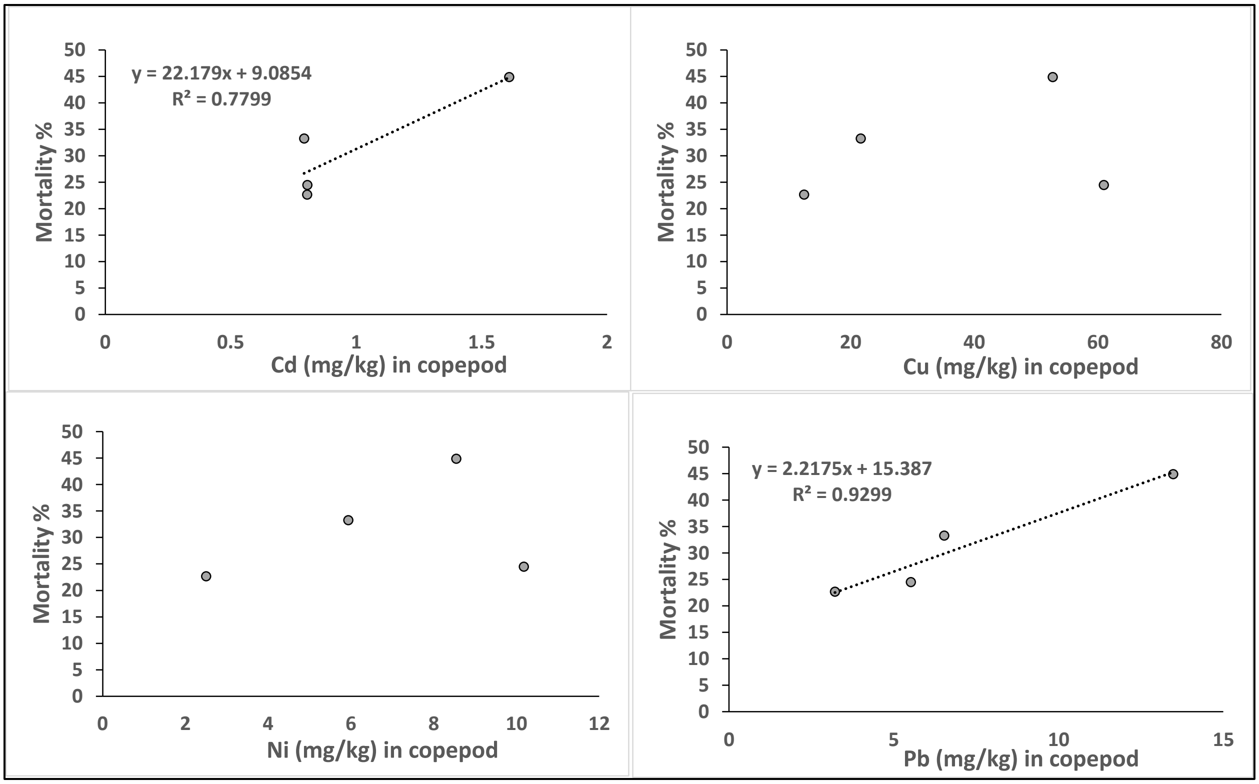

Fig 5.8 c-1 Relationship between metals bioaccumulated in copepods and mortality in extract treatment. Linear trend was noticed for the Lead (Pb) and Cadmium (Cd) through simple regression.

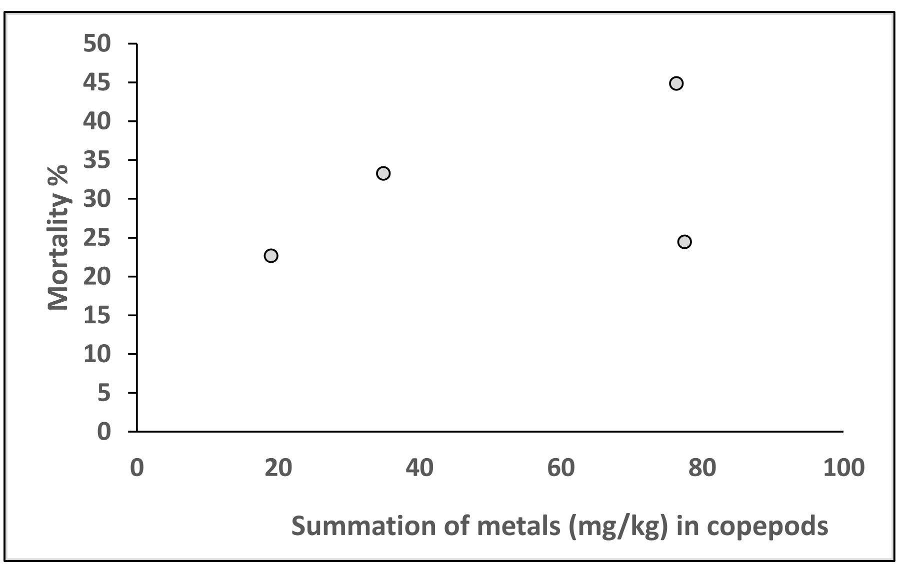

Fig 5.8 c-2 The comparison between mortality and the summation of the concentration of metals in *E. affinis* in extract treatment. No linear trend as such could be noticed.

5.3.6 Bioaccumulation effect of the heavy metals on the clutch size

The bioaccumulation in *E. affinis* of each metal when mixed together was analyzed and compared with the clutch size in each generation and treatment. Fig 5.9 a-1 shows the relationship between the clutch size and the bioaccumulation of the 4 metals namely, Cd, Cu, Ni and Pb in *E. affinis* for the sediment treatment. The results show that when the sediment in re-suspension was exposed to copepods, it affected the clutch size of the copepods along with bioaccumulating trace metals. The higher amount of metals led to decrease in clutch size for the sediment treatment. However, lowest clutch size was observed in F1 along with highest accumulation of all the 4 metals and thereby justifying lowest population in this generation. A significant negative correlation was established between the bioaccumulation of Pb and the clutch size (p value 0.05) in each generation for the sediment treatment. However, a strong correlation was also depicted when all the metal concentration were added and compared with the clutch size in each generation (Fig 5.9 a-2). Similar observation was noticed in case of the heavy metal treatment (Fig 5.9 b-1), where a negative correlation was established between the clutch sizes of the exposed copepods with the amount of metals accumulated in *E. affinis* over the generations. In both the sediment and heavy metal treatment, the clutch size was lowest in F1 and interestingly the clutch sizes increased in F3 when no exposure but only autoclaved seawater was used. A trend was reflected when the metal concentration bioaccumulated in *E. affinis* was added and compared with the clutch size for the heavy metal treatment (Fig 5.9 b-2). For the extract treatment (Fig 5.9 c-1) however we did not notice any correlation between clutch size and bioaccumulation of the four metals and even when all the metal concentrations were summed up and compared with the clutch size in every generation (Fig 5.9 c-2).

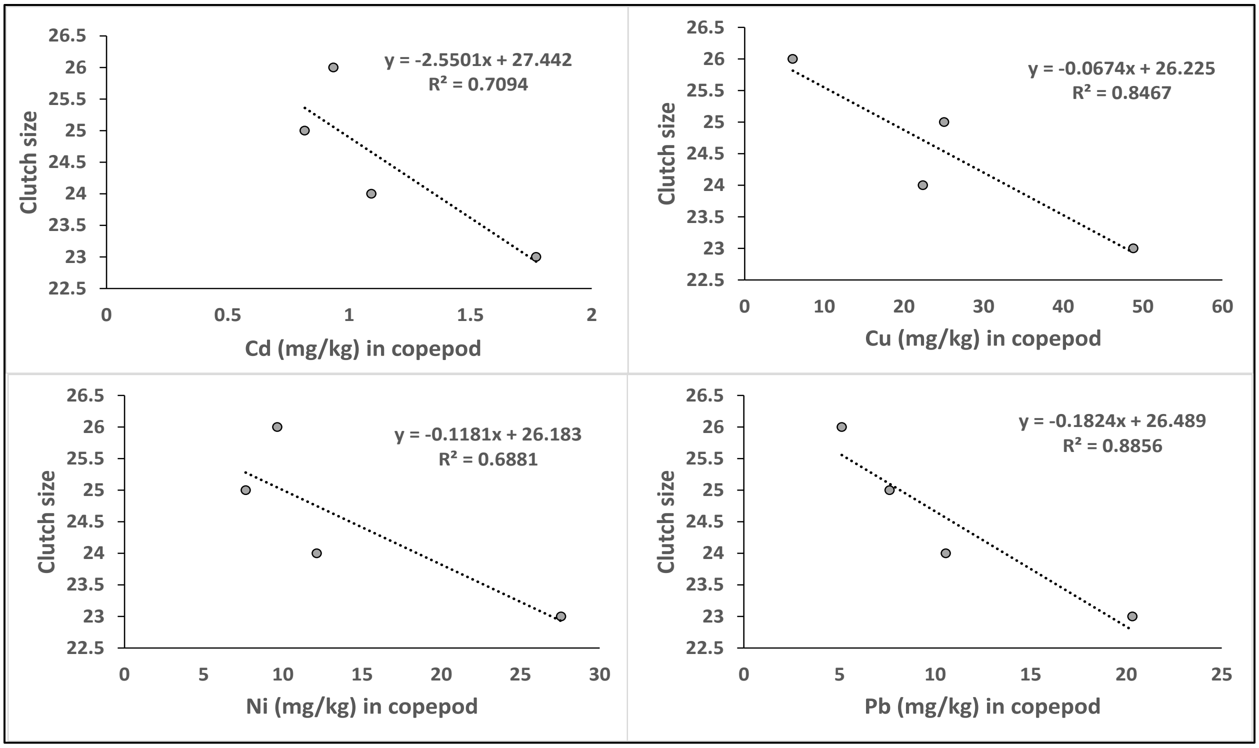

Fig 5.9 a-1 Relationship between metals individually bioaccumulated in copepods and clutch size in sediment treatment. All the heavy metals showed a linear trend to decreasing values of clutch size obtained through simple regression.

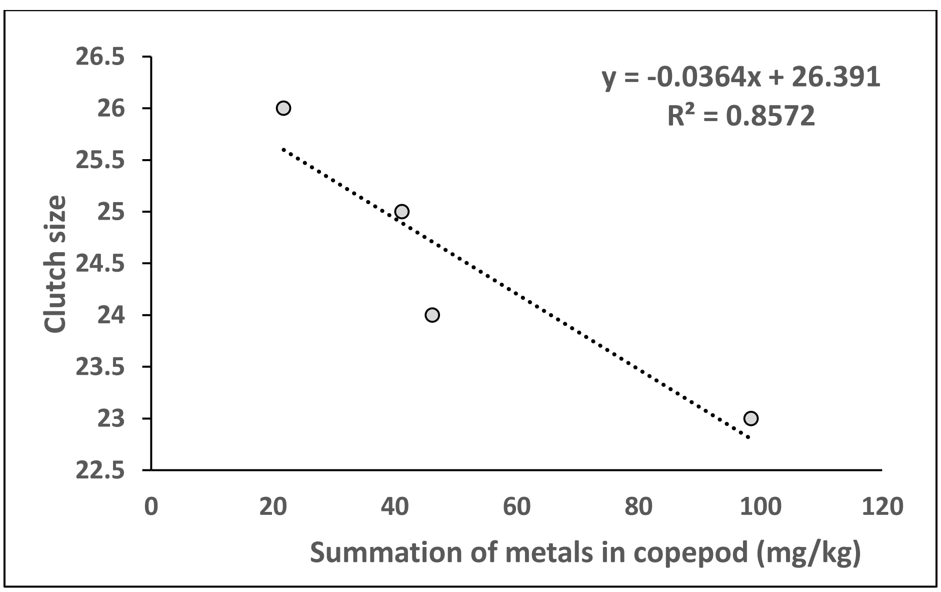

Fig 5.9 a-2 The comparison between clutch size and the summation of the concentration of metals in *E. affinis* in sediment treatment. A linear decreasing trend is noticed for the summation of all the metals when plotted against clutch size through simple regression.

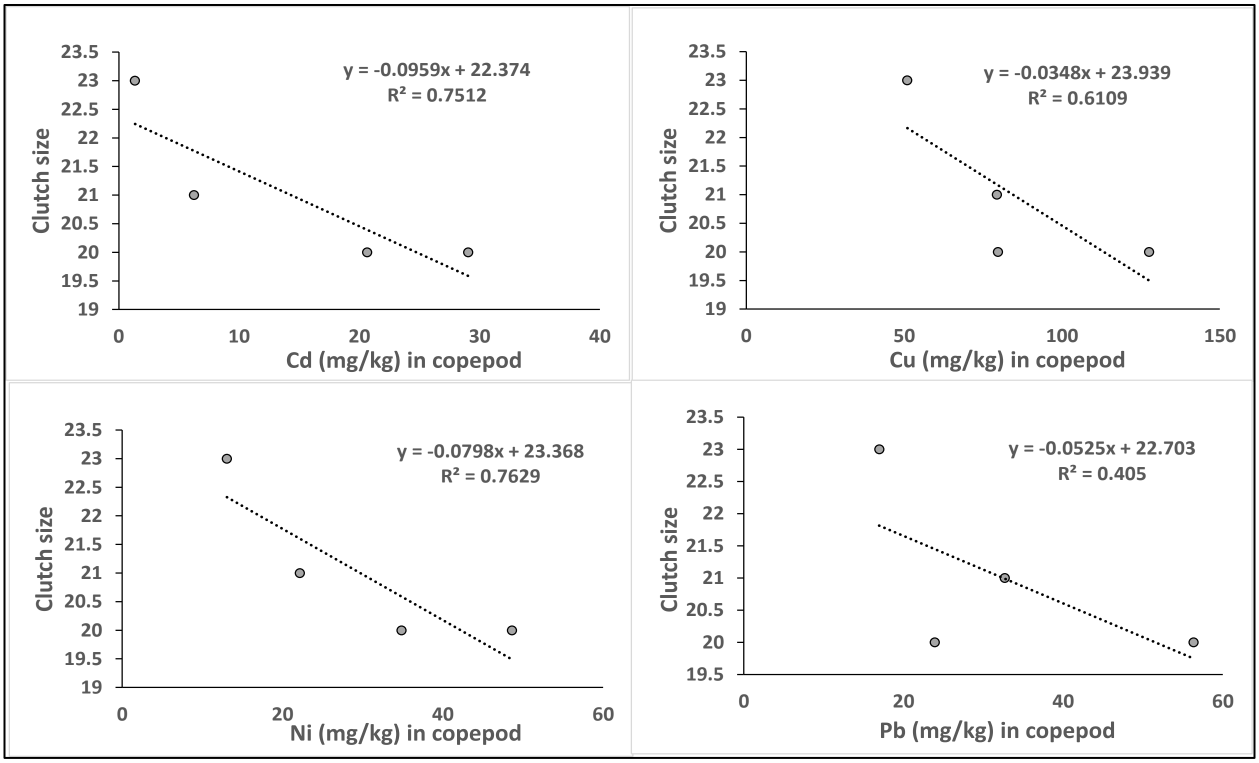

Fig 5.9 b-1. Relationship between metals bioaccumulated in copepods and clutch size in heavy metal treatment. All the metals showed a linear decreasing trend of clutch sizes with increase of bioaccumulated metals.

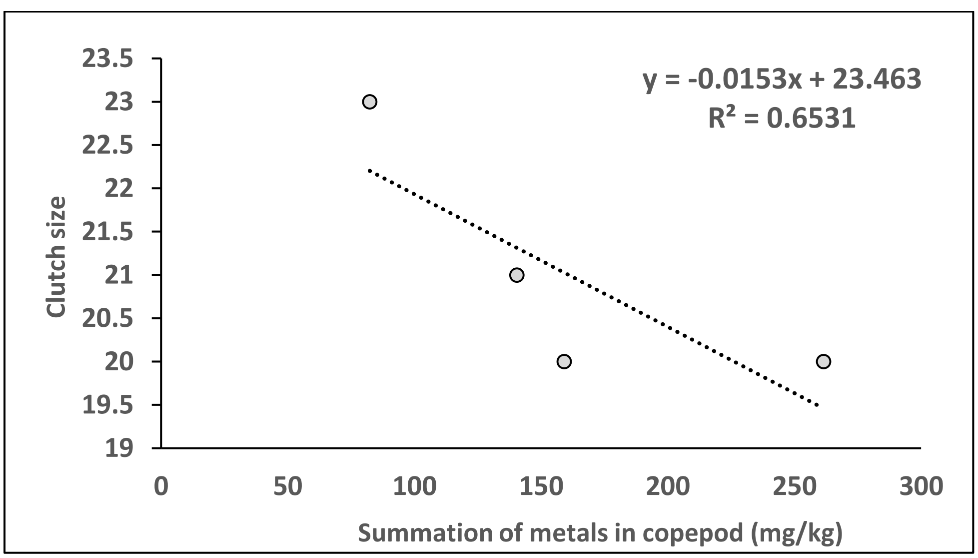

Fig 5.9 b-2 The comparison between clutch size and the summation of the concentration of metals in *E. affinis* in heavy metal treatment. A linear decrease is noticed through simple regression.

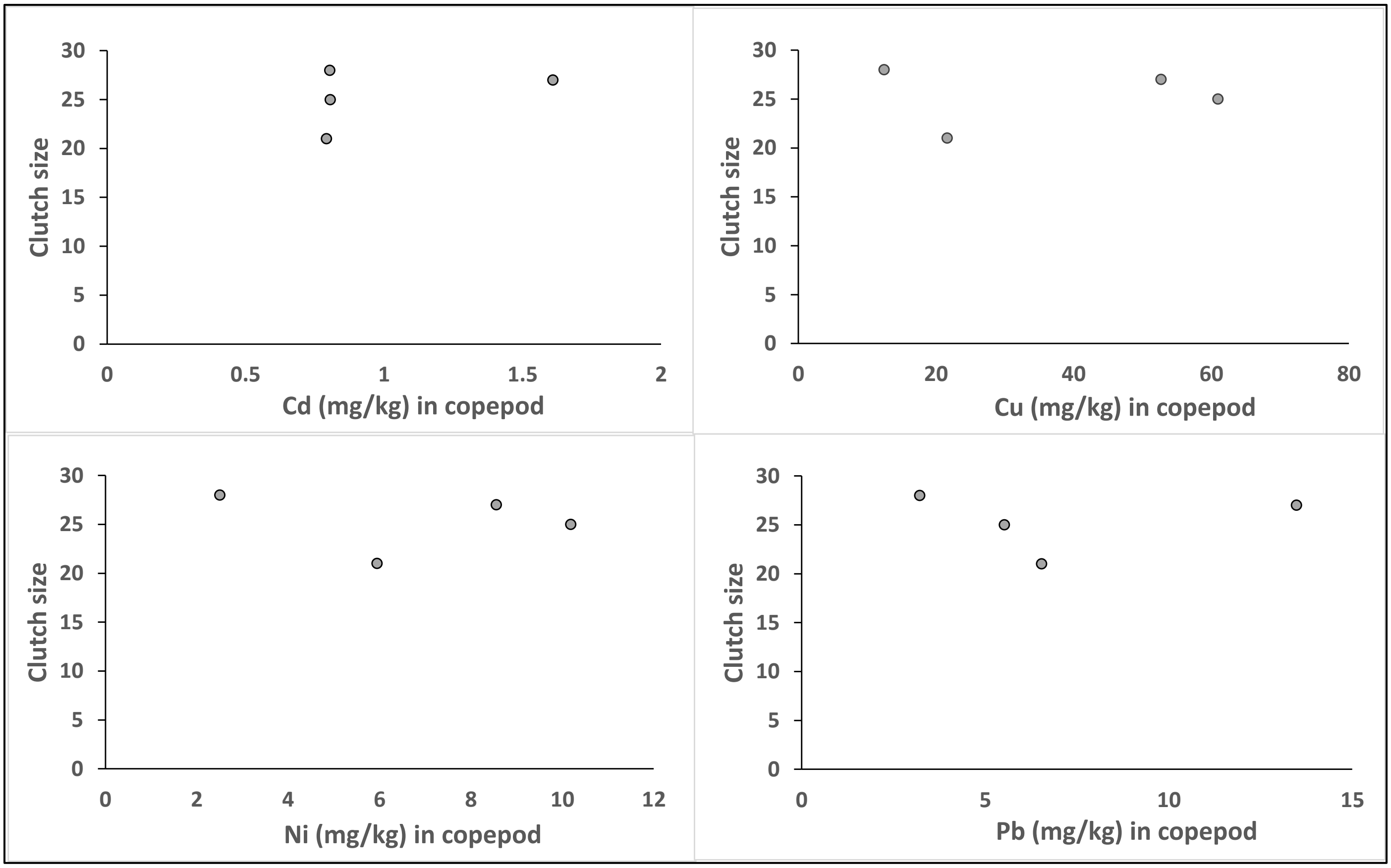

Fig 5.9 c-1 Relationship between metals bioaccumulated in copepods and clutch size in extract treatment. No trend in general was observed.

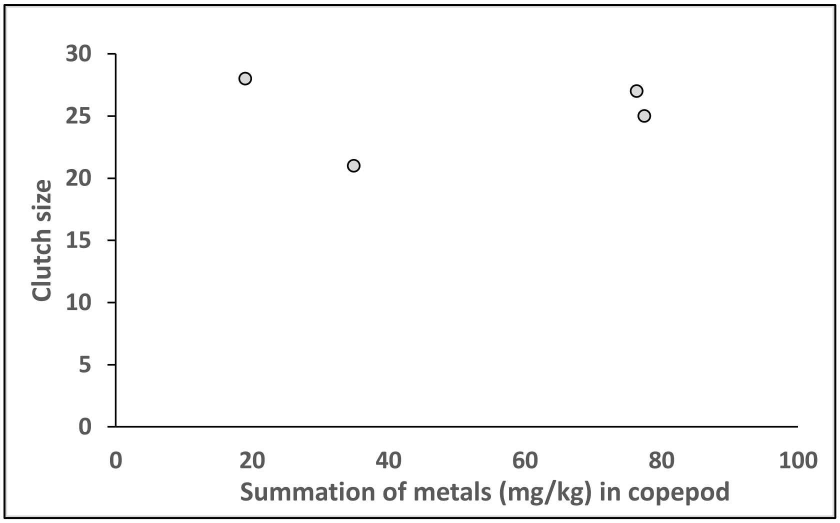

Fig 5.9 c-2 The comparison between clutch size and the summation of the concentration of metals in *E. affinis* in extract treatment.

Chapter 6 – General conclusion

To summarize the answers of few scientific questions those were raised in this thesis is a compilation of the diverse responses of the two crustacean models that was chosen in response to several environmental factors. We tried to say the story of their stress, their current state in the natural habitat, their surrounding ecosystem in general, few changes that they encountered with growing pollution thereby underlying the struggle of such communities in brief.

6.1 The habitat

We began the thesis by carefully observing and understanding the habitat of *A. edulis* (mud shrimp) and depicting some interesting facts and figures that was documented for the first time through this thesis (Chapter 2). Although *A. edulis* is both economically and ecologically an important member of the benthic diversity in South East Asia, not much work has been done based on their ecology, physiological aspects or even feeding strategies because of the difficulties in culturing them. Through this thesis, we tried to comprehend the role of this shrimp as an ecological engineer and the phenomena of how they accumulate finer particles in their burrow. Further to dig deeper, we compared all the morphological characteristics of the outer wall of the burrow and the burrow lumen, which led us to very precise information on the organic content, the maximum width, height, burrow diameter, void ratio etc. We showed statistically as well as experimentally that *A. edulis* selected specific sediments based on particle size and they changed the sediment characteristics by their burrowing activity. This unique ability to either choose or produce finer sediments for building their burrow was noticed. The outer morphological structure of the burrow wall differed greatly from the inner structure. The outer wall was thick with an accumulation of clayey particle. These clayey particles formed the burrow wall, which showed a low void ratio, thereby indicating a low permeability and higher sheer strength to protect the mud shrimp living inside the burrow. The burrow wall of *A. edulis* had almost 24 times higher organic content than one individual of mud shrimp. The shrimp might sustain its life with the available organic matter inside the burrow. These findings about the unique behavior of the mud shrimp *A. edulis* reflected change or alteration of the mud flat characteristics and as a consequence a huge ecological impact. The particular mechanisms of fine sediment acquisition while building the burrow and the quantification of burrow strength of the mud shrimp *A. edulis* demands for in depth follow-up studies.

6.2 Effect of Cadmium exposure

Back in 1817, Cadmium (Cd) was discovered by Friedrich Stromeyer in Germany and after several investigations he considered this element to be toxic for human health that often can lead to digestive disorder affecting kidneys, pulmonary damages and also affect the bones. Since then this element has been a major concern for every environmentalist and toxicological researchers attracting huge scientific attention. It is known that Cd is implemented in industries for over a long period and is still in use although knowing the fact that it has severe toxicity and can harm the organisms in the ecosystem. As mentioned in chapter 2 and chapter 3, *A. edulis* is a seafood delicacy in South East Asia and is largely consumed by many section of the society. The western Coast of Taiwan is one such place where there is abundance of *A. edulis* on one hand but on the other the vicinity is also enclosed by several industrial parks. Studies have shown that a lot of trace metals are released from the nearby industries in this area amongst which Cd is abundant and is much more bioavailable to sediment bioturbators actively increasing the enrichment factors over the past decades (Huang and Lin 2003; Peng et al. 2006; Chen et al. 2007). Through this thesis we noted the alteration of oxidative enzymes including the morphological damage that increasing concentration of Cd can potentially do to benthic crustaceans. In conclusion, the activities of the antioxidant enzymes (SOD, CAT, and GPx) in all the three organs namely, gills, hepatopancreas and muscles, decreased with increasing Cd concentration and extended exposure time in the mud shrimp *A. edulis*. Increasing Cd concentration led to an increase in ROS and resulted ultimately in membrane lipid peroxidation. Significant damage to the membrane structure of the hepatopancreas of *A. edulis* was noticed at a higher concentration of Cd, thereby proving that histological analysis was highly sensitive for water quality and benthic sediment composition assessment in aquatic systems. Conclusively, this study demonstrated that Cd could induce structural and biochemical changes in *A. edulis.* Changes in the activities of antioxidant enzymes and damage of cellular structure affecting the physiological functions with the exposure of cadmium indicate that mud shrimp populations are threatened at the industrial site where they were collected.

6.3 Role of Organic Pollutants

According to the aim of our thesis (section 1.6) as we proceed answering different facts about the macro-crustacean mud shrimp, we even thought of analysing Persistent Organic Pollutants (POPs) in that area. This idea was primarily cultivated to comprehend the other stresses on *A. edulis* apart from heavy metals as discussed in chapter 3. Some monitoring programs showed lower density of *A. edulis* in the recent past, thus a holistic toxicological study along with bioaccumulation of POPs was worthwhile and is first documented through this thesis. The western coast of Taiwan being a hub of industrial parks can perhaps benefit from the figures and facts laid in this thesis and undergo a habitat conservation plan for these benthic engineers. Conclusively, this paper presents the first comprehensive survey of PAHs, PBDEs, Organochlorine pesticides and PCBs in sediments from Changhua County, Taiwan providing useful information on concentrations, composition and sources. The spatial distribution of POPs showed that the proximity to sources was the most important determining factor for the distribution of these contaminants. In general POPs concentrations were greater in those samples collected near the industrial area (C and D) when compared with those from the non-industrial locations (A, B and E). Molecular indices such as Fl/202, IP/276, An/178 determined the existence of both pyrolitic and petrogenic inputs in the area; further, the use of PCA enabled the classification of sampling sites in accordance to their main PAHs source. Considering PCBs, Aroclor 1016 and 1260 were assessed as the main Technical sources for the area. Although levels were below the scientific sediment guidelines, DDT inputs were demonstrated to be quite recent for the area. Beyond the anthropogenic impact on the sediment, POPs appeared to pose a rudimentary or marginal risk in regards to its effect on the benthic organisms.

6.4 Effect of combined heavy metals and sediment in resuspension

Conclusively, the overall response or the sensitivity of *E. affinis* is diverse towards different types of pollutants and environment depending on various physical and chemical parameters. Exposure to the mixture of trace metals showed varied levels of toxicity in different stages of copepod and in different generation. The ability to accumulate trace metals in each generation also showed varied levels of trace metal concentration in *E. affinis.* The total population was found to be the lowest in the 2^{nd} generation (F1), in all the treatments universally and thereby mortality was found to be the highest in this generation for all treatments. In fact, also the bioaccumulation of all the metals was found to be the highest in this generation. Thus, as a link we assume that perhaps the bioaccumulation of metals from the exposed conditions like heavy metal or even from sediment was higher in this generation and as a result we found higher mortality of copepods thereby reducing the total population of *E. affinis* in this generation. Further when the clutch size of ovigerous females were analyzed we found lower clutch sizes in the 2^{nd} generation (F1) for control and sediment treatment but not for the heavy metal and extract, thereby partially confirming the assumed link between higher bioaccumulation and higher mortality with reduced clutch size and total population, in general. We found that in case of sediment, the % of males was less than the heavy metal treatment and also the control. This observation can slightly indicate the different ways of copepod sensitivity to heavy metals and sediment in re-suspension when exposed for multiple generations. A morphological inspection of the female ovigerous copepods in each generation showed us varied prosome lengths and other factors. Furthermore a comparison between the prosome length and the clutch size gave significant correlations showing higher values of prosome length in control and the lowest in the heavy metal treatment. The result overtone that perhaps the toxicity from the heavy metals affected the size of the copepods, which in turn slightly affected the number of egg production and the total population in each treatment and generation. Thus, fecundity and survival seems to be linked to the bioaccumulation of heavy metals thereby concluding that the sensitivity or fitness of *E. affinis* was directly connected to the trace metal accumulation in the copepod. Hence, the use of *E. affinis* as a biological tool to analyze various environmental factors and their relative toxicity to this calanoid copepod was the underlying inference obtained through this study.

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2013 - 2014	Project Assistant Fellowship	CSIR- National Institute of Oceanography, Goa. Council of Scientific and Industrial Research (CSIR), India
2015 (September) - 2018 (March)	PhD scholarship	National Taiwan Ocean University (NTOU), Taiwan, R.O.C
2018 (April) - 2018 end	NTOU President Scholarship for Co-tutorial research scholars	National Taiwan Ocean University (NTOU), Taiwan, R.O.C
2019 (Jan) – 2019 (June)	International mobility grant of University of Lille	University of Lille, France

Awards

Year of conduct	Name of Conference	Organization	Position
October 2015	The Crustacean Conference (Oral presentation)	Shanxi University, Taiyuan, Shanxi province, China	Best presentation award
November, 2016	IMB Academic Poster Competition	National Taiwan Ocean University, Taiwan	2nd position
May, 2017	Aspects of Oceanography (Oral presentation)	Oceanographic Society of Republic of China (OSROC), Kaohsiung, Taiwan	1st position
October, 2017	IMB Academic Poster Competition	National Taiwan Ocean University, Taiwan	2nd position

Research Experience

Master thesis: -

Impact of Gangasagar festival on the hydrological parameters and phytoplankton and micro zooplankton abundance in the mouth of the Ganges estuary, West Bengal, India.

*The main objective of our case study is to find out the ultimate stress of this large event on the adjacent biodiversity of phytoplankton and tintinnids. The hydrological parameters were also analyzed and correlated with the phytoplankton and tintinnids abundance.

Doctoral thesis: -

Effect of environmental factors on the behavior and life traits of a macro-crustacean (shrimp) and micro-crustacean (copepod)

"Burrow characteristics of the mud shrimp *Austinogebia edulis*, an ecological engineer causing sediment modification of a tidal flat"

*Our study highlights the potential of mud shrimps to modify sediment characteristics of the tidal flat by its burrowing behavior.

"Effects of cadmium exposure on antioxidant enzymes and histological alterations in the mud-shrimp *Austinogebia edulis* (Crustacean: Decapoda)"

*Our study shows the change in the enzyme activity and the tissue damage because of cadmium exposure.

"Spatial and temporal distribution of persistent organic pollutants (POPs) in sediment near Changbin Industrial park, Changhua, Taiwan"

*Our study highlights the POPs concentration in the sediment around an the western coast of Taiwan, along with sources and composition of POPs. It also reports on the ecological risk assessment of ***Austinogebia edulis*** in that area.

"Bioaccumulation of a combination of heavy metals in *Eurytemora affinis* as a bioassay to assess marine sediment quality"

*Our study highlights the potential of micro crustaceans (copepod, *Eurytemora affinis*) to bio-accumulate a pool of heavy metals from water and/or sediment or elutriate of sediment in a multi-generation approach.

"Enzymatic alteration in Copepod *Eurytemora affinis,* when exposed to a pool of Heavy metals (Pb, Cd, Cu and Ni)"

*We shall try to find out any alteration in enzymatic activity after exposing the copepods to a pool of heavy metals. Precisely, we shall find out changes in Acetylcholinesterase (AChE), GSH, digestive enzymes, lipid contents etc.

Experimental Skills

Flow cytometer, Gas Chromatography Mass Spectrometry, Spectrophotometer, Scanning electron Microscopy, Transmission electron Microscopy, Micro plate reader for enzyme activity.

Computing Skills

Writing packages: Microsoft office, power point, excels, Mactex (Latex)

Plotting packages: SPSS.

Field Work Experience

- I have experience in sailing several times to the shallow Hydrothermal Vent area (Guishan Island) in the northern part of Taiwan.

- I have fieldwork experiences in the intertidal area of the western coast of Taiwan in order to sample the benthic organisms in the mud, clayey or sandy tidal flats. We majorly collect shrimps living in the burrows, which can be around 1m deep or even more.

- I have gone to Sundarbans, the largest mangrove forest (Lothian island), which is situated in West Bengal, India. This wild life sanctuary includes estuarine crocodile, olive ridley sea turtle, deer, king cobra etc.

- For my Master's thesis I have extensively visited and resided at interior parts of Sagar Island in the Ganges delta, lying on the continental shelf of Bay of Bengal, India, for vigorous sampling at regular intervals.

Publications

- Das S, Tseng L-C, Wang L, Hwang J-S (2017) Burrow characteristics of the mud shrimp *Austinogebia edulis*, an ecological engineer causing sediment modification of a tidal flat. PLoS ONE 12(12): e0187647. https://doi.org/10.1371/journal.pone.0187647

- Das S, Tseng L-C, Chou C, Wang L, Souissi S, Hwang J-S (2018) "Effects of cadmium exposure on antioxidant enzymes and histological alterations in the mud-shrimp *Austinogebia edulis* (Crustacean: Decapoda)". Environ Sci Pollut Res (2019) 26: 7752. https://doi.org/10.1007/s11356-018-04113-x

- Shagnika Das, Andres Arias, Jing-O Cheng, Sami Souissi, Jiang-Shiou Hwang, Fung-Chi Ko (2019). Spatial and temporal distribution of persistent organic pollutants in sediment near Changhua Industrial park, Taiwan. (Under review).

- Tseng L-C, Huang S-P, Das S, Chen I-S, Shao K-T, Hwang J-S (2019). The hidden symbiotic slender goby found in burrows of mud shrimp *Austinogebia edulis* in western Taiwan: Exploration of symbiosis and ecology. PLoS ONE 14(7): e0219815. https://doi.org/10.1371/journal.pone.0219815

- Shagnika Das, Baghdad Ouddane, Jiang-Shiou Hwang, Sami Souissi (2019). Effects of sediment in re-suspension and mixture of heavy metals on the calanoid copepod *Eurytemora affinis*- a multi generation approach. (In Preparation).

Appendix 2

A near future perspective

To further get a comparison between macro and micro crustaceans, a follow up study similar to the third chapter is almost on the verge of the analysis. Copepods (micro-crustacean, *E. affinis*) shall be exposed to a mixture of heavy metals along with a negative control of Cadmium (Cd). Thereafter an acute exposure of 96 hours, AChE (Acetylcholinesterase, a neurotransmitting enzyme) and GST (Glutathione *S*-transferases, a detoxifying enzyme) activity along with digestive enzyme activities has been of our interest. Enzyme activity will be measured each day at 24 h, 48 h, 72 h and the end point 96h to get the daily variation of the activities when exposed to combined metals and only Cd along with the residual metal concentration in the water of each experimental tank. All these results shall be compared with the T=0 individuals, which means the batch of copepod from where we shall start the experiment. A pool of T=0 individuals shall be initially analyzed for all the enzyme activities and also the amount of heavy metals bioaccumulated in the copepods body as well as in the culture water. We target to show that functional indicators of environmental stresses could be highly useful in estimating how environmental changes affect the lower levels of the food web. These above issues have been scarcely investigated considering their undeniable significance for the correct functioning of the whole ecosystem, and also for directing proper actions for the protection and management of the marine environment.

Exposure	**Concentration**	**Replicates**	**Period of exposure**	**Sampling time of water and copepod**	**No. of individuals****	**Enzymes to be tested**
Control Seawater	Autoclaved seawater (salinity-15)	2	96h	24h—48h—72h-96h (end point)	A pool of 100 copepods at each sampling	AChE +GST expressed in ml/mg of protein
Cadmium (Cd)	Seawater + 10% lc50 of Cd	3	96h	Same as above	Same as above	Same as above

Cd+ Lead(Pb)+ Copper(Cu)+ Nickel(Ni)	Seawater + 10% lc50 of all metals (mixed)	3	96h	Same as above	Same as above	Same as above

**No. of copepods to be sampled in each day is to be finalized after trials.

***Copepod at t=0 and copepod at t= 96h has to be stored in filter for analyzing bioaccumulation of heavy metals. The water samples will also be analyzed for metal concentrations.

****Copepod remaining at 96h in all the treatments will be divided into two halves (by volume). One half will be stored for counting population and the other half will be filtered for bioaccumulation.

Appendix 3

Parallel work on symbiotic fish in shrimp's burrow-

<PLOS ONE>

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The hidden symbiotic slender goby found in burrows of mud shrimp *Austinogebia edulis* in western Taiwan: Exploration of symbiosis and ecology

Li-Chun Tseng[1,2,+], Shih-Pin Huang [3,+], Shagnika Das[1], I-Shiung Chen[1], Kwang-Tsao Shao[1], Jiang-Shiou Hwang[1,2] *

Overview

This investigation documents a unique and rare symbiotic goby *E*. cf. *gilli* associated with the mud shrimp *A. edulis* from the mudflat in western Taiwan. The population size and biogeographic distribution of *E*. cf. *gilli* in Taiwan needs to be studied in details in the future to provide its sustainable conservation. Furthermore, previous records demonstrate that *E*. cf. *gilli* is distributed in the marine waters of China, Korea, Japan, and the present report from mudflats of the western Taiwan coast. Studies of the phylogenetic relationships of *E*. *gilli* are needed, to better understand their population differentiation and geographic distribution.