Behavioural ecotoxicology of the brown shrimp, *Crangon crangon*: changing colour in polluted environments

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This thesis is presented to the School of Environment & Life Sciences, University of Salford, in fulfilment of the requirements for the degree of Ph.D.

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October 2019

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Al = Aluminium

As(III) = Arsenite

As(V) = Arsenate

As = Arsenic

AsB = Arsenobetaine

B to W = black to white direction

Ba = Barium

BPDH = Black pigment dispersing hormone

Ca = Calcium

cAMP = Cyclic adenosine monophosphate

Cd = Cadmium

cGMP = Cyclic guanosine monophosphate

Cr = Chromium

CRM = Certified reference material

Cu = Copper

DMA = Dimethylarsinic acid

Fe = iron

FZD= Furazolidone

Gt= Gentamicin

H_2O_2 = Hydrogen peroxide

HNO_3 = Nitric acid

HPLC-ICP-MS = High-performance liquid chromatography-inductively coupled plasma mass spectrometer

iAs = Inorganic Arsenic

ICP-AES = Coupled Plasma Atomic Emission Spectrometry

ICP-MS = Inductively coupled plasma mass spectrometry

ICP-OES = Inductively Coupled Plasma Optical Emission Spectrometry

K = Potassium

Li = Lithium

LOD = Limit of detection

MBC = Mersey Basin Campaign

MMA = Monomethylarsonic acid

Mn = Manganese

Mg = Magnesium

Na = Sodium

orgAs = Organic Arsenic

OTC = Oxytetracycline

OXA = Oxolinic acid

Pb = Lead

PCB = Polychlorinated biphenyl

ppm = Part per million

psu = Practical salinity units

W to B = white to black direction

Zn = Zinc

Dedication

This thesis is dedicated to my wonderful and supportive parents, brothers and sisters.

Acknowledgments

First and foremost, I am grateful to the Almighty Allah for bestowing His blessings on me and giving me the strength, knowledge, ability and opportunity to successfully undertaking this research study with perseverance, determination and tenacity.

I would like to express my sincere appreciation and thanks to my supervisors **Dr Debapriya Mondal** and **Dr Chiara Benvenuto** for their help, support, motivation and guidance throughout my PhD journey. I would like to thank you for steering me in the right direction during my research and for allowing me to grow as a research scientist. Your advice on research has been invaluable and inspirational.

My sincere thanks also go to my family, especially **Parents**, for their continuous support and encouragement during my research time. I hope that I have made you proud. Furthermore, I extend my thanks to my brother **Omar** for helping me collect the samples, and his wife **Wajd** for being beside me and cooking for me if needed. Thank you both for everything.

I would also like to acknowledge **Dr Chloe James's** contribution to chapters 2. The Arsenic speciation part in chapter 4 would not have been possible without the help of **Professor Jörg Feldmann** and **Dr Andrea Raab**. I greatly appreciate the opportunity they offered me to work with them at the University of Aberdeen. I would like also to thank **Savarin Sinaviwat** for her help in the laboratory while I was working at the University of Aberdeen.

I would also like to thank **Paulo Marini** and **Mike Ratcliffe** from the Aquaponic laboratory for their contribution in building up the new rearing system for the shrimp and **Paul Oldfield** for his help in accessing the Mersey Estuary.

I also take this opportunity to thank **Hector A. Velencoso**, **Andjin Siegenthaler**, **Michelle Santos, Chinedu Ifeoma, Rachael Fraser**, and **Chayan Munshi** for their help in the laboratory and collecting the shrimp from the field.

My deep appreciation goes out to my best friend ever **Sultana Al Harthi** for her loving support and without her motivation, I would not have had the strength to finish this journey. I am also very grateful to all my friends here in the UK and in Saudi Arabia for their support.

I would like to thank the UK **NHS** and especially all those people working in the transplant unit in Manchester Royal Infirmary for taking care of me and supporting me back in 2016 when I was diagnosed with a kidney failure, and when I had a kidney transplanted in 2018 and until now.

Last but not least, I sincerely thank **The Ministry of Higher Education in Saudi Arabia** for funding my research.

Abstract

The brown shrimp, *Crangon crangon* has a great ecological value in UK estuaries and coastal areas and it is also a commercially important species. It utilizes chromatophores (specialized cells containing pigments) to match its background, changing its colour either from pale (pigments contracted) to dark (pigments expanded) or vice versa, thus becoming almost invisible in its environment. Chromatophores also protect the shrimp (at least in the larval stages) from UV light. The control of pigments depends on hormones, secreted by the shrimp as a response to different stimuli (e.g., light, temperature and colour of substrate). Contaminants are found often and consistently in estuaries, due to human activities along the coasts and can potentially affect behavioural responses, acting as anthropogenic stressors. The use of colour change as a behavioural marker of pollution is considered here, as a potential effective tool to assess the initial stages of biological alteration in aquatic organisms. To test the efficiency of this novel technique, brown shrimp have been treated with non-lethal concentrations of cadmium (Cd) plus two different antibiotics [Gentamicin (Gt) and Furazolidone (FZN)] and arsenic (As). A great interindividual and interpopulation variability in response to the use of heavy metals and antibiotics was found. Therefore, colour change ability in the brown shrimp is not a sensitive behavioural ecotoxicological marker to detect early stages of contaminations in the environment, at least for Cd, As, Gt and FZD. Finally, given the high level of total As often detected in seafood, a speciation analysis was performed in the dosed specimens. The quantification of the various As species through time suggest the possibility of the brown shrimp to bio-transform inorganic arsenic (iAs) into the less toxic organic form [arsenobetaine (AsB)]. This has relevant implications for aquaculture and fisheries of crustaceans in areas contaminated by inorganic As: shrimp can be consumed by humans even if grown in contaminated areas, and potentially could detoxify water from iAs. However, more investigations are needed, leaving this field open to applied studies in the field.

1. Chapter One: Introduction and background

1.1 General introduction

It has become imperative to better manage wild caught food sources, due to the gradual depletion of natural food resources, including marine, and the requirement to feed the ever-expanding global population. The brown shrimp, *Crangon crangon* L. , which like many shrimps is exploited as a valued commercial species, is found in most aquatic environments, including the waters of the Atlantic Ocean, White Sea, Baltic Sea, Mediterranean Sea, North sea and Black Sea (Chak, Bauer, & Thiel, 2015; Gibson, Yin, & Robb, 1995; Kuipers & Dapper, 1984; Spaargaren, 2000); more recently its range has expanded to Icelandic waters (Gunnarsson, Ásgeirsson, & Ingólfsson, 2007). In the UK, the brown shrimp is one of the most important commercial species of caridean shrimp and is considered an important source of income (Tulp et al., 2016). It inhabits estuaries and shallow waters, which are often subject to environmental or anthropogenic pollution (Buccolieri et al., 2006). Heavy metals are often found in trace amounts in estuarine and marine environments and are transported to the sea through rivers by erosion of natural sediments and rocks. Cadmium (Cd) is a heavy metal with significant acute toxic effects on aquatic life due to its bioaccumulation in the food chain (Das & Khangarot, 2010). Another pollutant also detected in aquatic environments is arsenic (As), in both inorganic and organic forms (IPCS, 2001). Its effect on marine organisms is dependent on its form and certain other factors such as salinity and the uptake route (Meharg & Hartley-Whitaker, 2002; Ng, 2005; Zhang et al., 2018).

Not only heavy metals of natural origin but also man-made emerging contaminants threaten the marine environment. For example, the excessive application of antibiotics in recent years, for a variety of purposes (e.g., in animal husbandry practises to promote growth (Meek, Vyas, & Piddock, 2015), have raised concern for the spread of antimicrobial resistance, which can be exacerbated by the presence of heavy metals. For instance, the combined selection and co-selection of antibiotic-resistant bacteria can occur when antibiotics used prophylactically in animal production are discharged and reach the aquatic environment where heavy metals are already present (Seiler & Berendonk, 2012). Thus, the

agricultural overuse of antibiotics can affect both animal and human health (Goldburg & Naylor, 2005; Naylor & Burke, 2005).

A range of behavioural changes occur in animals exposed to pollutants, which may impact on their feeding, reproduction and predator evasion (Scott & Sloman, 2004). A fairly common behavioural response is feeding depression, a physiological/behavioural endpoint which allows quick examination when animals are subjected to chemical contamination (Barata & Baird, 2000; McLoughlin, Yin, Maltby, Wood, & Yu, 2000; Taylor, Baird, & Soares, 1998). Colour change is another sensitive, yet underused, indicator of response to contamination in some aquatic organisms (Meidivanto et al., 2018). The effects of contaminants on organisms in the aquatic environment cannot be assessed by mere chemical analyses, which can only determine the concentration of selected contaminants in the water and/or tissues (Oliveira et al., 2009; Palma et al., 2010; Wang et al., 2014). The combined use of biomarkers to detect the effects of contamination on aquatic organisms, along with chemical analyses to quantify environmental pollution, is a much more efficient strategy, because it can evaluate both the biochemical effects and the primary stage of biological alterations, including behavioural, in the organisms (Osman, Heuvel, & Van Noort, 2007).

Public awareness of the widespread use of heavy metals and antibiotics and their presence in the environment has stimulated interest in addressing the adverse effects, especially on commercially valuable species, of concentrations of these pollutants far exceeding background levels (Seiler & Berendonk, 2012).

While some promising advances have been made, very few studies have focused on specific behavioural responses that can be easily quantified and linked to physiological pathways, as biomarkers of toxicity in crustaceans in general and in *C. crangon* in particular. This multidisciplinary research will thus combine ecotoxicological (analysing natural and dosed concentration of Cd and As), behavioural (studying the ability to change colour to match the background) and microbiological (investigating the effects of antibiotics) aspects in the brown shrimp, *C. crangon*, using this species as a promising study system for behavioural ecotoxicological studies, while considering food safety.

1.2 The study organism – *Crangon crangon* L.

1.2.1 Habitat and characteristics

The brown shrimp, *Crangon crangon* (Figure 1.1), also called the common bay shrimp, is an oceanic coastal species which is widely distributed along European coasts from the Mediterranean and Black seas to the White Sea in the north of Russia (Gelin, Crivelli, Rosecchi, & Kerambrun, 2000; Muus, 1967; Figure 1.2) and has recently reached Iceland (Gunnarsson et al., 2007). Together with lobsters, various types of shrimps and prawns, crabs and crayfish, it belongs to the order Decapoda, one of the major crustacean groups. The name of the order is derived by the presence of five pairs of ambulatory thoracopods termed pereiopods, behind three pairs of thoracopods called maxillipeds because they serve as mouth parts and before the abdominal pleiopods, which females use to carry their eggs (Brusca & Brusca, 2003; Martin & Davis, 2001; Figure 1.4). The brown shrimp is included in the infraorder Caridea, family Crangonidae.

Figure 1.1 The brown shrimp, *Crangon crangon* L. (Eucarida: Caridea: Crangonidae)

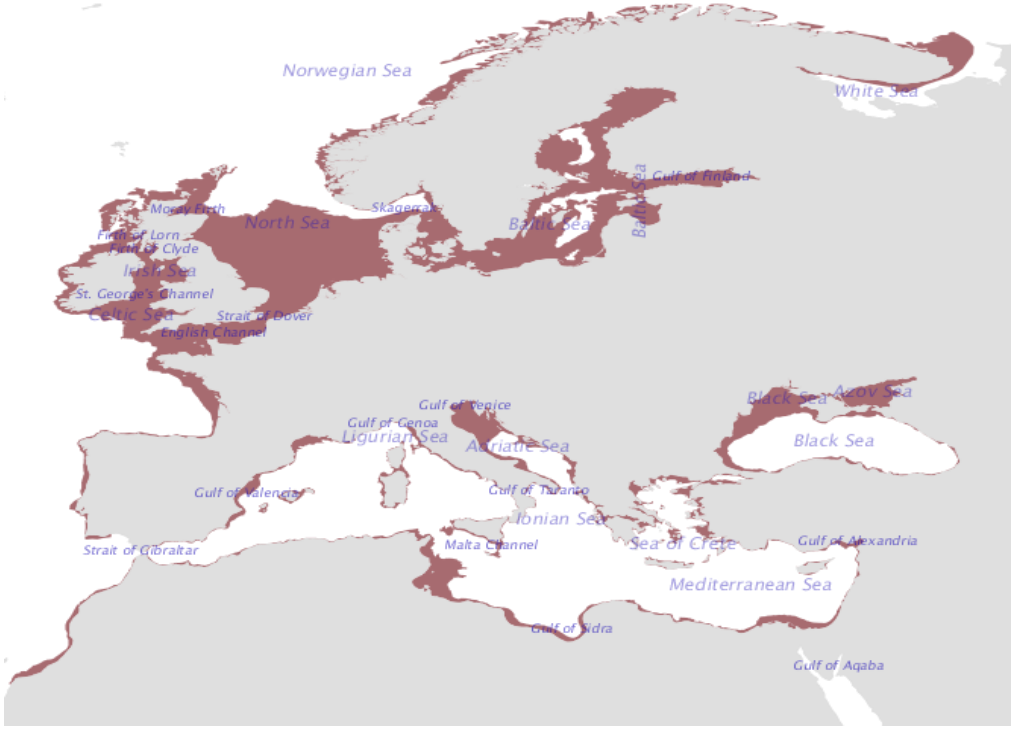

Figure 1.2 Distribution of the brown shrimp (FAO, 1980)

According to studies of its lifecycle, production and landings (Boddeke, 1979; Kuipers & Dapper, 1984; Tulp et al., 2016), the brown shrimp has been very heavily exploited by coastal fisheries in the North Sea, thus boosting the economy of this region significantly. Since 2000, landings have increased 40% compared to the 1980s and 1990s. In 2015, 31,375 tonnes of *C. crangon* were caught in the North Sea alone (ICES, 2016). Indeed, *C. crangon* is the basis of a large commercial fishery in northern European waters, particularly in Britain (Temming & Damm, 2002), where such fisheries are located in Morecambe Bay, the Solway Firth, the Wash, the Bristol Channel and the Thames Basin. The brown shrimp has not yet been used for aquaculture (Delbare, Cooreman, & Smagghe, 2015) but it can be used as a model system in the laboratory to check for emerging issues in the aquaculture of crustaceans. *Crangon crangon* is categorised as either a carnivorous opportunist (Pihl & Rosenberg, 1984), an omnivore (Kühl, 1972; Lloyd & Yonge, 1947; Muus, 1967) or a trophic generalist (Evans, 1983; Pihl & Rosenberg, 1984). Recent metabarcoding analyses of its diet (Siegenthaler, Wangensteen, Benvenuto, Campos, & Mariani, 2019; Siegenthaler, Wangensteen, Soto, et al., 2019) indeed reveal a great variety of items in the gut of this opportunistic and generalist predator/scavenger shrimp.

The abundance of *C. crangon* specimens in European estuaries makes it an essential constituent of those ecological units. In addition, it makes it a widespread food source for a large number of predators, such as flatfishes (order Pleuronectiformes), cod and other gadoids, wading birds, and other crustaceans (Del Norte-Campos & Temming, 1994; Henderson, James, & Holmes, 1992; Pihl, 1985; Walter & Becker, 1997). Conversely, numerous benthic species, including juvenile plaice and bivalve spat, are key food sources for *C. crangon* (Amara & Paul, 2003; Oh, Hartnoll, & Nash, 2001; Leif Pihl & Rosenberg, 1984; Siegenthaler, Wangensteen, Benvenuto, et al., 2019; Siegenthaler, Wangensteen, Soto, et al., 2019; Van der Veer, Bergman, Dapper, & Witte, 1991).

Given its life cycle (see section 1.2.2), *C. crangon* is tolerant of great variations in salinity (Mees, 1994; Mouny, Dauvin, & Zouhiri, 2000); it can live in a range of 0 to 35 psu (practical salinity units; Practical Salinity Scale 1978 [PSS-78]) and frequently occurs in waters of moderately low salinity between 1 and 5 psu (Boddeke, 1976; Criales & Anger, 1986). The ability of the brown shrimp to live in water with a wide range of salinity depends on a variety of factors, including sex (males are less adaptable than females), age and water temperature (Campos, Moreira, Freitas, & Van Der Veer, 2012). Thus, it is described as a euryhaline species (Campos & Van der Veer, 2008), even though larval stages do not tolerate well salinities below 25 psu (Delbare et al., 2015).

Jeffery & Revill (2002) and Lloyd & Yonge (1947) have shown that brown shrimp can live at temperatures ranging from 6 to 30 °C. In addition, this shrimp expresses an inclination to move towards deep waters during adverse winters as it favours high salinity at lower temperatures (Campos & Van der Veer, 2008). It is normally found at a depth of 20 m and tends to burrow into the sand as self-defence against predators (Lloyd & Yonge, 1947). Temperature and salinity also affect the migration and distribution of juvenile and adult shrimp in estuaries (Culshaw, Newton, Weir, & Bird, 2002).

1.2.2 Lifecycle

Reproduction takes place in more saline waters offshore, about 10 to 20 m deep, commonly in muddy or sandy areas (Henderson & Holmes, 1987). Fertilisation is internal and can occur only on recently moulted females (Delbare et al., 2015); females keep sperm and host their inseminated eggs until they are hatched (Boddeke, 1991). Breeding takes place numerous

times each year until the shrimp attain their maximum age of three to five years. Adult females are easy to identify when carrying eggs (Figure 1.3; Campos & Van der Veer, 2008; Muus, 1967).

Females migrate back towards coastal areas and estuarine nursery grounds, carrying their eggs attached to the pleopods rather than releasing them to float freely in the plankton. The size of the eggs depends on the size of the female and the season (Henderson, Seaby, & Somes, 2006). The eggs can mature faster at high temperatures. While an increase in temperature is thus important for egg development, a high temperature in low salinity (below 15 psu) leads to egg loss (Campos & Van der Veer, 2008).

Figure 1.3 Ovigerous *Crangon crangon* female

The eggs hatch as a simply-floating planktonic larval stage, then, two to five months later, the larvae settle and grow in shallow nursery regions in estuaries (Beukema, 1992; Boddeke, Dijkema, & Siemelink, 1976; Campos & Van der Veer, 2008; Heerebout, 1974). As they continue to grow, the adults migrate to offshore waters, where they breed. Due to the inflexibility of the brown shrimp's exoskeleton, growth is uneven and occurs through numerous moulting cycles, in which the exoskeleton is shed, an expansion in body volume follows and a new soft skeleton is formed then quickly toughens with deposition of calcium and magnesium carbonate ($CaCO_3$ and $MgCO_3$; Delbare et al., 2015; Smaldon, 1978). In their vulnerable soft condition, moulted shrimp can be easily cannibalised by conspecifics (Delbare et al., 2015). The status of moult and moulting itself are affected by several factors;

moulting increases when the water temperature is high, from an average of 5.9 at 12 °C to 7 at 18 °C (Criales & Anger, 1986).

The morphological differences between sexes, especially under 20 mm in length, are not instantly noticeable (Meredith, 1952). The three central morphological characteristics of pleopods by which the sexes are differentiated in adults are the outer (olfactory) branch of the first antenna (shorter in females and with no olfactory hairs) and particularly the endopod of the first pairs (longer in females) and second pairs (characterized by an appendix masculine in males, not present in females; Figure 1.4; Campos & Van der Veer, 2008).

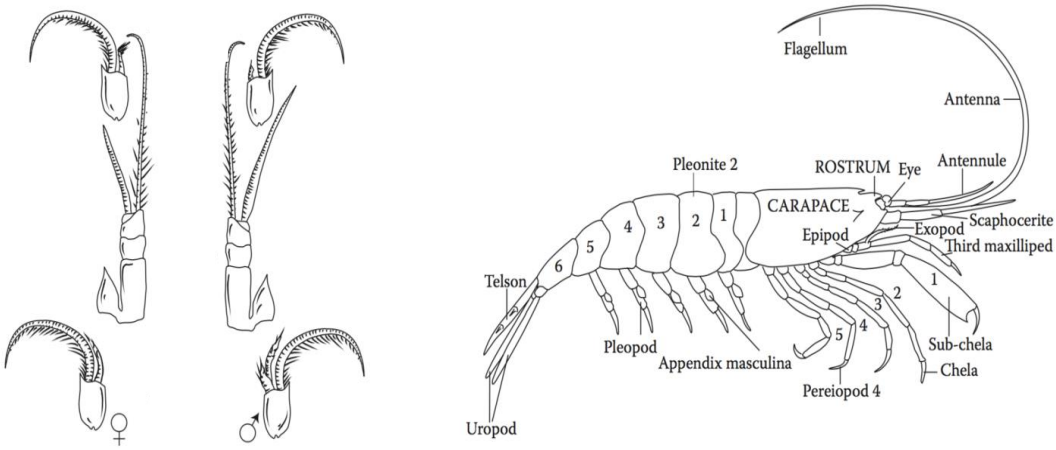

Figure 1.4 Morphological differences between male and female in *Crangon crangon* (Campos & Van der Veer, 2008)

Depending on temperature conditions, *C. crangon* males generally mature in the first year between 22 and 43 mm total length, whereas females reach 55 mm (Campos & Van der Veer, 2008). All shallow coastal waters such as estuaries serve as nursery grounds for brown shrimp throughout the primary stages of life (Amara & Paul, 2003; Cattrijsse, Dankwa, & Mees, 1997; Kuipers & Dapper, 1984). Their abundance is affected by seasons and migration patterns (Campos et al., 2010). Typically, the quantity of *C. crangon* in shallow water is larger in summer/spring (for juveniles) and smaller in winter/autumn, when adults longer than 50 mm in total length migrate to the North Sea (Rudolf Boddeke, Driessen, Doesburg, & Ramaekers, 1986; Campos et al., 2012). Their estuarine and shallow-water habitat often exposes brown shrimp to environmental or anthropogenic pollution, particularly by heavy

metals (Dauvin, 2008). Hence, it is important to investigate the impact of heavy metals and/or antibiotics on these crustaceans.

1.3 Colour change in animals

In order to avoid being spotted by predators, some species have developed different defence techniques involving camouflage and background matching (Booth, 1990; Merilaita, Scott-Samuel, & Cuthill, 2017; Price, Green, Troscianko, Tregenza, & Stevens, 2010; Stevens, 2016; Stevens & Ruxton, 2019). Colour change is one strategy to conceal an animal presence in the environment and it is widely used among animals (Figon & Casas, 2018; Stuart-Fox & Moussalli, 2009), from arthropods (Umber, Fabricant, Gawryszewski, Seago, & Herberstein, 2014) to cephalopods (Gonzalez-Bellido, Scaros, Hanlon, & Wardill, 2018), reptiles (Stuart-Fox & Moussalli, 2008), fish and amphibians (Nilsson, Aspengren, & Wallin, 2013) It is ecologically important since it allows animals to adjust with spatial and temporal environment changes (Duarte, Flores, & Stevens, 2017). The background of the environment changes rapidly, sometimes within 24 hours, or longer (e.g., seasonally) which requires change in appearance especially in animals that have less mobility than the ones that can move and change their environment (Caro, Sherratt, & Stevens, 2016). Colour change ability in animals depends on how fast the environment changes, animal mobility, the presence of alternative habitat and the spatial scale of change (Caro et al., 2016). Colour change is not only restricted to animal, but also includes plants (Cuthill et al., 2017).

Hence, studying colour change systems is important to understand camouflage's adaptive value and animals' diversity, shaped by physiological processes (Duarte et al., 2017).

Colour change strategies differ among species. Coohill, Bartell and Fingerman (1970) studied the pigmentary effectors that impact colour change in crustaceans. The sinus gland is considered to be a storehouse for hormones that play a role in colour change when released (Fingerman, Jackson, & Nagabhushanam, 1998). Changing light is considered to be a stimulus that affects the release of these hormones (Coohill et al., 1970), as does circadian rhythm (Brown Jr & Sandeen, 1948; Darnell, 2012), temperature (Silbiger & Munguia, 2008), physical activity (Herreid & Mooney, 1984), environmental conditions (Hemmi, Marshall, Pix, Vorobyev, & Zeil, 2006), tides (Brown & Sandeen, 1948; Darnell, 2012) and stress (Zeil & Hofmann, 2001).

Crustaceans have two types of pigmentary effectors which controlled by neurosecretory products: retinal cells and chromatophores (Fingerman, 1966). Retinal pigments regulate the quantity of light hitting the rhabdom, a transparent crystalline structure located in the compound eyes of arthropods and forming the photosensitive part of each ommatidium. They perform this regulation by obscuring the rhabdom when the light is strong and exposing it in weak light or in the absence of light. Migration of the retinal screening pigments occurs as an adaptation to light/dark photomechanical fluctuations in the compound eye; this may be limited to photoreceptor cells. In some species, it can include distal pigment cells and/or reflecting pigment cells (Rao, 2001). Chromatophores normally exist in the external cuticle; they make it possible for an organism to alter its colour as a result of the aggregation or dispersal of pigments (Reddy, Nguyen, Obih, & Fingerman, 1997).

Crustaceans have been found to have many pigments in their chromatophores: pteridines in leucophores (yellow, orange and red), carotenoids in erythrophores and xanthophores (yellow, orange and red) and ommochromes in melanophores (black; Czerpak & Czeczuga, 1969; Grynbaum et al., 2005; Nakagoshi & Negishi, 1992). However, true crabs (Brachyura) normally use melanin in their melanophores (Green, 1964).

Based on the findings reported in many studies, it is now evident that in a broad selection of decapod crustaceans, the chromatophores that are responsible for integumentary colour modifications are managed by antagonistically-acting pigment-spreading and pigment-aggregating neurohormones (Fingerman et al., 1998). Changes in the concentrations of intracellular couriers, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and Ca^{2+}, control the pigment migration in chromatophores. However, details of these processes are still unclear (Ribeiro & McNamara, 2007).

Normally, increases in the cAMP levels result in pigment dispersal, whereas aggregation occurs when the cAMP levels decrease (Nascimento, Roland, & Gelfand, 2003). Augmented cGMP levels (Ribeiro & McNamara, 2007) and intracellular free Ca^{2+} stimulate the accumulation of red chromatophores in caridean shrimp (Lambert & Fingerman, 1978, 1979; McNamara & Ribeiro, 2000); this causes blackening in crabs due to pigment dispersion (Ranga Rao & Fingerman, 1983). However, aggregation is not always correlated with an increase in Ca^{2+} (Kotz & McNiven, 1994). Another mechanism of colour change

ability in some crustaceans occurs as a result of combining morphological and physiological mechanisms. Wade et al. (2011) reported expanding in the pigment of *Penaeus monodon* alongside the accumulation of crustacyanin protein connected with free astaxanthin in the hypodermal when they were placed in a dark substrate. Whereas, the opposite was reported as an adaptation to a white substrate.

Colour change in organisms is normally influenced by environmental circumstances; for example, cloudiness impacts the light spectrum and an animal's vision in the water. Some types of fish such as *Perca fluviatilis*, which lives in water darkened by algal blooms, have been found to have dark body colouration, while the coloration of the same species living in experimental water containing clay tends to be lighter (Gusén, 2010; Hidayati, Sulaiman, Ismail, Shuhaimi-Othman, & De Bellard, 2017).

Colour changes can occur in organisms due to the presence of pigments as well as micro-scale structures. Moreover, colour can play both physiological and signalling roles (Tibbetts & Dale, 2004). Some colours are unchangeable and some changes are irreversible, including ontogenetic colour changes, which occur in some species as a result of individuals' normal progressive development (Booth, 1990). For the most part, however, it is beneficial for animals to have their colours revert to normal after stimulation by external or internal factors. An example of reversible change is the camouflage that some species use to conceal their location from prey or predators (Umbers et al., 2014). The changing of colour in animals is thus a suitable topic to study as it can be easily manipulated experimentally and it can cover both behavioural and physiological aspects, including the dynamics of community, behavioural ecology and animal physiology (Endler & Mappes, 2017; Gagliano, Depczynski, & Siebeck, 2015).

The range of techniques and roles of reversible colour alteration vary greatly among arthropods, more than in any other animal phylum, although all rely mostly on chromatophores, perhaps due to their unique possession of an exoskeleton, which may constitute an extra substrate where pigmentation can be altered (Umbers et al., 2014). In particular, crustaceans have been a valuable study system, and works on *C. crangon* have been pioneer in the field (Bomirski & Klek, 1974; Brown, 1941; Chassard-Bouchaud, 1965; Czerpak & Czeczuga, 1969; Elofsson & Kauri, 1971; Fingerman & Fingerman, 1972; Koller, 1927; Pautsch, 1953; Skorkowski, 1971; Skorkowski, 1973; Skorkowski & Kleinholz, 1973)

with a gap till more recent years (Siegenthaler, Mastin, Dufaut, Mondal, & Benvenuto, 2018; Siegenthaler, Mondal, & Benvenuto, 2017)

In all organisms, colour change can be classified into two main types, morphological and physiological, less commonly described in the literature as chromomotor and chromogenic (Umbers et al., 2014). Physiological colour change does not involve an alteration in pigment amounts, but merely their redistribution within the chromatophore. On the other hand, morphological colour change entails a quantitative increase or decrease of pigmentation. Integumentary pigments are now recognized as having beneficial biological functions which include protection against deleterious radiation, thermoregulation, camouflage and courtship (Fingerman, 1970). Colour change can be under hormonal control and is also affected by external factors such as temperature, colour of the background, time and light (Brown & Sandeen, 1948; Fingerman, 1970).

1.3.1 Physiological colour changes

Physiological colour changes are quick; they normally occur in a matter of minutes or hours after an animal has experienced internal or external stimulation (Filshie, Day, & Mercer, 1975; Key & Day, 1954; O'Farrell, 1964; Sumner, 1939; Umbers, 2011; Veron, 1973). This type of colour change can result from the movement of intracellular reflective granules and the dispersal and/or concentration of pigments in chromatophores (Filshie et al., 1975; Hadley & Oldman, 1969; Umbers et al., 2014; Vigneron et al., 2007). There are typically changes in the location of nanostructures or pigments and in the refractive index of the layers of the epidermis.

In arthropods, physiological colour alteration can be caused by five main factors: a) movement of the granules, as described in stick insects (*Carausius morosus*), b) hydraulic techniques, when water is used to change the refractive index of multiple layers, c) amoeboid chromatophore migration, found in tracheal air sacs, d) pigment diffusion and aggregation in the chromatophores and e) guanocyte retraction (Umbers et al., 2014). In crustaceans, the outlines of the chromatophores vary widely and the pigments aggregate and diffuse within their confines (Fingerman, 1970; Josefsson, 1975; Perkins, 1928; Stephens, 1962).

1.3.2 Morphological colour changes

Morphological colour change refers to alterations in any of the constituents that are directly connected to the visible colour of an animal by catabolism or anabolism. It occurs when one or more of the layers of the structures or pigments that are responsible for the colour of the animal are modified (Grether, Kolluru, & Nersissian, 2004). In general, morphological colour changes take place when the colour constituents are altered, for instance by minimising the oxidation of ommochrome pigments or by modifying the concentration of these constituents via combination, sequestration, deposition or even collapse. Morphological colour change tends to take a longer time to occur than physiological colour change; in fact, it can take a few days and, in some cases, weeks. Animals that undergo morphological colour change usually have the corresponding ability to retain their colour for a longer time than those that use physiological colour change (Umbers et al., 2014).

1.4 Contaminants - Heavy metals

Heavy metals, either essential in trace quantities or non-essential, are important pollutants in various aquatic systems, where they are introduced partially through natural and mainly through anthropogenic sources (for instance mining and industrial outputs; Marsden & Rainbow, 2004). Essential metals [manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), molybdenum (Mo), vanadium (V) and nickel (Ni)] are important micronutrients for many organisms because they are integrated into cofactors and enzymes (Matyar, Kaya, & Dinçer, 2008). However, since they bind to enzymes and DNA and produce oxygen radicals through the Fenton reaction, they are often poisonous in high concentrations. Non-essential toxic metals (As, Cd, etc…) are called instead ‘heavy metals’ (based on their high atomic weight; López-Maury, García-Domínguez, Florencio, & Reyes, 2002; Matyar et al., 2008). According to Nies (1999), Mo, Mn and Fe are physiologically essential with partial toxicity. The toxicity of trace elements often essential for metabolic purposes, such as Cu, Zn, chromium (Cr), Ni, Co, tungsten (W) and V, is highly dependent on concentration. On the other hand, the non-essential metals, such as mercury (Hg), Cd, As, silver (Ag), lead (Pb), uranium (U) and antimony (Sb), are toxic even at trace levels and some heavy metals (Hg, As and Cd) can generate harmful complexes (Fakhri et al., 2018; Nies, 1999). Thus, the excess presence in the environment of anthropogenic heavy metals is a considerable issue. The various uses of metals in anti-fouling products, pesticides, inorganic and organic fertilizers,

and in feed additives for agricultural (Han et al., 2002; Nicholson, 2003) and aqua cultural purposes (Burridge, Weis, Cabello, Pizarro, & Bostick, 2010) contribute to the increased contamination of the environment. The bioaccumulation and stability of heavy metals make them a very serious threat to aquatic animals (Fakhri et al., 2018).

Cadmium, one of the heavy metals investigated in the present study, is a major toxic threat to aquatic organisms. It is a non-essential toxic trace element, with naturally low concentrations of 0.001 to 0.0001 ppm near coastal areas and between 0.00025 to 0.0003 ppm in estuaries and rivers (Pavlaki, Morgado, Soares, Calado, & Loureiro, 2018). It occurs naturally and one of the most important natural sources that releases significant amount of Cd into the water environment is non-ferrous metal mines (Rainbow, 2007).

Normally, living creatures absorb Cd from the environment in two different ways: directly from the water and/or by consuming contaminated food. Gardner & Yevich (1969) and Jennings & Rainbow (1979) report that Cd has a negative impact on some marine animals. Most recent study has reported that Cd affect antioxidant enzymes of mud shrimp *Austinogebia edulis* (Das et al., 2019).

Earlier studies found that Cd affected the nervous system and sensory ganglia in mammals (Gabbiani, Baic, & Deziel, 1967). Jacobson & Turner (1980) report that Cd, Hg and Pb accumulate in the brain and that these metals prevent the release of sulfhydryl group-containing enzymes. Reddy & Fingerman (1995) found that when fiddler crabs were exposed to 10 ppm Cd for 10 days, the neurosecretory cells in the brain and the eyestalk ganglia were damaged.

Owing to its acute toxicity and potential bioaccumulation in invertebrates, especially molluscs, Cd is perceived to be one of the main risks to organisms' health (Das & Khangarot, 2010). While LC_{50} (the concentration that could kill 50% of a sample population) values are variable between freshwater and marine benthic species, Cd is lethal to all crustaceans at specific doses (Guner, 2010). In experiments studying the effect of Cd on *C. crangon*, it has been observed that increased levels of dissolved Cd in the water correlate with increased concentration of Cd in tissues and with increased mortality (Szaniawska, 1985). Jung & Zauke (2008) have shown that *C. crangon* is sensitive to the increase of external concentrations of Cd and Pb. Some studies have used Cd to examine different behavioural

changes in crustaceans (Table 1.1**Error! Reference source not found.**) and report negative effects on marine organisms.

Table 1.1 Effects of Cd and As on some of behavioural and physiological aspects in crustaceans

Species	**Heavy metal**	**Concentrations**	**Physiological and behavioural endpoint**
Atyaephyra desmarestii	Cd + Zn	0.042 ppm Cd 5.43 ppm Zn	Feeding behaviour[1]
Echinogammarus meridionalis	Cd + Zn	0.036 ppm Cd 4.61 ppm Zn	
Macrobrachium sintangese	Cd	0.086 ppm	Structure of gills and hepatopancreas[2]
Balanus amphitrite (larvae)	Cd	Range from 0.20 to 1.36 ppm	Development and swimming behaviour[3]
Macrobrachium dayanum	Cd	Acute concentration: 0.15 ppm for male and 0.16 ppm for female Sub-acute concentration: 0.0375 ppm for male and 0.04 ppm for female	Behaviour, scaphognathite oscillation and heart rate[4]
Daphnia, Bosmia, Eudiauptomus and cyclopoid copepods	Cd	0.010–0.100 ppm	Feeding[5]
Hippolyte inermis	Cd	1, 2, 3.5 ppm	Locomotory activity[6]
Acartia tonsa, Palaemon varians	Cd	Range from 0.00059 to .00957 ppm	Adult survival, hatching success and larval development ratio[7]
Macrobrachium sintangense	Cd	0.00001 and 0.00002 ppm	Survival, osmoregulation and gill structure[8]
Crangon crangon	As(V)	Range from 0.1 to 20 0 ppm	Survival and metabolisms[9]
Artemia franciscana	As(V)	4, 8, 15, 31 and 56 ppm	Growth, survival, and reproduction[10]
Gammarus pulex	Cd and As(V)	Cd: 0.028 and 0.054 ppm As(V): 1.12 and 1.65 ppm	Ventilatory, locomotor and iono-regulation of $[Na^+]$ and $[Cl^-]$ in haemolymph[11]
Scylla serrata	As(III)	1, 2 and 3 ppm	Avoidance, hypersecretion of mucoid element and release of excess excretory products[12]

[1]Pestana, Ré, Nogueira & Soares, (2007); [2]Soegianto, Winarni, & Handayani, (2013); [3]Lam, Wo, & Wu, (2000); [4]Tripathi & Pandey, (2014); [5]Gulati, Bodar, Schuurmans, Faber, & Zandee, (1988); [6]Untersteiner, Gretschel, Puchner, Napetschnig, & Kaiser, (2005); [7]Pavlaki et al., (2016); [8]Putranto,

Andriani, Munawwaroh, Irawan, & Soegianto, (2014); [9]Madsen, (1992); [10]Brix, Cardwell, & Adams, (2003); [11]Vellinger et al., (2012); [12]Saha, Ray, & Ray, (2018)

However, no study has investigated the effect of Cd, either as a single contaminant or in combination with other contaminants like antibiotics, on the behavioural responses of the brown shrimp. Therefore, Cd was used in the present study, as reported in Chapters 2 and 3.

Another pollutant present in aquatic environments alongside Cd is As (Ji et al., 2016; Smedley & Kinniburgh, 2002), which is widely distributed and ranked 22nd in terms of its abundance in seawater (Azizur Rahman, Hasegawa, & Peter Lim, 2012; Neff, 1997; Smedley & Kinniburgh, 2002). Arsenic can be found in both sea and freshwater, but its average concentration in seawater, at around 0.0017 to 0.002 ppm (Madsen, 1992; Neff, 1997), is more consistent than in freshwater, the average in river water being 0.0008 ppm (Azizur Rahman et al., 2012).

There are many sources of As, both natural and anthropogenic. Among the latter, As is used as an agent for drying cotton, preserving food or wood, and in smelting and coal burning (Hutton & Symon, 1986; Madsen, 1992; Sanders, 1985). In the United Kingdom, the primary source of As is industrial riverine waste discharged into estuaries, where concentrations range from 0.00054 to 0.0041 ppm, followed by the emissions of coal-burning power stations into the air (Murcott, 2012). As most factories' waste is released into the water, aquatic organisms are exposed to high levels of As. While As has been detected in land animals, levels in marine animals are higher (Doyle & Spaulding, 1978). This may be due to the ability of aquatic organisms to accumulate As in their bodies, regardless of its form (De Gieter et al., 2002).

Figure 1.5 depicts some of the main species of As in the aquatic environment, showing a fundamental distinction between organic As (orgAs) and inorganic As (iAs) forms. Inorganic As is found mainly in natural water and sediment as arsenate [As(V)] or arsenite [As(III)], which are both highly toxic species with the potential to cause cancer, especially in humans (Foulkes, Millward, & Rattanachongkiat, 2004; Francesconi, Hunter, Bachmann, Raber, & Goessler, 1999; Petursdottir et al., 2012). In contrast, most orgAs is less toxic than iAs and typically predominates in marine organisms, mainly as arsenobetaine (AsB), beside some fractions of dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) (Madsen, 1992; Ng, 2005; Smedley & Kinniburgh, 2002). Organic As may be found in water that is

profoundly affected by industrial pollution (Smedley & Kinniburgh, 2002), while it is possible to find it in surface water due to biological activities (Smedley & Kinniburgh, 2002). Organic As species (particularly AsB) that could be in the water can be transformed into arsenate if present in oxic sediment and seawater (Neff 1997). Arsenite is more likely to occur where the environment is less oxidative, whereas As(V) is often present in more oxic waters (Azizur Rahman et al., 2012). Thus, As has a complex cycle in water and in the biota (Hasegawa et al., 2001). It has known for phytoplankton and marine macroalgae the ability of transforming iAs to orgAs and since it is a food source for some higher trophic level organisms (Al Mamun et al., 2019; Azizur Rahman et al., 2012), this may explain to the high level of orgAs found in aquatic organisms. Nevertheless, the metabolism, accumulation and toxicity of As varies, depending on several factors: the route of absorption, the presence and concentrations of various As species in the environment, temperature, salinity, moult status and the organisms' body size (Fowler & Yaşar Ünlü, 1978; Hasegawa et al., 2001; Madsen, 1992; Ng, 2005; Zhang, Chen, Zhou, Wu, & Zhang, 2016).

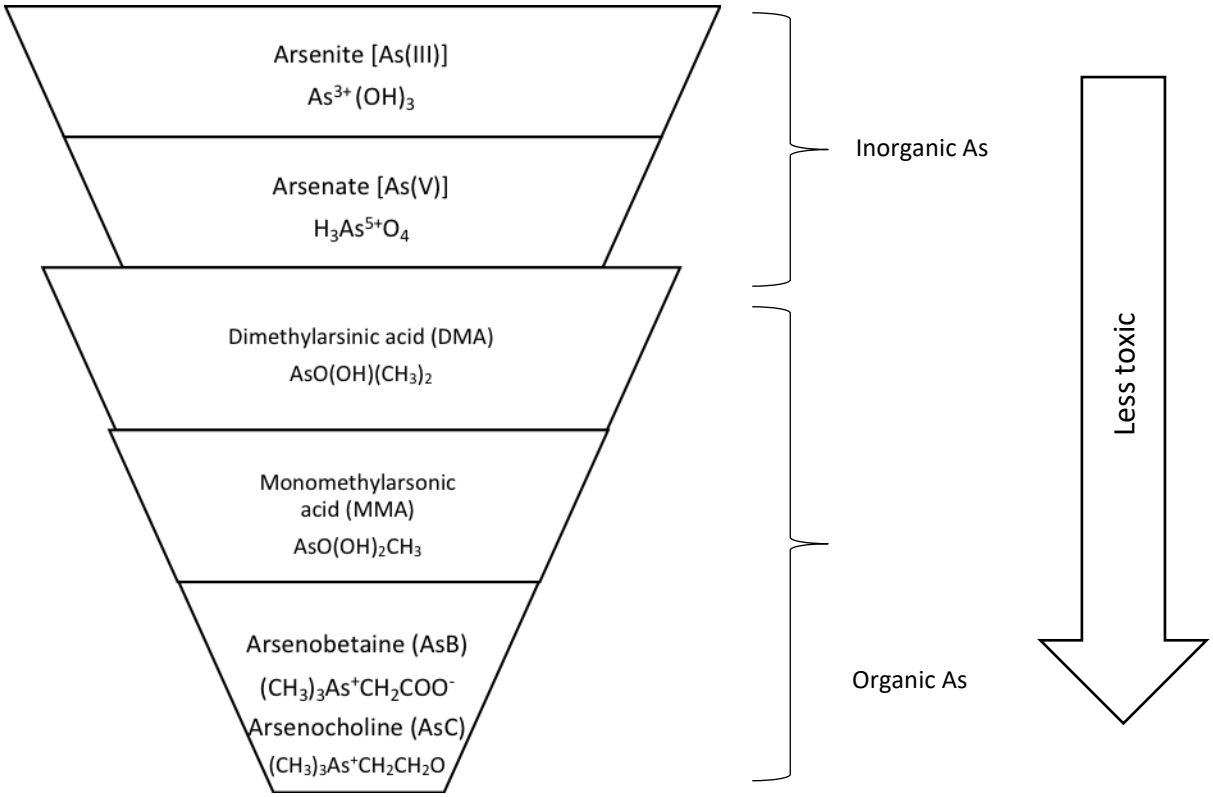

Figure 1.5 Summary of the main arsenic species found in the aquatic environment

Consumption of seafood is considered the main source of As for humans, especially in areas where it is not present naturally in drinking water or the food chain. The United States Food and Drug Administration (US FDA, 1993) reports that 90 % of the As in humans comes from consuming seafood, except in areas where drinking water is contaminated (Gao et al., 2018). Thus, As may affect human health indirectly through the food chain.

Some researchers have studied the effects of As at different concentrations on aquatic animals' behaviour and physiology. Erickson et al. (2010) report that As affected the growth of rainbow trout (*Oncorhynchus mykiss*) when they were exposed to iAs at 26–77 μg dm^{-3} through their diet for 30 days. Another study, conducted by Madsen (1992), found that survival of brown shrimp could be significantly affected if they were kept in a concentration over 25 ppm of As(V). It also found that smaller shrimp were more affected by iAs in terms of As accumulation, survival and respiration level. Hunter, Goessler and Francesconi (1998) evaluated the ability of *C. crangon* to accumulate As when exposed to iAs or orgAs. They found that As absorption depended on its chemical form and on route of exposure, whether through water or diet. These studies indicate that As toxicity experiments in aquatic organisms can be considered of great importance. It is also important to study As speciation in order to determine which species are toxic and which are less toxic to them (Zhang et al., 2018).

Although the toxicity of arsenic in aquatic organisms has been studied extensively, its ecotoxicity and especially its effects on colour change and its bio-transformation in *Crangon crangon* have not yet been investigated.

1.5 Role of antibiotics in aquaculture

Alongside the heavy metals discussed above, antibiotic-resistant bacteria are found in marine and freshwater environments, aquaculture, and in the soil (Kang, Shin, Yu, Kim, & So, 2018). The excessive use of antibiotics, including their use as growth promoters in animal husbandry, has raised concern for the spread of antibiotic resistance in the marine environment, since this can be exacerbated in the presence of heavy metals. For example, the combined effect of selection and co-selection of antibiotic-resistant bacteria may occur

when antibiotics from animal production and agriculture are released into the aquatic environment alongside heavy metals (Seiler & Berendonk, 2012).

In several developing and developed countries, industrial aquaculture is a fast-expanding industry (Cabello, 2006). It is anticipated that this development will intensify at a more rapid rate in the future, inspired by the market forces that globalize the food supply sources and the exhaustion of fisheries (Goldburg, Elliott, & Naylor, 2001; Goldburg & Naylor, 2005). There has been a quadruple increase in industrial aquaculture globally over the last 20 years (Naylor & Burke, 2005). This remarkable industrial growth has resulted in the release of large quantities of veterinary medicines into the ecosystem (Boxall et al., 2004); these prophylactic practices can negatively affect animal and human health (Cabello, 2006; Goldburg & Naylor, 2005; Naylor & Burke, 2005). The use of antibiotic prophylaxis in shrimp and fish aquaculture has led to an increased environmental presence of antibiotic-resistant bacteria (Alcaide, Blasco, & Esteve, 2005; Miranda & Zemelman, 2002b, 2002a; Petersen, Andersen, Kaewmak, Somsiri, & Dalsgaard, 2002). Increasing antimicrobial resistance in fish pathogens then stimulates the overuse of antibiotics in aquaculture, since their effectiveness decreases with increasing resistance (Davies et al., 1999). Thus, the occurrence of resistance to antibiotics in fish pathogens thwarts the success of the prophylactic application of antibiotics in aquaculture (L'Abée-Lund & Sørum, 2001; Sørum, 2006). Those present in aquatic systems can transmit their resistance by horizontal gene transfer to bacteria infecting humans and terrestrial animals (Kruse & Sørum, 1994; L'Abée-Lund & Sørum, 2001; Sørum, 2006). *Vibrio cholerae*, responsible for the Latin American epidemic of cholera that began in 1992, for example, seems to have developed antibiotic resistance due to interaction with antibiotic-resistant bacteria selected by means of the intensive use of antibiotics in the Ecuadorian shrimp industry (Weber et al., 1994).

Industrialised nations have regulated the use in aquaculture of quinolones, a class of artificial antibiotics used to treat bacterial infections, since each member of this group has the capacity to stimulate cross-resistance with other antibiotics, with the risk of creating a highly active group of antibiotic-resistant bacteria for human infections (Cabello, 2006; Gorbach, 2001; Moellering, 2005; Sørum, 2006). Flaherty, Szuster, & Miller (2000) assert that as many commercial shrimp feeds are supplemented with antibiotics, there is a strong

possibility that antibiotics are contained in some of the commercial feeds used in aquafarms.

In recent years, shrimp farming has become a major part of tropical aquaculture and this achievement has amplified the need to improve farming practices to capitalize on profits (Suzuki & Nam, 2018; Tendencia & Dela Peña, 2002). These developments have often been accompanied by increased risk of infections, as ecological conditions have declined and the impulse to increase production has led to dependence on antibiotics. Diverse medications are usually prescribed to farmed shrimp to enhance development and to prevent or treat diseases (Ali, Rico, Murshed-e-Jahan, & Belton, 2016; Tendencia & Dela Peña, 2002). In Philippine grow-out ponds, for example, synthetic feeds have been supplemented with furazolidone (FZ), oxolinic acid, chloramphenicol and oxytetracycline (Cruz-Lacierda, De la Peña, & Lumanlan-Mayo, 2000). Antimicrobial agents are generally employed in aquaculture throughout the production phases, both in hatcheries and grow-out processes.

In the European Union, the antibiotics permitted for use in aquaculture are trimethoprim, quinolones, tetracyclines, sulphonamides and penicillin, whereas the nitrofuran antimicrobials (FZ), chloramphenicol, nitrofurantoin, nitrofurazone and furaltadone have been barred from use in food manufacture for several years, because of their effects associated with drug resistance and aplastic anaemia, mutagenicity and severe nephrotoxicity (Conti et al., 2015).

1.6 Effects of contaminants on the behaviour of crustaceans

Behavioural responses constitute a sensitive biomarker in crustaceans. A possible biomarker is any measurable alteration resulting from stressors, such as disease states, xenobiotics and environmental changes (e.g. in temperature or salinity), which provoke an organism to adapt to cope with such conditions (Allen, Awasthi, & Rana, 2004).

A number of biomarkers are not sufficiently sensitive to distinguish contaminant contact or to reflect the effects of stressors that are present in the environment (Tu, Silvestre, Phuong, & Kestemont, 2010). It is worth mentioning that every broad-spectrum biomarker of pollution has some specific constraints; thus, it can be more efficient and successful to use a set of biomarkers (Smolders, Bervoets, Wepener, & Blust, 2003). The presence of several toxic compounds is specifically and quickly detected through physiological or biochemical

indicators (Tu et al., 2010). Using such behavioural responses can combine exogenous and endogenous aspects and therefore, provide a better understanding of the effects of environmental contamination not only at the individual level but at the community level as well (Little, Dwyer, Fairchild, Delonay, & Zajicejk, 1993). Many studies have investigated the behavioural effects of heavy metals on crustaceans. Those summarised in Table 1.1 are particularly concerned with Cd and As, being the target metals of the study reported in Chapters 2, 3 and 4.

When exposed to pollutants, animals exhibit a range of behavioural changes which affect predator evasion, reproduction and feeding, including avoidance of the contamination (Scott & Sloman, 2004). Behavioural analyses offer organically significant endpoints to assess sub-lethal contact effects and to complement standard toxicity tests. There are currently no adequate explanations of the connections between many exposure-related behavioural changes detected in the laboratory and significant ecological effects observed in the field. Numerous studies have shown that penaeid shrimp subjected to sub-lethal levels of pesticides display a number of behavioural changes, including hyper-excitability and restlessness, uncoordinated swimming motions, tremor in the appendages, sudden movements of chelate legs and spasms (García-de la Parra, Bautista-Covarrubias, Rivera-de la Rosa, Betancourt-Lozano, & Guilhermino, 2006; Reddy & Rao, 1990). In general, these changes are more obvious at higher concentrations of pesticides and are believed to result directly from the effects of pesticides on the nervous system. The following section examines further the effects of pollutants on behavioural responses.

1.6.1 Feeding rates and contaminants

A significant endpoint for estimating the response of an organism to chemical exposure has been found to be feeding depression, which can be rapidly assessed (Barata & Baird, 2000; McLoughlin et al., 2000; Taylor et al., 1998). Food is an essential requirement for development and other bodily processes (McWilliam & Baird, 2002). Many studies have found changes in feeding rate to be a sensitive marker of toxic stress in both marine and freshwater species (Maltby, Naylor, & Calow, 1990; McLoughlin et al., 2000).

Contamination causes stress, which produces nutritional changes leading to serious effects on many vital processes. It has been shown that deviations in the amounts of digestive

enzymes may be related to external factors and developmental cycles (Perera et al., 2008). Therefore, alterations in the rate of utilization of reserves or in the concentration of digestive enzymes may partially explain the major changes in feeding rate resulting from contact with contamination. On the other hand, a number of studies have found that feeding rate can be increased by exposure to contaminants, although this reaction may be due not to stimulatory effects but instead to the necessity for more energy to manage metabolic impairment (Bodar, Van Leeuwen, Voogt, & Zandee, 1988). Jensen, Forbes and Parker (2001) have shown that a reduced feeding rate at the individual level in some gastropod species is related to increased Cd pollution.

Food reserves constitute a significant environmental endpoint, as persistent energy shortage can cause a decrease in egg laying activity after starvation of 1 to 7 days in the snail *Lymnaea stagnalis* (Das & Khangarot, 2010).

1.6.2 Colour change and contaminants

Another sensitive biomarker of response to contaminants in some aquatic organisms is colour change. Its purpose varies among animals, which may use it as camouflage in order to evade predators (Llandres, Figon, Christidès, Mandon, & Casas, 2013), or conversely to warn them of their toxicity (Stevens & Ruxton, 2011), as a signal in social interactions (Tibbetts & Dale, 2004) and in some animals as a means of sexual attraction (Allen, Zwaan, & Brakefield, 2011).

The outer surfaces of marine animals tend to be anatomically and physiologically sensitive, especially in comparison to land-based animals (Akarte & Agnihotri, 2013). The colour of shallow-water shrimp is shaped by chromatophores (cells containing pigments responsible for body colouration) often combined in larger structures normally referred to as chromatosomes), which are found under the transparent exoskeleton (Siegenthaler et al., 2017). The alterations in colour are linked to the animal's age and gender. In addition, colour is articulated by chromatosome pigment diffusion, compactness and the order of the pigments (Bauer, 1981).

Brown & Wulff (1941) have reported that the chromatophore in *C. crangon* is one of the most complex components in terms of function and structure in crustaceans. Indeed, it has been found that even the same type of pigment in the same species under the same

experimental conditions can react differently if located in different parts of the body (Brown & Wulff, 1941).

Hormones in crustaceans have been found to be affected by organic or inorganic pollutants and contaminants in the environment. Pigmentary effectors, which are among the functions regulated by hormones, are also affected by heavy metals (Fingerman et al., 1998). Some studies have used the brown shrimp as a model organism to examine the toxicity of heavy metals, but none has studied the effects of As or Cd on colour change. Therefore, the effects of Cd and As on colour change in the brown shrimp are of interest.

Table 1.2 Effect of selected contaminants on colour change of aquatic organisms

Contaminant	**Species**	**Results**
Cadmium chloride	Fiddler crab, *Uca pugilator*[1]	Exposure by injection (8.5 ppm) or immersion (10 ppm) prevents the expansion of black pigments, because Cd affects the neuroendocrine complex in the eyestalk, reducing the release of black pigment dispersing hormone
Polychlorinated biphenyls (PCBs)	Fiddler crab, *Uca pugilator*[2]	Exposure to PCB preparation, Aroclor 1242 (2, 4 and 8 ppm) reduced the ability to disperse black pigments, due to the effects of PCB on the level of melanin-dispersing hormone in the eyestalk
Polychlorinated biphenyls (PCBs)	Fiddler crab, *Uca pugilator*[3]	Fiddler crabs exposed to PCB were paler than the control group, because they were unable to disperse black pigments
Cadmium chloride	Catfish, *Heteropneustes fossilis*[4]	Chromatophores decreased significantly in number and the fish became paler when exposed to acute and sub-acute doses of $CdCl_2$ (392.92 and 98.23 ppm) for four days
Arsenic trioxide	Spotted snakehead, *Channa punctatus*[5]	Arsenic trioxide affected the body colouration of freshwater fish at a concentration of 6 ppm for 30 days by causing pigment aggregation
Arsenic trioxide	*Channa punctatus*[6]	Exposure to 1 ppm of As trioxide reduced the melanophore index in freshwater fish for the first 30 days only when they were exposed for 90 days

[1]Reddy & Fingerman, (1995); [2]Fingerman & Fingerman, (1978); [3]Hanumante, Fingerman, & Fingerman, (1981); [4]Ahmad et al., (2018); [5]Akarte & Agnihotri, (2013); [6]Allen et al., (2004)

Previous studies have investigated the impact of contaminants in the environment on colour change in aquatic organisms, focusing mainly on fish and the fiddler crabs (Table 1.2). The ability to swiftly classify harmful chemicals in water supplies can be a pivotal aspect of early warning systems that detect chemical pollutants (States, Scheuring, Kuchta, Newberry, & Casson, 2003).

All studies in Table 1.2 report the same effect on body colouration, irrespective of the organisms tested and the contaminants used: shrinkage in the chromatophores, which resulted in paleness in the animals.

1.6 Aim and objectives

The aim of this research is to determine the effects of heavy metals on the behavioural responses in the brown shrimp, *C. crangon*

The objectives are:

- To assess the individual and combined effects of Cd and antibiotics on the feeding rate of the brown shrimp (Chapter 2)
- To determine the individual and combined effects of Cd and antibiotics on the ability to change colour in the brown shrimp (Chapter 2)
- To compare two different populations of *C. crangon* to determine whether the effect of Cd on colour change is influenced by their natural habitats, as different sites can have different contamination profiles (Chapter 3)
- To determine effect of As on colour change and evaluate how arsenic metabolism (the bio-transformation of iAs to organic arsenicals) governs behavioural response in the brown shrimp (Chapter 4)

2. Chapter Two: Effects of cadmium and antibiotics (Furazolidone and Gentamicin) on feeding behaviour and colour change in the brown shrimp (*Crangon crangon*)

2.1 Introduction

The brown shrimp (*Crangon crangon*), is an oceanic coastal species which is widely distributed along the European coast from the Mediterranean and Black Seas to the White Sea in the north of Russia (Gelin et al., 2000; Muus, 1967). *Crangon crangon* is a key component in the aquatic ecosystem and one of the most important commercial species in many countries, especially in the UK and North Sea (Temming & Damm, 2002). Besides being easy to catch in high number, it can adapt easily to the lab conditions. Although it has not been yet used for aquaculture (Delbare et al., 2015), it can be used as a model system in the laboratory to check for emerging issues in aquaculture of crustaceans. The brown shrimp inhabits estuaries and shallow waters, which are often subject to environmental or anthropogenic pollution. One of the main toxic heavy metals to aquatic organisms is cadmium, Cd, a non-essential toxic trace element, with naturally low concentrations in ponds, lakes and rivers (see section 1.4 Contaminants - Heavy metals for more detail; Thorp & Gloss, 1986). Besides being present in the environment even recently (Enya, Lin, & Qin, 2019) and threat to organisms' life (Mahmood, Asif, Shaheen, Hayat, & Ali, 2019), Cd in this study has been selected over other based on the previous studies that have been proved its effect on behaviour of crustaceans including colour change and feeding rate in some animals (See Table 1.1, Table 1.2).

Furthermore, another emerging pollutant risk that could affect marine organisms is the increase of incorrect use of antibiotics (from clinical and agricultural overuse). High use of antibiotics may lead to the spread of drug resistant bacteria.

For more than 30 years, Furazolidone (FZD), specifically 3-(5-nitrofurfurylideneamino)-2-oxazolidinone, has been administered as an antibacterial and anti-protozoal drug for human and animals (Zhang, Niu, Yin, Liu, & Chen, 2013). Aqua cultured animals can be protected against red skin disease, bacterial gill-rot disease and protozoiasis using FZD (Meng, Mangat, Grudzinski, & Law, 1998). The World Health Organization (WHO) recognized FZD as an

essential human medication during its Expert Consultations in 2005 in Canberra, Australia and in 2007 in Copenhagen, Denmark on “Critically Important Antimicrobials for Human Medicine” (WHO, 2005, 2007). In terms of human usage, FZD is prescribed to patients who suffer from *Helicobacter pylori* suppression (Zhang et al., 2013).

Furazolidone, which has been broadly used in the form of premix food additives for the treatment of gastrointestinal infections in poultry, pigs and cattle and has also been extensively used in aquaculture for the cure of fish diseases or other diseases caused by bacteria, and it falls under the class of nitrofuran antibacterial agents (Balizs & Hewitt, 2003; Hoogenboom et al., 2002). It can potentially cause genotoxic and carcinogenic effects (Jin et al., 2011) and it can be removed from contaminated systems using *Acinetobacter calcoaceticus* T32, which is likely to degrade 99% of FZD successfully from the environment (Zhang et al., 2013).

Gentamicin (Gt) is an aminoglycoside antibacterial drug that hinders bacterial protein production: this agent possesses a wide range of activities on Gram-negative bacteria. In spite of the fact that Gt is used commonly at therapeutic doses, the regular use of it may cause severe nephrotoxicity (Augusto, Smith, Smith, Robertson, & Reimschuessel, 1996).

The presence of resistant bacteria to both heavy metals and antibiotics has a significant impact on human health (Sharma, Agrawal, & Marshall, 2007). The multiple antibiotics resistance (MAS) hypothesis was proposed since 1983 to indicate the overall environment polluted with multiple antibiotics, and then it has been used also to assess their influence on human health (Krumperman, 1983). Hence, the combination of heavy metal and antibiotics resistance should be studied especially in marine organisms. It has been detected that *Vibrio parahaemolyticus* strains isolated from oysters (*Crassostrea gigas*) have a predominant tolerance to heavy metals with two or more antibiotics resistant phenotypes (Kang et al., 2018).

One way to understand the negative impact of environmental pollutants on organisms is to study the effects of them on organisms' behaviour. The excess presence of both heavy metals and antibiotics could affect negatively the behaviour of aquatic animals. Indeed, only a few studies have illustrated the effect of antibiotics on the behaviour of crustaceans (Tu et al., 2010, 2009). Thus, it is important to address the impact of heavy metals and antibiotics on the brown shrimp and in particular their combined effects, which have not been fully investigated yet.

A sensitive biomarker in some marine animals against any external stresses is colour change (Umbers et al., 2014). The integument of caridean shrimp is feebly calcified (Hung, Chan, & Yu, 1993) and the low sclerotization results in a transparent exoskeleton (Flores & Chien, 2011). Colour change in many crustaceans and fishes occurs as a result of reflection, absorption and scattering of the light on the body due to the distribution of the chromatophores on the surface of the body (Flores & Chien, 2011). In addition, the spread or contraction of pigments, as well as the alteration in the density of chromatophores, play a role in the change of colour (Tume, Sikes, Tabrett, & Smith, 2009). However, morphological and physiological adaptations control the time needed for these changes to happen. The brown shrimp uses colour change (Figure 2.1) as a camouflage to escape from the predators through its ability to match the substrate of its environment (see section 1.5.2 in Chapter 1; Pinn & Ansell, 1993).

Figure 2.1 The brown shrimp *Crangon crangon* show different body colouration (pale and dark) depending on the colour of the background (white and black sand, respectively)

In addition to colour change, feeding behaviour in marine animals is another biomarker that can be used to assess the contamination of the aquatic environment (García-de la Parra et al., 2006; Gulati et al., 1988; Pestana et al., 2007; Pynnonen, 1996; Santos, Troca Da Cunha, & Bianchini, 2000). By studying these two biomarkers (colour change and feeding), it would be possible to have an important insight into how the brown shrimp reacts in response to the contaminant in the environment.

The aim of this chapter was to investigate the effect of Cd and two selected antibiotics (FZD and Gt) on feeding rate and colour change ability of the brown shrimp. The combination of heavy metal and antibiotics in this study was considered because of the recent concern about co-selection of heavy metal and antibiotics resistance bacteria which have an influence on aquatic organisms' health. Some recent studies have been investigated co-selection resistance bacteria in different organisms (Ding, 2019; He et al., 2017; & Lloyd, Janssen, Reinfelder, & Barkay, 2016), however none have been conducted to illustrate the effect of them on the behaviour of marine animals (Dual stress was expected for the use of both Cd and antibiotics). Our hypothesis was that the brown shrimp form Mersey pool would not be able to consume the normal food rate, and they would not be able also to change colour to match the substrate when they were exposed to heavy metal and antibiotics separately or in combination. In addition, the Mersey population would be less affected by Cd and antibiotics than the Dale population, considering being in contaminated water for a long time in their environment (see 2.3.1 Study sites below).

2.3 Methodology

2.3.1 Study sites

For this experiment two study sites were considered: a) Upper Mersey Estuary and b) Dale Cleddau Ddu estuary. In North West England, the Mersey Estuary runs from Warrington, where the Mersey River drains freshwater into it, to Liverpool Bay (47 kilometres westwards; Figure 2.2). The Mersey Estuary is separated into four regions: The Inner, Upper and Outer Estuary and the Narrows. For long time, the Mersey Estuary has been considered as one of the most contaminated estuaries in Europe (Burton, 2003). The Mersey Basin was contaminated to such a level that in 1985 a corporation composed by the local and central government, the Mersey Basin Campaign (MBC) was founded with the goal to raise the

quality of water inherited from the area's previous industrialization (Mersey Strategy, 1996). Over the last two decades, there has been some positive actions put in place to resolve the perpetual problem of pollution in Mersey, which has been a cause for distress in Liverpool over the last 150 years (Burton, 2003). This site, a semi-isolated pool, was chosen based on its historical past, as it was assumed to be highly contaminated (see 2.3.2 Sampling).

In South West Wales, Dale is located in the south west tip of Pembrokeshire (51.708284, -5.168316; Figure 2.3), in the Pembrokeshire Coast National Park. The site was supposed to be a "clean" site even though the area faced a major ecological disaster in 1996 when the oil tanker "Sea Empress" spilled 73,000 tons of crude oil in the water. However, it was supposed to be less contaminated than the Mersey pool since it is an open area.

2.3.2 Sampling

Crangon crangon specimens were collected from the two above mentioned sites. The first collection was from a semi-isolated pool in Widnes, Cheshire (Figure 2.2), Upper Mersey Estuary in February 2016. This is important because it implies that some shrimp stay in here and do not go in and out the estuary (at least not too easily): this will make them more resistant to salinity changes (if it rains the pool becomes less saline than the river), and if there is historical contamination, this population might be more used to high levels of contaminants, more than in Liverpool estuary.

The second collection was performed in the Cleddau Ddu estuary (Lower Waterway) close to Dale (Figure 2.3), Pembrokeshire in April 2016. The samples were collected by using push nets (mesh size: 6mm). Water and sediment samples (at least two replicates per site) were also gathered from each location, labelled and stored in the fridge for heavy metal analysis. Water salinity and temperature for each site were also measured using salinity meter. All the shrimp were kept in a bucket with seawater and a portable oxygenator while being transferred to the lab. Then, all the shrimp were placed in glass aquaria with artificial seawater and a 2 cm thick layer of black or/and white sand (Pettex Roman Gravel), gently aerated, to be acclimated to the lab environment for one week. A specific code was given to each sample (e.g. WP1: Widnes Pool 1; D1: Dale 1).

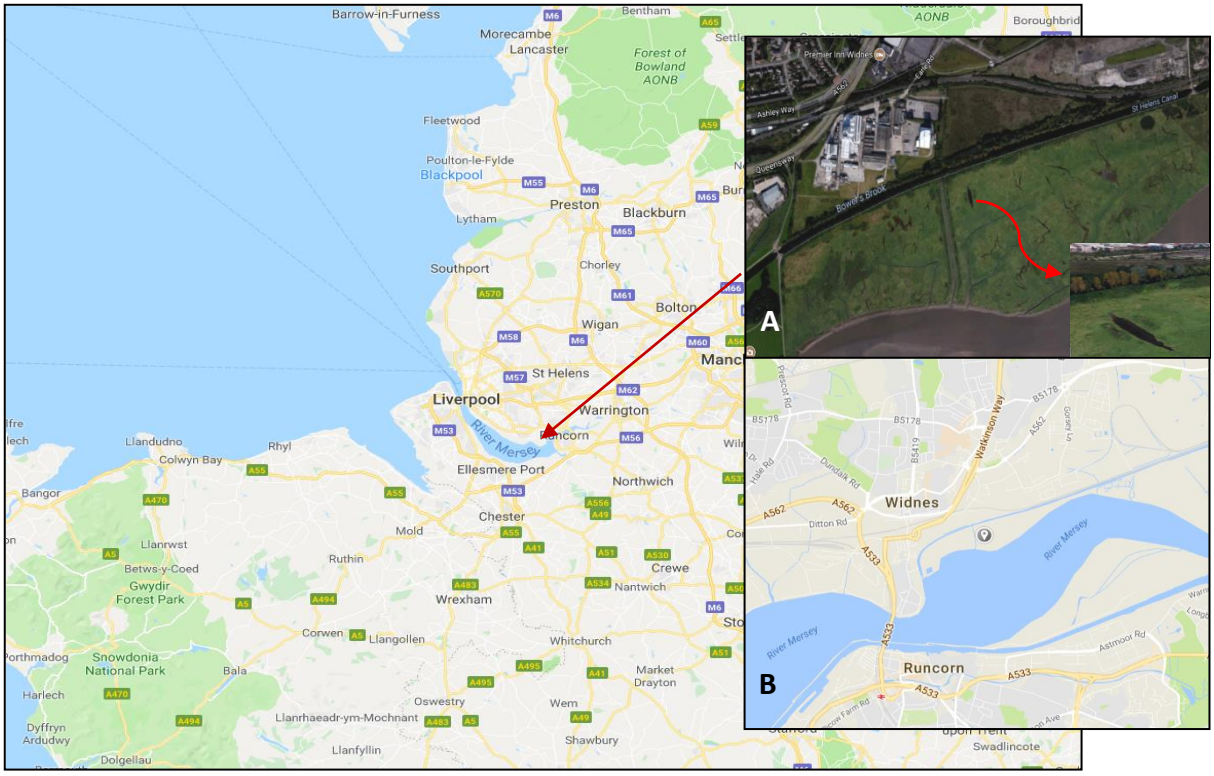

Figure 2.2 Sampling pond to the south of Widnes, Cheshire, England. A) Satellite map; B) General map (Google Maps, 2019)

Figure 2.3 Sampling site in Dale, Pembrokeshire, South West Wales. A) Satellite map; B) General map (Google Maps, 2019)

2.3.3 Experimental design

To prepare the artificial seawater, 160 and 213 grams of aquarium systems instant ocean salts respectively were added to 10 litres of deionised water to obtain a salinity of 15 psu (shrimp from Mersey) and 20 psu (shrimp from Dale) corresponding to the salinity in their original environment. Salinity and temperature (~17˚C) were maintained constant throughout the experiment. Four glass aquaria containing 12 sterile polystyrene petri dishes surrounded by a cylindrical mesh to avoid cannibalism were used as experiment vessels (Figure 2.5 A). Each one was filled with 1 cm thick layer of black sediment, air bubblers and artificial seawater. Twenty-four small beakers (600 ml beakers, Ø: 10.4 cm), filled with clean artificial seawater 1 cm of white sediment and aerated were also prepared for feeding time (Figure 2.5 B, C).

Depending on the treatment, the following solutions were prepared:

- 0.04 ppm of Cd (prepared dissolving 0.4 g of Cd chloride in 10 litres artificial seawater)
- 10 ppm FZD (prepared dissolving 0.1 g of FZD into 10 litres of artificial seawater)
- 2 ppm Gt (prepared dissolving 400 μl of Gt into 10 litres of artificial seawater)

2.3.4 Pilot experiments

Two pilot experiments were performed, one to test the dose of antibiotics (based on literature) and the other one to determine the dose of Cd.

2.3.4.1 Test the antibiotic dose

The choice of antibiotics and concentrations were established by a pilot study performed in collaboration with Dr Chloe James (presented at Salford Postgraduate Annual Research Conference (SPARC) 2016, Appendix Figure A.4). We tested the growth of bacteria obtained from the dissected gut of *C. crangon* on microbiology discs having 10 ppm FZD and 2 ppm Gt (

Figure 2.4). These concentrations have been used with fish, molluscs and crustacean to identify antibiotics resistance (Guo, Chou, & Chiu Liao, 2003; Manjusha & Sarita, 2013).

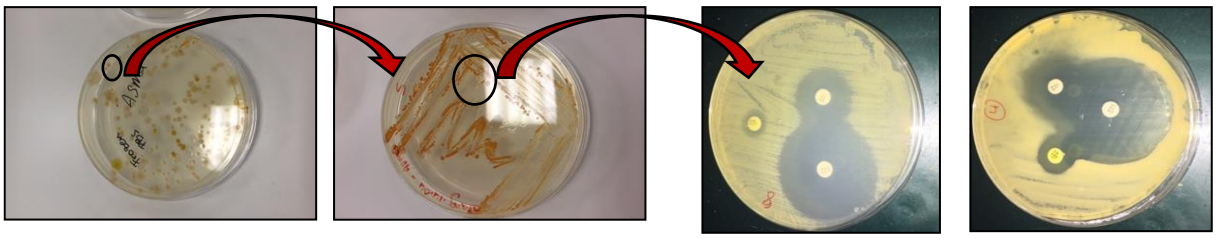

Figure 2.4 Diffusion assay technique with gut extract from *Crangon crangon* used to test the resistance of some antibiotics

2.3.4.2 Test Cd dose (Mersey experiment)

Twenty-four shrimp from Mersey pool were randomly divided into two groups (12 shrimp each) and placed individually into labelled chamber with 1 cm thick layer of black sediment (Figure 2.5 A).

Control group: 12 shrimp were kept individually in a glass aquarium filled with 10 litres of artificial seawater for 3 weeks.

Cd group: 12 shrimp were exposed to 0.02 ppm of Cd in a glass aquarium filled with 10 litres of artificial seawater for two weeks and the concentration in the last week was increased to 0.04 ppm. A dose of 0.04 ppm Cd is known to affects colour fading and other behavioural aspects such as scaphognathite oscillation and heart beat rate in the prawn *Macrobrachium dayanum* (Tripathi & Pandey, 2014). However, due to the small size of the brown shrimp that collected, the initial test was done with a decreased dose of 0.02 ppm. After two weeks of the experiment, the dose was increased to 0.04 ppm because no causality was observed, and we wanted to go closer to the dose used in literature.

Figure 2.5 Set up of the experiment: A) shrimp were housed individually in a common environment; B, C) shrimp were fed in small beakers with white sediment

2.3.5 Main experiment (Dale experiment)

Prior to the acclimatization period (one week), about 10 to 12 shrimp were sacrificed, labelled and frozen to assess the initial baseline concentration of heavy metals in the field. The level of the water in the aquaria was checked carefully during the experiment and clean artificial seawater added if needed (to account for evaporation) to ensure that the shrimp were exposed to the same level of the Cd and antibiotics every day.

In this experiment, 32 adult shrimp from Dale were divided randomly into 4 groups (8 in each) and placed individually into labelled chamber with black sediment (Figure 2.5). The experiment lasted 3 weeks.

All solutions were prepared in the day one of the experiment to avoid change in concentrations by evaporation.

- Control group: 8 shrimp were kept in 10 litres of artificial seawater.
- Cd group: 8 shrimp were exposed to 0.04 ppm of Cd in 10 litres of artificial seawater.
- Antibiotics group: 8 shrimp were exposed to 10 ppm FZD and 2 ppm Gt in 10 litres of artificial seawater.
- Antibiotics+Cd: 8 shrimp were exposed to 0.04 ppm of Cd plus 10 ppm FZD and 2 ppm Gt in 10 litres of artificial seawater.

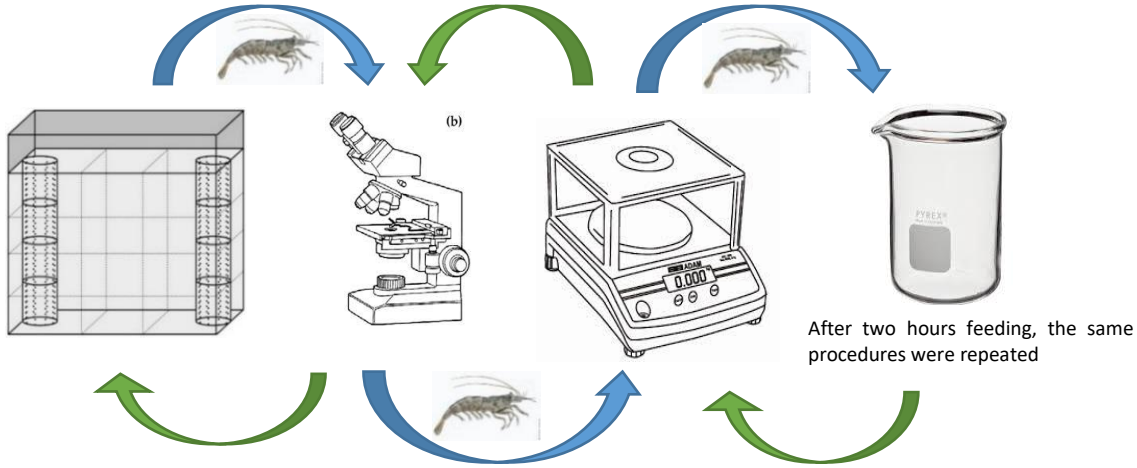

Figure 2.6 Experimental design: blue arrows show the initial steps of the experiment (shrimps were taken from the aquaria and a picture of the right exopod was taken using microscope and then the shrimps were measured then transferred to a small beaker to be fed and to allow them to change their colour since the sediment in the beaker is different from the aquaria); green arrows show the final steps after the shrimp were feed and left in different substrate colour for two hours

2.3.6 Behavioural responses

2.3.6.1 Shrimp weight and food intake

Every other day of the experimental period, all shrimp were moved individually from the glass aquaria (with black sediment) to a small beaker (600 ml) that contained non-treated artificial seawater and different sediment colour (white sediment) in order to feed them and to assess their colour change ability (Figure 2.6). Shrimp's wet weight was measured before offering the food and then one piece of known-weight fish muscle was given to each shrimp individually. All shrimp were kept in the feeding beakers for two hours following (Siegenthaler et al., 2018), to allow them to eat and at the same time to change colour. After two hours, all remaining food was re-measured and then disposed. Wet weight of the shrimp was measured again after feeding. All shrimp were then transferred to the main glass aquaria (Figure 2.5 A). Initial body weight was used to determine the effect of Cd or/and antibiotics on body weight over time and the difference between shrimp's wet weight before and after feeding (final- initial) was used to assess the amount of food intake. Due to the difficulties in taking all the food out of the beakers and in measuring them, the weight of given food was not used in the analysis.

2.3.6.2 Colour change measurement

Colour change measurement in animals is often conducted by measuring the dark pigment in a specific part of the body instead of the whole animal (Darnell, 2012; Siegenthaler et al., 2017; Stevens, Rong, & Todd, 2013). In fish, the scales can be separated from the animal before measuring the colour change to reduce the stress that could affect the measurements (Nguyen, Sugimoto, & Zhu, 2006). In caridean shrimp, the fan tail was found to be the most appropriate part of the body to assess colour change, as it is flat and transparent thus allowing a precision and standardization identification of the pigments (Brown & Wulff, 1941; Flores & Chien, 2011; Siegenthaler et al., 2017). Besides that, it is harmless to the shrimp. Before transferring shrimp from the glass aquaria to the small beaker (to be fed), a photo of the right exopod in the telson (Figure 2.7) for each individual shrimp was taken by placing the shrimp on a white background, to see the dark pigment clearly, under a Leica DFC295 camera using a dissecting microscope (Leica S6D). Two led spotlights (JANSJÖ; 88 lm; 3000 Kelvin) were provided in both side of the microscope. A second photo of the same area was taken again in two hours' time, after feeding was over.
All shrimp were then returned back to the divided aquaria (Figure 2.5 A). The same protocol was performed every other day for every individual shrimp. At the end of the experiment, all the shrimp that still alive from Cd group and some from control group were sacrificed by freezing and placed individually in labelled bag.

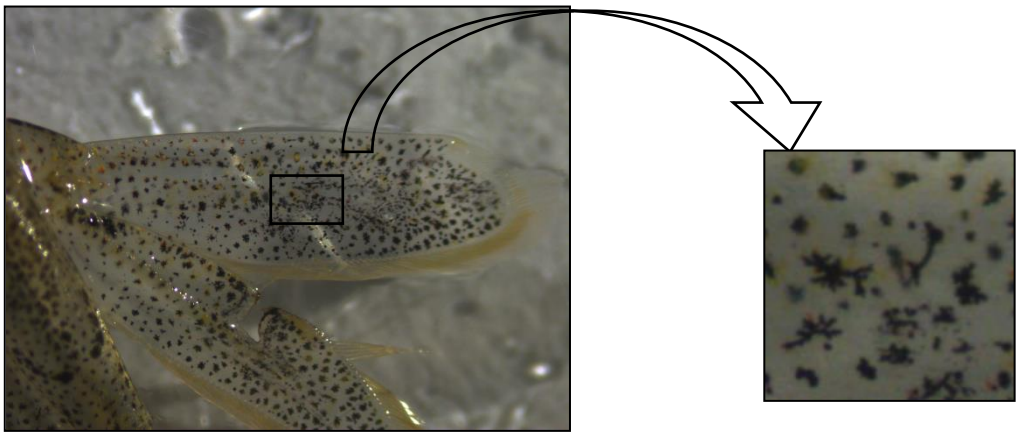

Figure 2.7 Right exopod in the telson and section showing the inset of the photo used to assess colour change

2.3.7 Brightness/ pigment analysis

All pictures collected from individual shrimp were analysed with ImageJ software (version 1.48, https://imagej.nih.gov/ij/plugins/color-space-converter.html; Schneider, Rasband, & Eliceri, 2012) using a protocol established by Siegenthaler et al. (2017). Briefly, the exact right exopod was cropped from the original picture and the area of interest was selected (1-mm^2; Figure 2.7). Dark pigments were selected by using threshold function in ImageJ, which segments the image based on pixel values (default thresholding algorithm was chose based on Siegenthaler et al. (2017); Figure 2.8). The contrast between the background and the pigment should be high and it could be achieved by choosing "enhance contrast" function in the software. The manual adaptation was performed if needed, otherwise it should be kept at a minimum. To minimize bias, all the pictures were analysed without knowing the treatment.

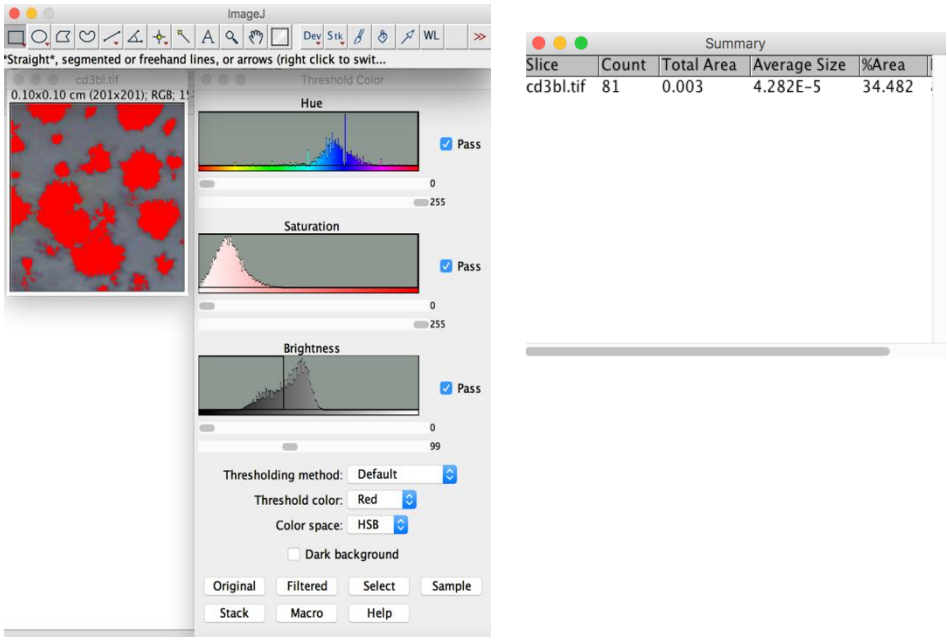

Figure 2.8 Analysis of photo with ImageJ (version 1.48), the left image showed how the dark pigment was selected by using threshold and the right image showed the results from the software

2.3.8 Heavy metal analysis

Metal analysis was outsourced and was conducted by SOCOTEC group (https://www.socotec.co.uk), instead of the inhouse Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) at the University of Salford. This is due to the low concentrations of Cd in the shrimp tissue which was beyond the detection limit of our instrument at the university. The following protocol was used as was provided by the

company: the samples were weighed into acid-cleaned beakers and ultra-pure (trace) nitric acid (HNO_3) was added. One reagent blank was added per 20 samples, which was a beaker containing only trace HNO_3. Also, one Method-Quality Control was added per 20 samples, which consisted of various elements spiked at a known concentration. The samples were put on a hotplate, at moderate heat, and heated for approximately 3 hours. Once the samples were digested, they were made to a known volume with deionised water (18.2MΩcm-1 resistivity). Certified reference material (CRM) used was ERM- CE278k (mussel tissue). The digested samples were then analysed via both Coupled Plasma Atomic Emission Spectrometry (ICP-AES) and Inductively Coupled Plasma Mass Spectrometers (ICP-MS). The dilution factors were applied to the raw data prior to reporting. Missing values that were below the limit of detection (LOD) was replaced by 50 % of LOD for each element (Płotka-Wasylka, Frankowski, Simeonov, Polkowska, & Namieśnik, 2018; Shrivastava & Gupta, 2011).

2.3.9 Statistical Analysis

The aims of the experiment were to test the change in body weight and colour change ability of the brown shrimp dosed with Cd and/or antibiotics for three weeks. The initial body weight (before feeding) and the difference between the wet weight before and after food (referred as food intake in this chapter) were used to assess the effect of the treatment on feeding behaviour. The difference between the dark pigment cover in black and white sediment (dark pigment cover in the final sediment colour – dark pigment cover in the initial sediment colour) was used to assess the ability of changing colour in the brown shrimp. Negative values suggested that the shrimp became paler and positive values suggested that the shrimp became darker. The average of dark pigment cover in either black or white sediment was also used in the analysis to detect the starting and ending point of dark pigment over time. These changes were tested using two treatments (control and Cd) in Mersey shrimp and four treatments (control, Cd, antibiotics and antibiotics+Cd) in Dale shrimp.

The comparison within treated group was performed using repeated measures ANOVA for all outcomes (Verma, 2015), followed by pairwise comparisons if the ANOVA was statistically significant. The pairwise comparisons based on multiple paired t-tests, with a Bonferroni correction to keep the type one error at 5% overall, was used to compare each

pair of days. The comparison between groups (control and Cd in Mersey and control, Cd, antibiotics and antibiotics+Cd in Dale) was performed using one-way ANOVA and t-test between independent groups for each outcome. Boxplots were used to display minimum, first quartile, median, third quartile and maximum of each data across time. Regression slopes were also performed to see the overall trend during the time of the experiment.

Normality assumptions were checked using K-S test, skewness and kurtosis for each group, and all data were found to be approximately normally distributed. For repeated measures ANOVA, another assumption checked was sphericity using Mauchly's test (testing for equal population variances of all possible difference changes in day 1, 4, 6, 8, 11, 13, 15, 18, 20 and 22; Field (2009). This assumption was violated in all groups, hence, F test based on Greenhouse-Geisser (Epsilon<0.75) was used (O´Brien & Kaiser, 1985).

Missing values were frequently encountered in data and can undermine the reliability of the data and cause a bias in the results. Data missing can be at random (such as death) without being influenced by other specific factors. This was tested using a chi-square test (Roderick, 2010). As the missing data were indeed random (since chi-square was not significant), imputation analysis was used to replace missing values with estimated values, computed using an expectation maximization algorithm of the likelihood distribution (Enders, 2010).

Spearman correlation and non-parametric t-test (Mann-Whitney) were used to check the relationship and the difference of the heavy metal elements among water, sediment and shrimp in both sites. All analyses were performed with IBM SPSS 24.

2.4 Results

In this chapter, we have looked at the responses of *C. crangon* to contaminants and antibiotics from two different angles: food intake and colour change ability.

2.4.1 Mortality

In the pilot study (Mersey experiment), one dead shrimp was recorded in the Cd group and 4 in control group out of 12 during the 22-day experiment. In the Dale experiment, there was no mortality in control group. In the Cd group and antibiotics+Cd group, there were 3 dead shrimp during the experiment in each group. The highest number of mortalities was found in the antibiotics group (4 out of 8).

2.4.2 Shrimp weight and food intake

Regarding initial body weight between days in each group, only the shrimp in Cd group in Mersey experiment appeared to have a significant decrease (Greenhouse-Geisser: F= 2.746; p-value=.043; Table 2.1) in body weight between some days (Table 2.1, Figure 2.9; Appendix Table A.1 for pairwise comparison). In Dale experiment, all treatment group including control had similar body weight over 22 days (Table 2.1, Figure 2.11).

The regression slopes showed that the overall trend in initial body weight of the shrimp decreased over time compared to the control group (Figure 2.10), in Dale experiment, shrimp in Cd group seemed to lose weight more than other group over time as well (Figure 2.12).

Table 2.1 Repeated measures ANOVA for initial body weight within group between days for Mersey and Dale

Site	**Group**	**F (Greenhouse-Geisser)**	**p-value**
Mersey	Control	2.937	.067
	Cd group	2.746	**.043**
Dale	Control	1.638	.212
	Cd group	2.337	.114
	Antibiotics	1.594	.230
	Antibiotics+Cd	1.752	.190

- Bold values were significant (p-value<.05)

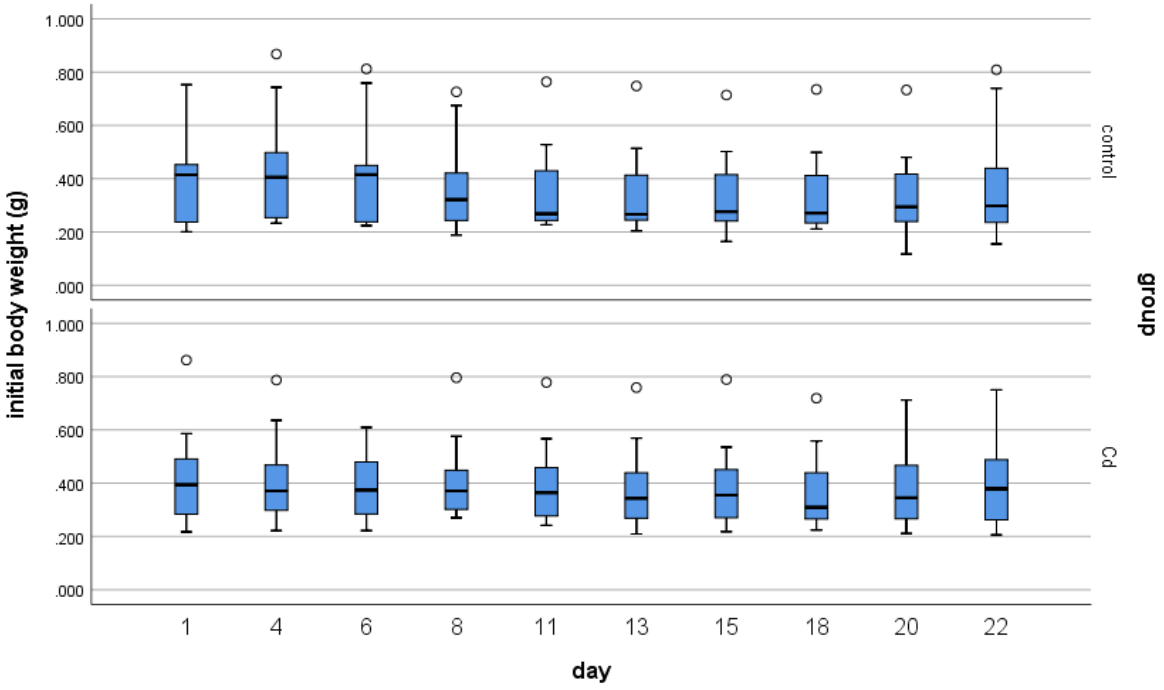

Figure 2.9 Initial body weight (wet weight) of each shrimp in gram before feeding over time in both control (top panel) and Cd group (bottom panel; Mersey experiment), box plots display minimum, maximum, median and first and third quartile; circles show outlier values

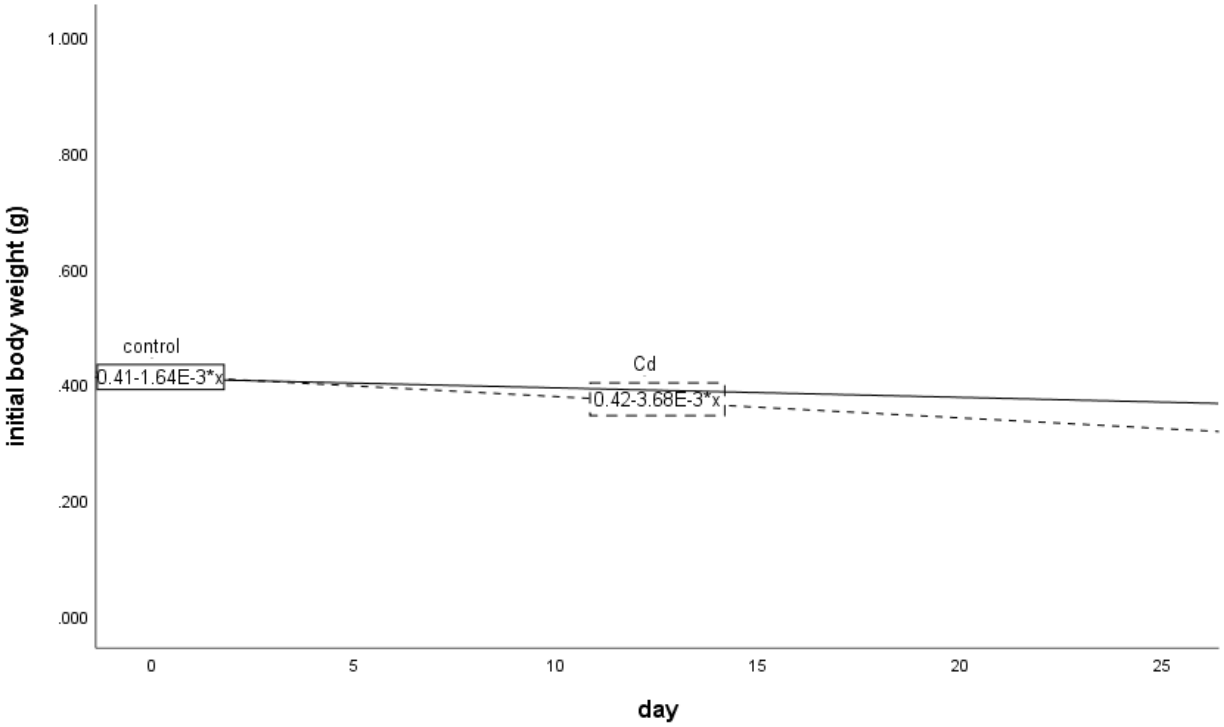

Figure 2.10 Regression slopes of initial body weight of the shrimp over time in both control and Cd group in the Mersey experiment, solid line represents control and dashed line represent Cd group

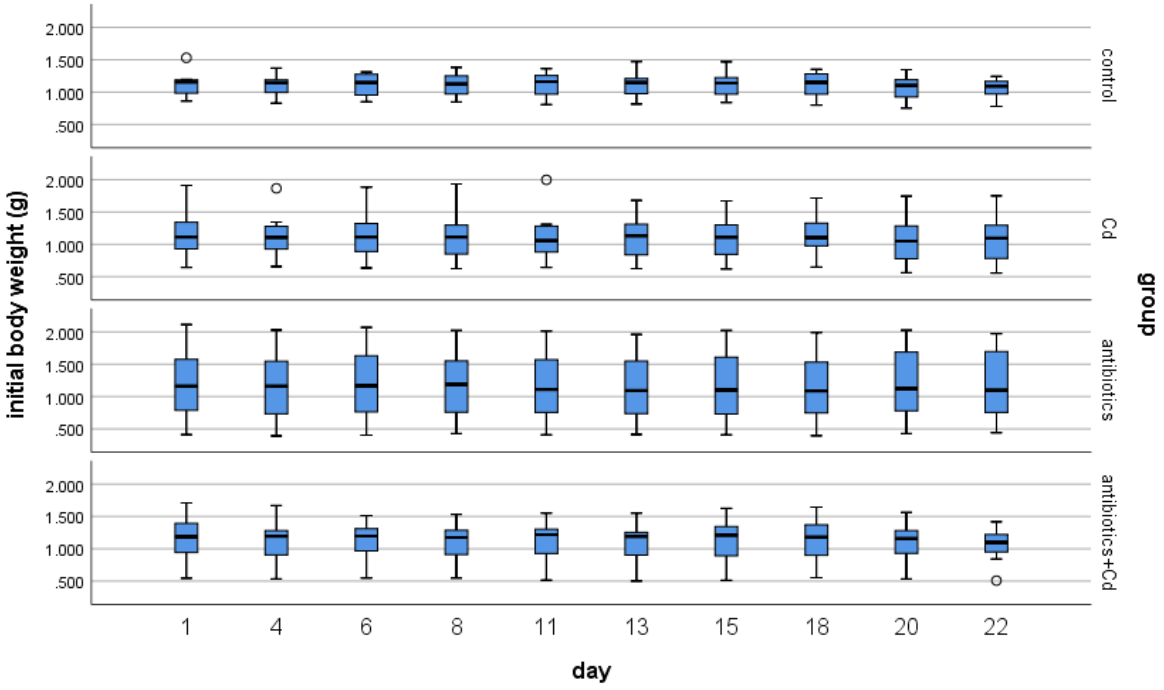

Figure 2.11 Initial body weight (wet weight) of each shrimp in gram before feeding over time in all groups (Dale experiment), box plots display minimum, first quartile, median, third quartile and maximum; circles show outlier values

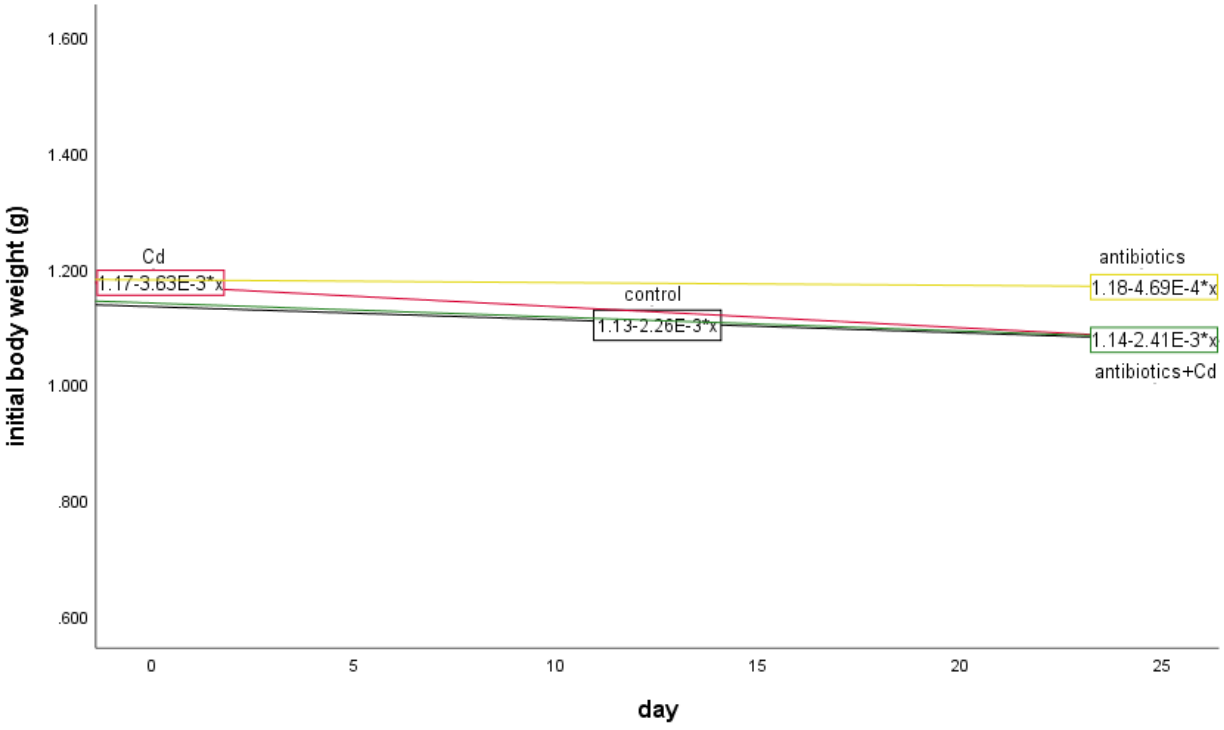

Figure 2.12 Regression slopes of initial body weight of shrimp over time in all groups group in Dale experiment, black line represent control, red represent Cd, yellow represent antibiotics and green represent antibiotics+Cd group

In terms of food intake (difference in body weight of shrimp after feeding – before feeding), results in Table 2.2 showed no difference in food intake between groups in Mersey and Dale (F=.285, p-value=.594 and F=1.520, p-value=.210 respectively, Table 2.2). The box plots showed that the change in body weight of the shrimp in Mersey experiment was less and much more consistent over time than in Dale experiment (Figure 2.13).

Table 2.2 Descriptive Statistics and ANOVA for food intake measured by the difference in shrimp wet weight (after food-before food) between groups for Mersey and Dale

group		Mean	Std. Error	F	p-value
Mersey	Control	.010	.003	.285	.594
	Cd group	.008	.003		
Dale	Control	.048	.011	1.520	.210
	Cd group	.050	.013		
	Antibiotics	.056	.012		
	Antibiotics+Cd	.080	.013		

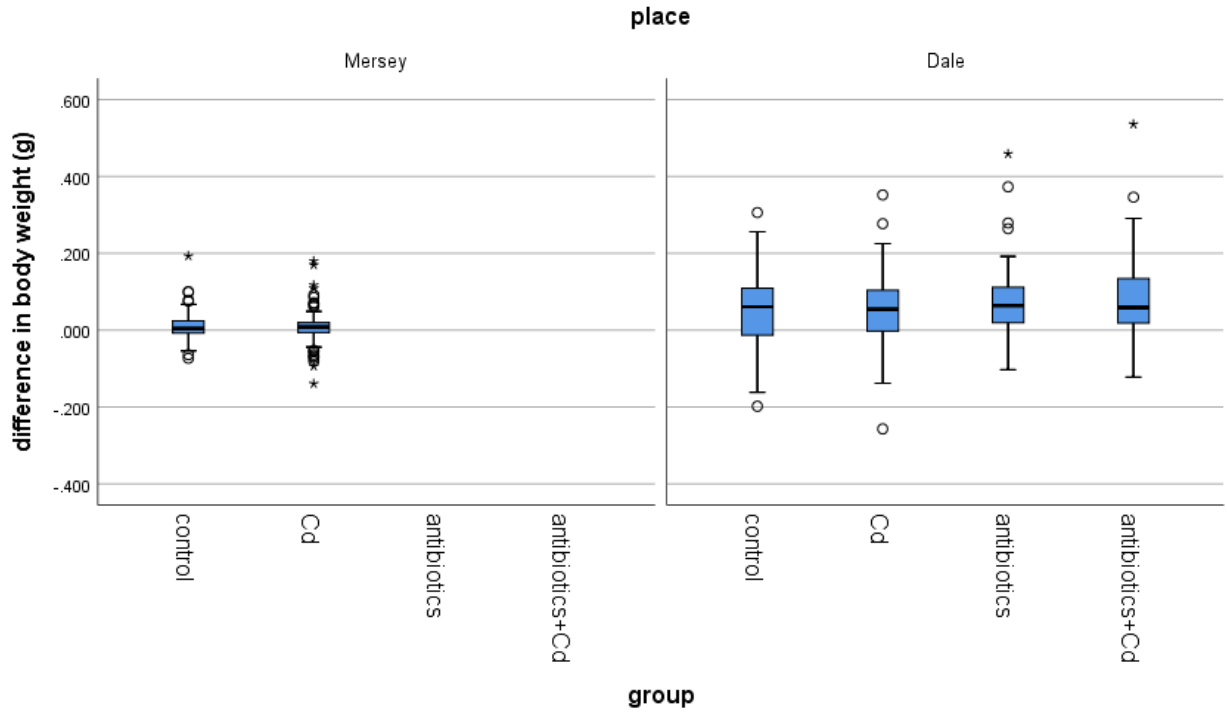

Figure 2.13 Food intake measured by the difference in shrimp's wet weight in gram before and after feeding between groups in Mersey (left panel) and Dale (right panel), box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

2.4.3 Dark pigment cover in black and white sediment

2.4.3.1 Dark pigment cover in black sediment

With regards to dark pigment cover in black sediment in both experiments, repeated measures ANOVA between days was significant in all groups (p-value<.002), with exception of control and antibiotics group in Dale experiment (Table 2.3). This indicates that the shrimp were not be able to control their pigment (getting darker in Mersey experiment; Figure 2.14 and Figure 2.15 but getting less dark in Dale experiment compared to day 1 of the experiment; Figure 2.16 and Figure 2.17) during 22 days.

Table 2.3 Repeated measures ANOVA for dark pigment cover in black sediment within group between days in Mersey and Dale

	Group	**F (Greenhouse-Geisser)**	**p-value**
Mersey	Control	10.731	**.000**
	Cd group	12.292	**.000**
Dale	Control	2.212	.098
	Cd group	9.609	**.000**
	Antibiotics	3.104	.050
	Antibiotics+Cd	4.322	**.012**

- Bold values were significant (p-value<.05)

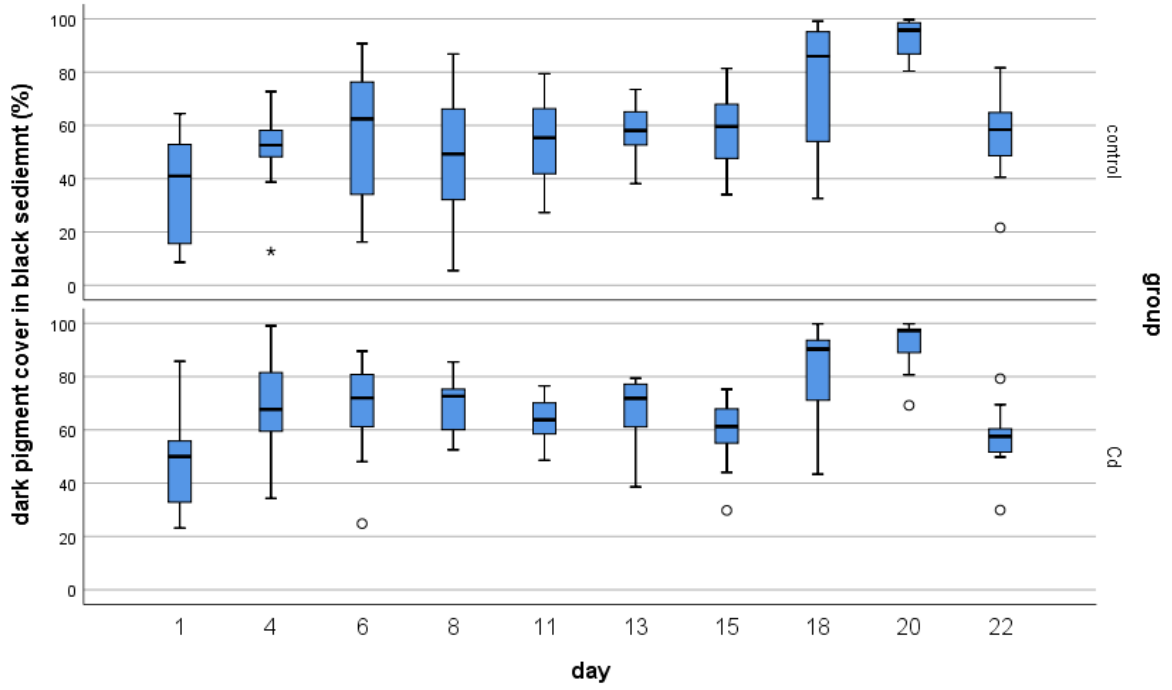

Figure 2.14 Dark pigment cover in black sediment in both control (top panel) and Cd (bottom panel) group in Mersey, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

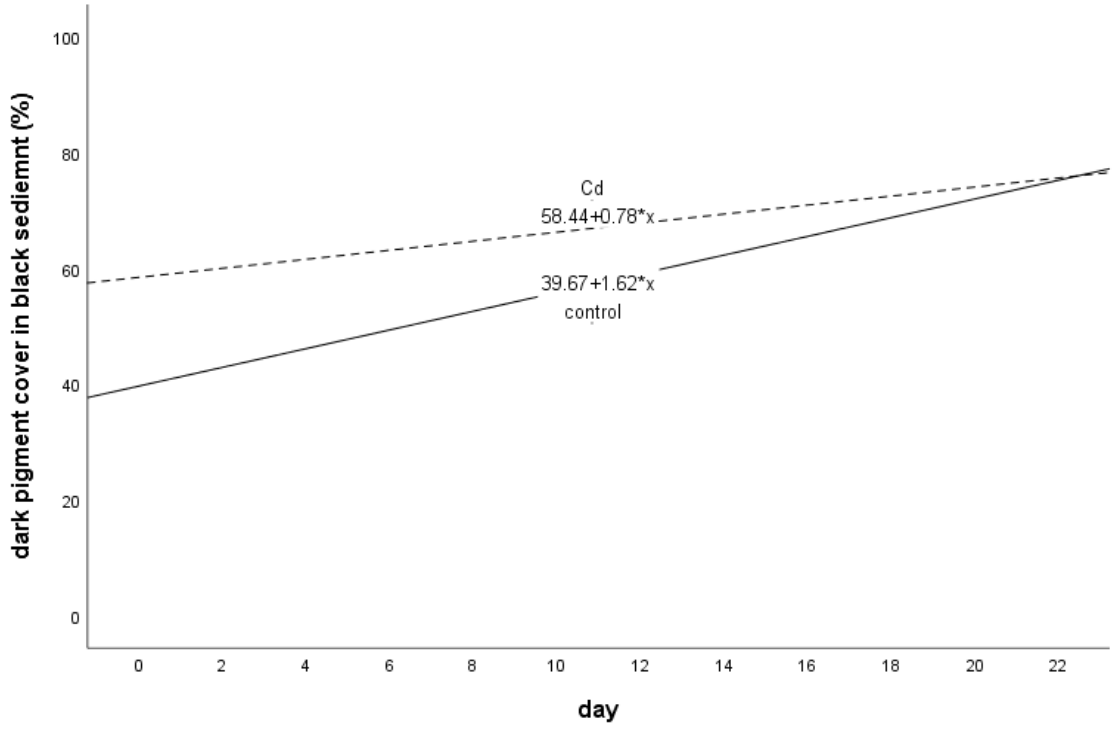

Figure 2.15 Regression slopes of dark pigment cover in black sediment over time in both control and Cd group in the Mersey experiment, solid line represents control and dashed line represent Cd group

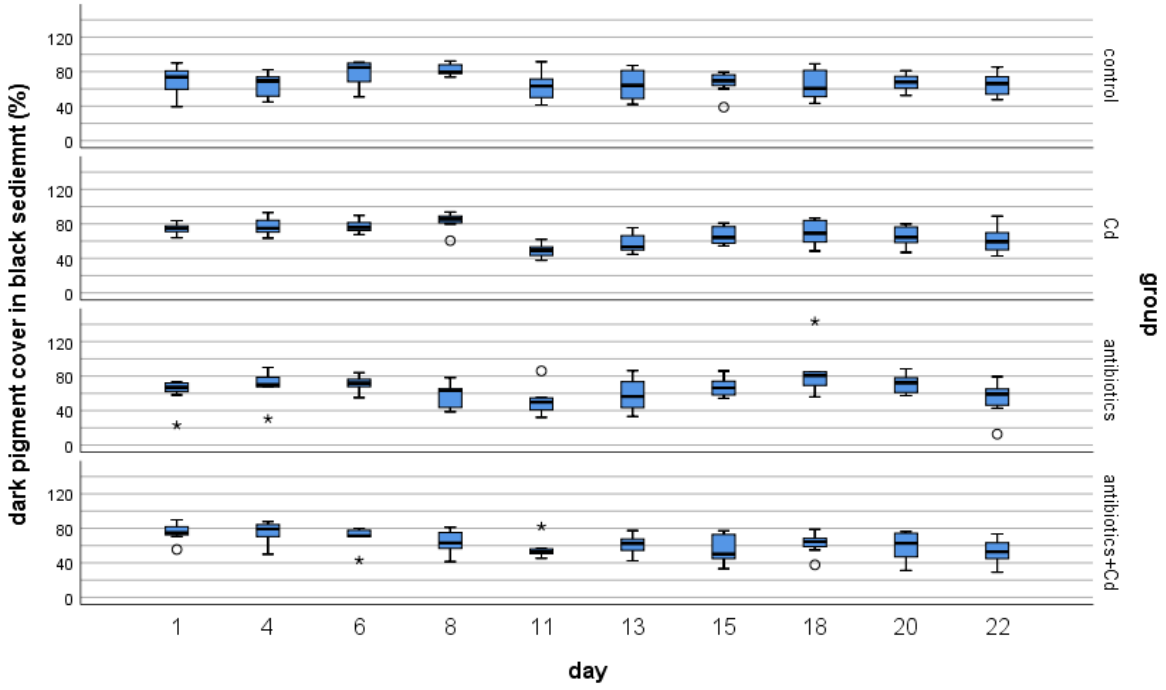

Figure 2.16 Dark pigment cover in black sediment in all groups in the Dale experiment, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

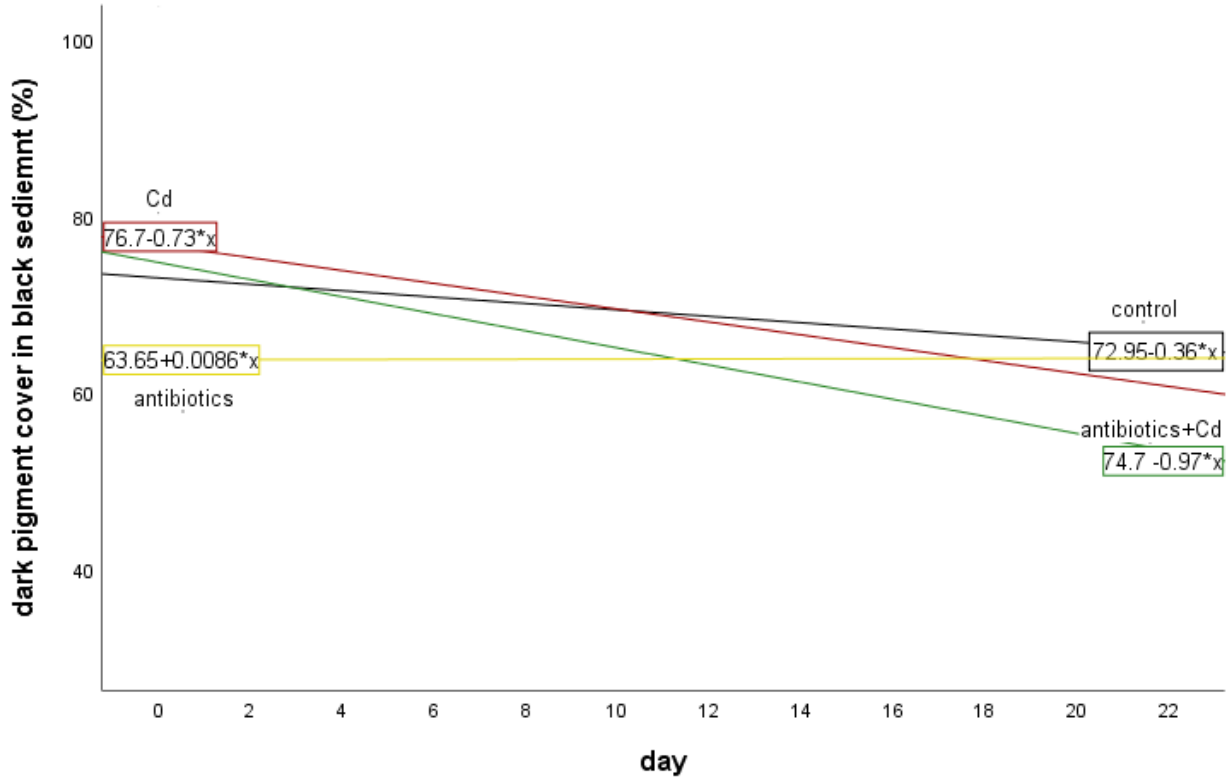

Figure 2.17 Regression slopes of dark pigment cover in black sediment over time in all groups in the Dale experiment, black line represent control, red represent Cd, yellow represent antibiotics and green represent antibiotics+Cd group

Concerning the difference between groups in dark pigment cover in black sediment, the results present in Table 2.4 showed a significant increase in the average of dark pigment cover in black sediment (p-value=.001) between control and Cd group in Mersey experiment. Indicating that the shrimp became even darker in black sediment compared to the shrimp in control group (Figure 2.18). However, in Dale experiment, the average of dark pigment cover in black sediment was similar in all groups (Figure 2.18).

Table 2.4 Descriptive Statistics and ANOVA for dark pigment cover in black sediment between groups in Mersey and Dale

group		Mean	Std. Error	F	p-value
Mersey	Control	57.85	2.20	11.602	**.001**
	Cd group	67.19	1.67		
Dale	Control	68.66	1.67	2.291	.079
	Cd group	69.10	1.91		
	Antibiotics	63.94	2.12		
	Antibiotics+Cd	64.21	1.66		

- Bold values were significant (p-value<.05)

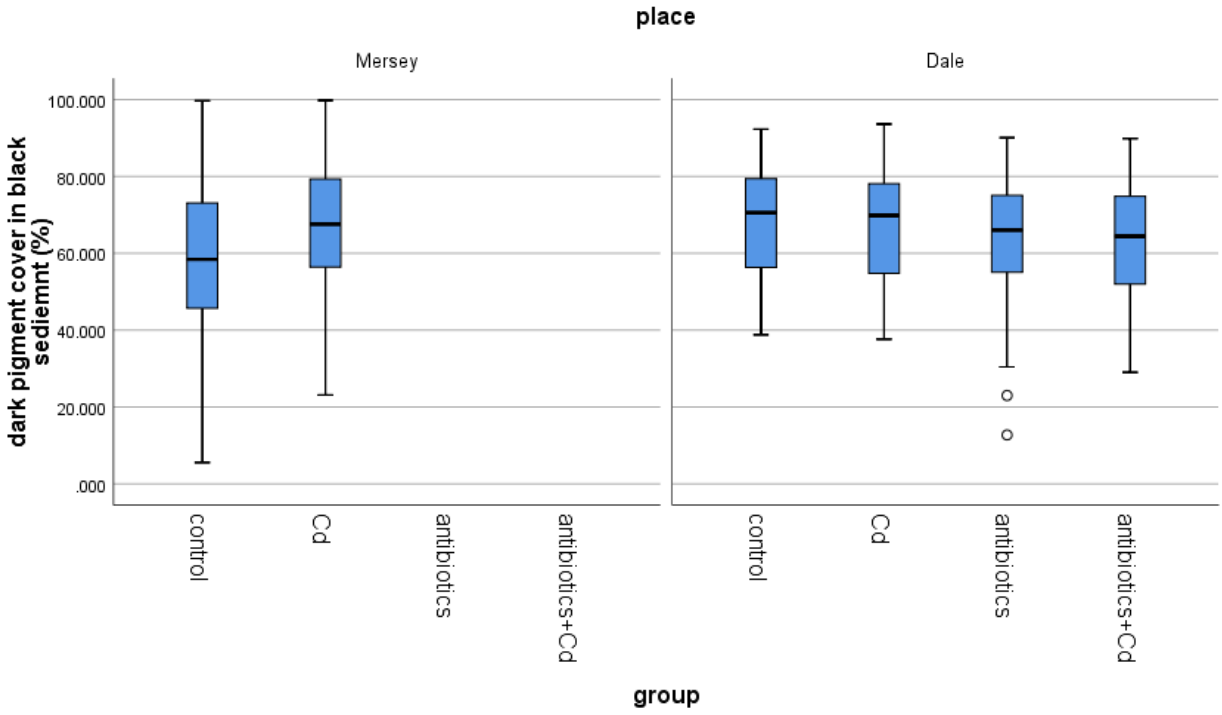

Figure 2.18 Dark pigment cover in black sediment between groups in Mersey (left panel) and Dale (right panel), box plots display minimum, maximum, median and first and third quartile; circles show outlier values

2.4.3.2 Dark pigment cover in white sediment

With respect to dark pigment cover in white sediment in Mersey experiment, repeated measure test showed significant changes in dark pigment cover in white sediment between days in control and Cd group (p-value<.01; Table 2.5). Whereas, the average of dark pigment cover in white sediment in Dale shrimp were similar between days in all groups (Table 2.5).

Table 2.5 Repeated measures ANOVA for dark pigment cover in white sediment within group between days in Mersey and Dale

Site	Group	F (Greenhouse- Geisser)	p-value
Mersey	Control	5.954	**.001**
	Cd group	3.827	**.006**
Dale	Control	0.773	.511
	Cd group	1.230	.324
	Antibiotics	2.201	.142
	Antibiotics+Cd	1.136	.350

- Bold values were significant (p-value<.05)

It appeared that Mersey shrimp in both control and Cd group had difficulties to contract their pigment when they were placed in white sediment at the beginning of the experiment, however, they get better at the end of the experiment (Figure 2.19, Appendix Table A.4 for pairwise comparison). With regards to Dale shrimp in all groups, there was no trend in the average of dark pigment cover in white (Figure 2.21). Overall slope showed a decrease in all groups in both experiment indicating that the shrimp were getting paler when they were placed in white sediment compared to their performance at the begging of the experiment (Figure 2.20 and Figure 2.22)

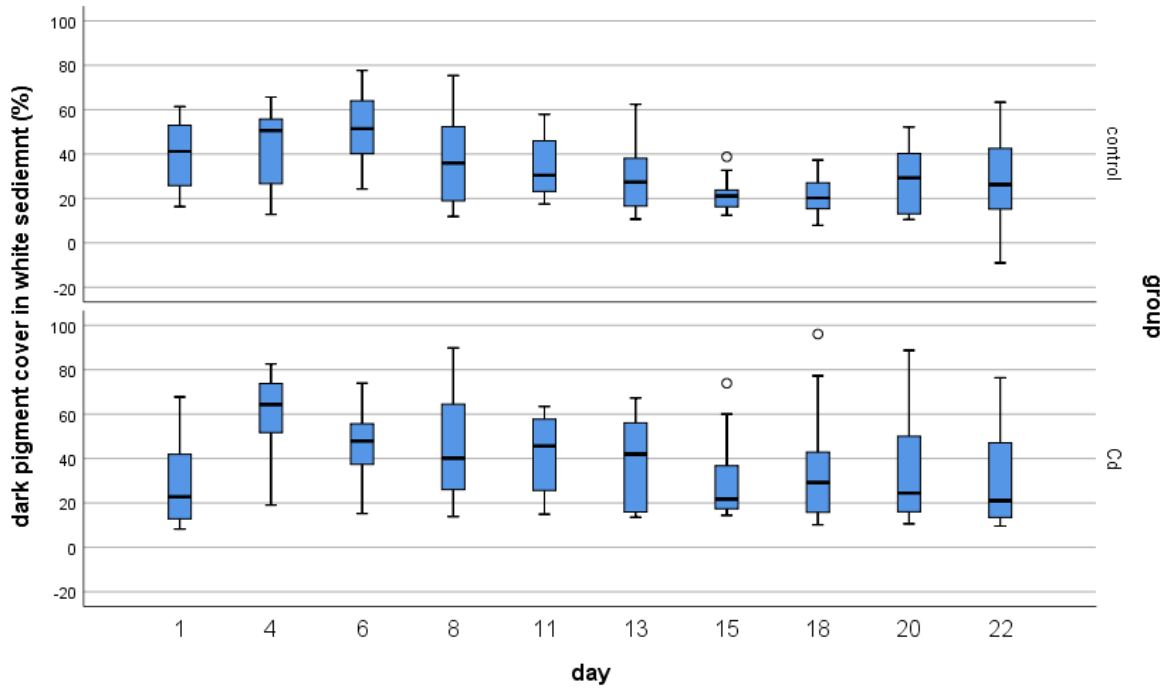

Figure 2.19 Dark pigment cover in white sediment between days in both control (top panel) and Cd (right panel) group in Mersey, box plots display minimum, first quartile, median, third quartile and maximum; circles show outlier values

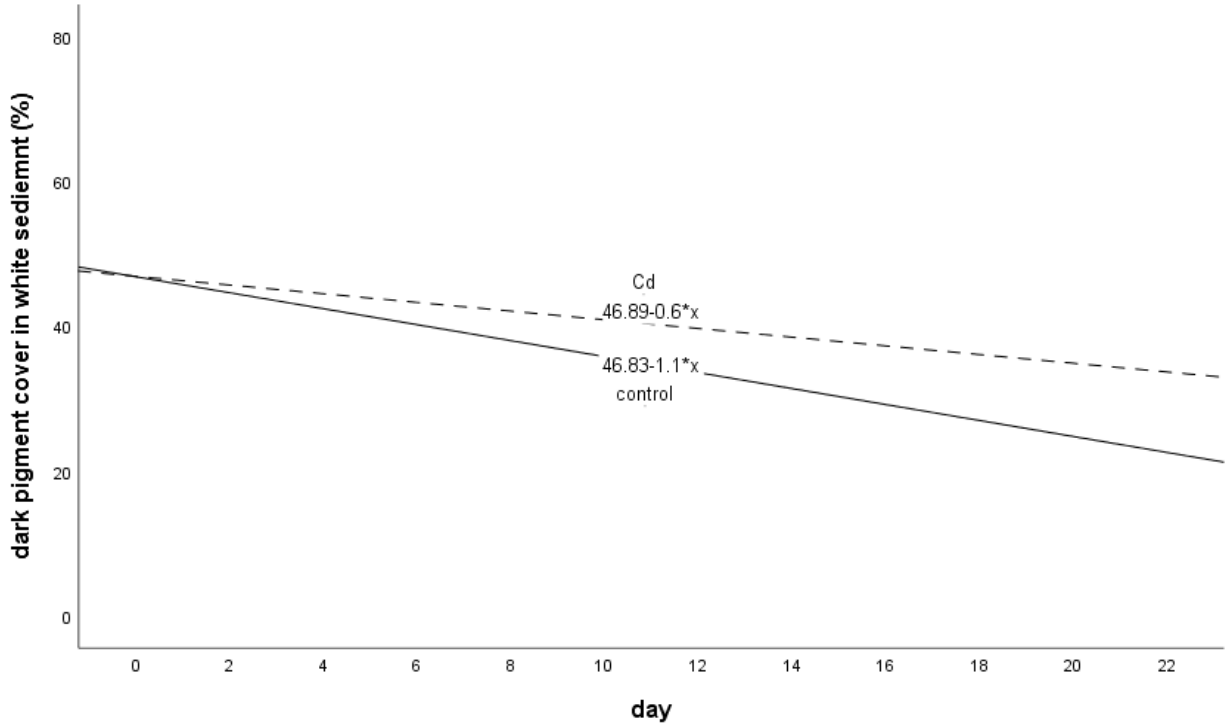

Figure 2.20 Regression slopes of dark pigment cover in white sediment over time in both control and Cd group in the Mersey experiment, solid line represents control and dashed line represent Cd group

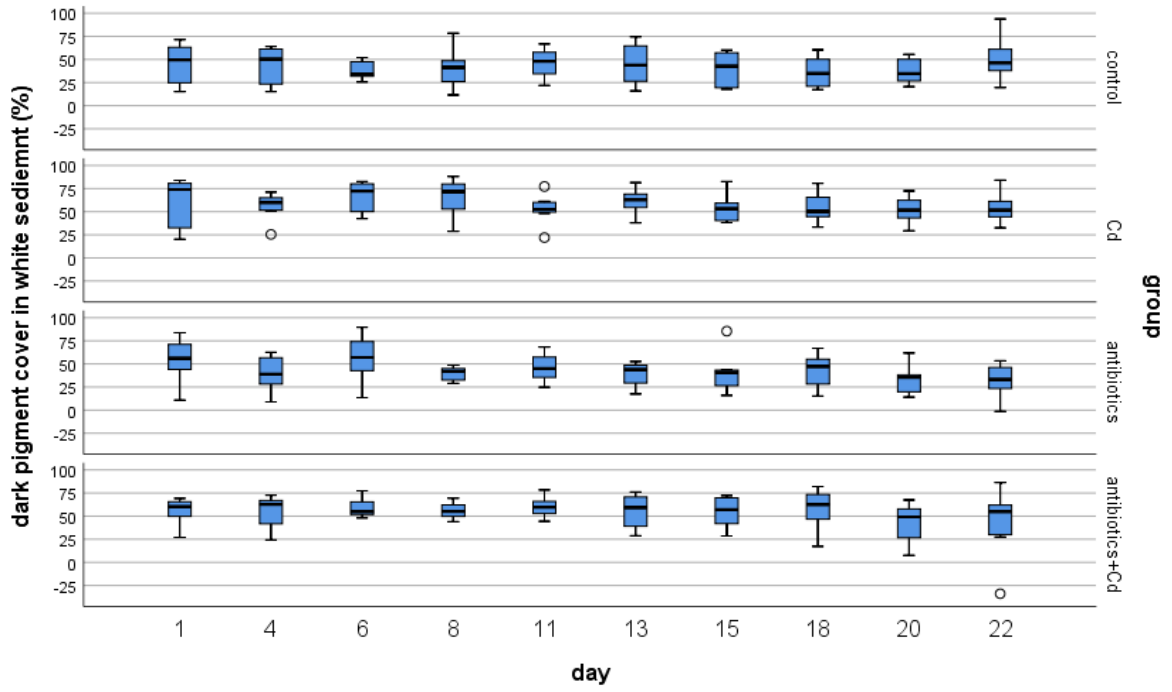

Figure 2.21 Dark pigment cover in white sediment between days in all groups in Dale, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

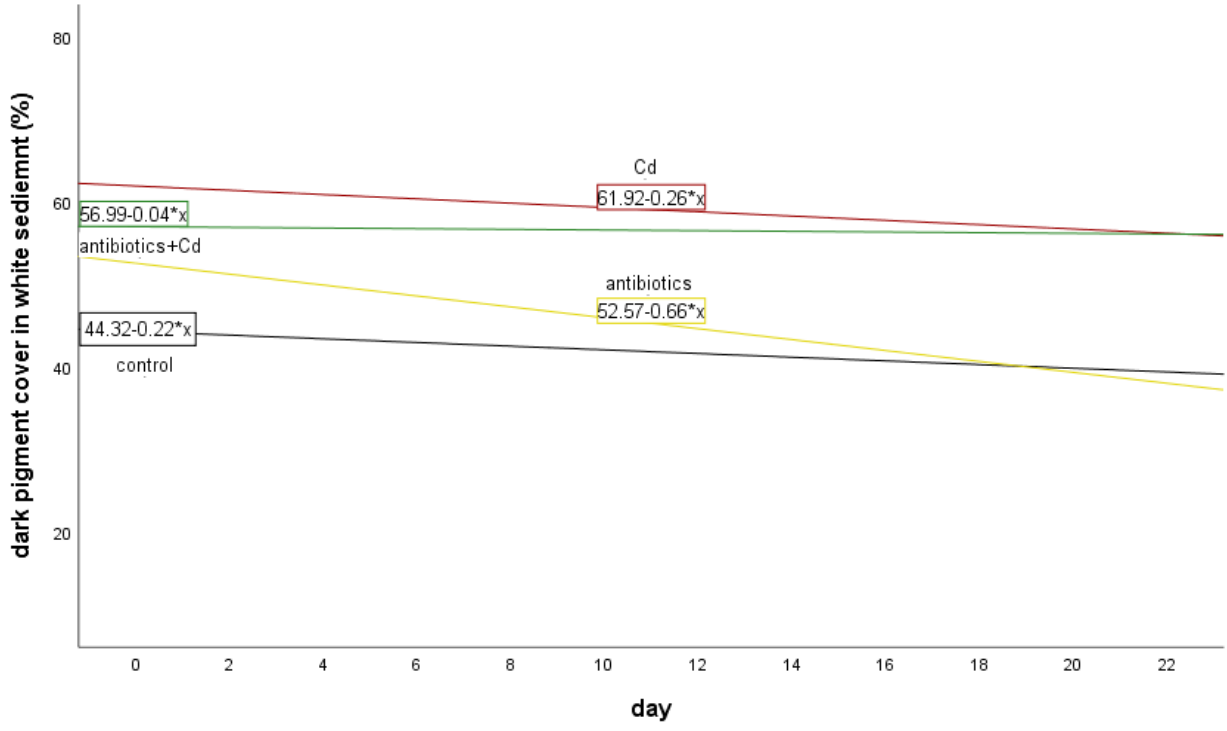

Figure 2.22 Regression slopes of dark pigment cover in white sediment over time in all groups in the Dale experiment, black line represent control, red represent Cd, yellow represent antibiotics and green represent antibiotics+Cd group

The ANOVA test conducted between groups showed significant difference in dark pigment cover in white sediment in between control and Cd in Mersey (Table 2.6; Figure 2.23). In Dale experiment, shrimp that treated with Cd alone or with antibiotics seemed to have higher average of dark pigment cover in white sediment compared to control and antibiotics group (p-value<.001; Table 2.6, Figure 2.23). This indicates that Cd may affect the ability of the brown shrimp to contract their pigment when they were place in light substrate.

Table 2.6 Descriptive Statistics and ANOVA of dark pigment cover in white sediment between groups for Mersey and Dale

group		Mean	Std. Error	F	p-value
Mersey	Control	50.48	1.16	4.61	**.033**
	Cd group	33.86	1.72		
Dale	Control	42.20	2.01	17.78	**.000**
	Cd group	60.04	2.28		
	Antibiotics	44.01	2.74		
	Antibiotics+Cd	56.70	1.55		

- Bold values were significant (p-value<.05)

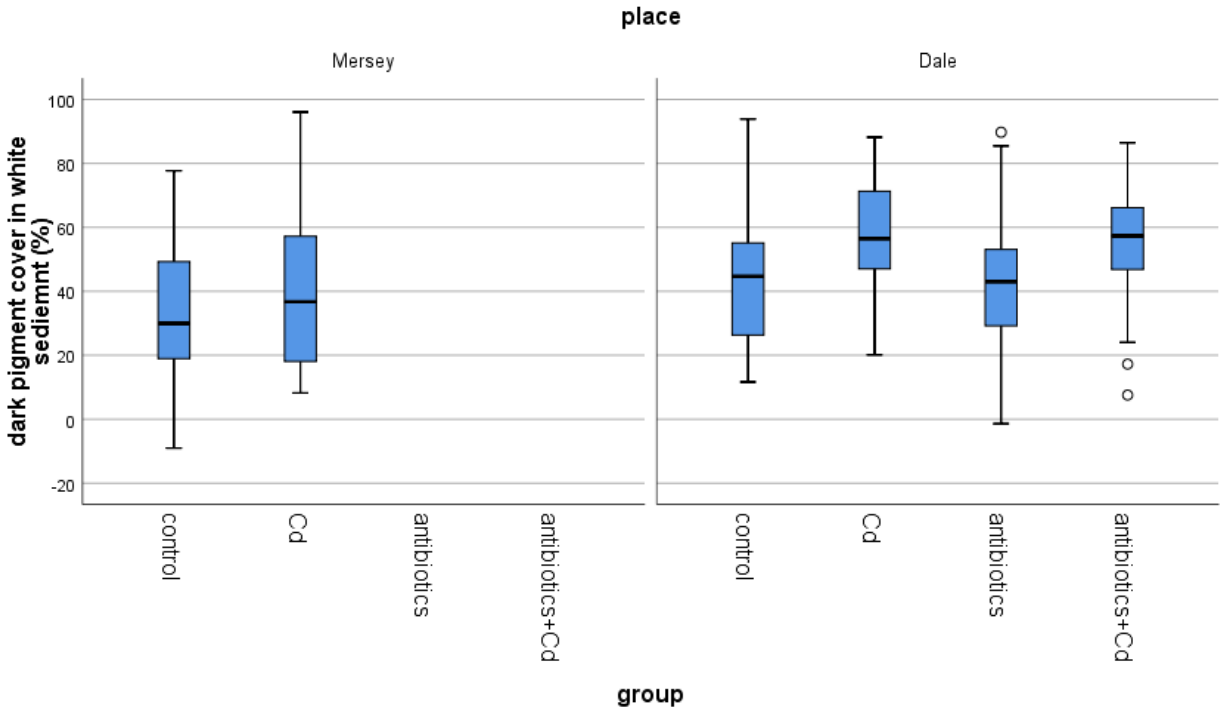

Figure 2.23 Dark pigment cover in white sediment between groups in Mersey and Dale, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

2.4.3 Colour change ability

Results of colour change ability measured by the difference between pigment cover in black and white sediment showed significant difference between days in Mersey experiment (both control and Cd group; p-value<.002; Table 2.7), whereas, no difference was found in all groups for Dale experiment (Table 2.7).

Table 2.7 Repeated measures ANOVA for colour change measured by the difference in dark pigment cover in black and white sediment within group between days in Mersey and Dale

Site	Group	F (Greenhouse-Geisser)	p-value
Mersey	Control	20.177	**.000**
	Cd group	5.090	**.001**
Dale	Control	2.311	.091
	Cd group	2.002	.139
	Antibiotics	3.048	.061
	Antibiotics+Cd	1.457	.254

- Bold values were significant (p-value<.05)

In general, colour change ability in Mersey experiment was increasing over time in both control and Cd group (Figure 2.24), indicating that the shrimp were getting better in changing colour. However, the regression slopes showed that control group was performing better than Cd group (
Figure 2.25)

Whereas, in Dale experiment, the colour change ability of the shrimp in each group were fluctuated during 22 days (Figure 2.26). Overall trend showed that control group was almost steady over time while Cd and antibiotics+Cd group were getting worse, indicating that the shrimp were less able to change colour over time (Figure 2.27).

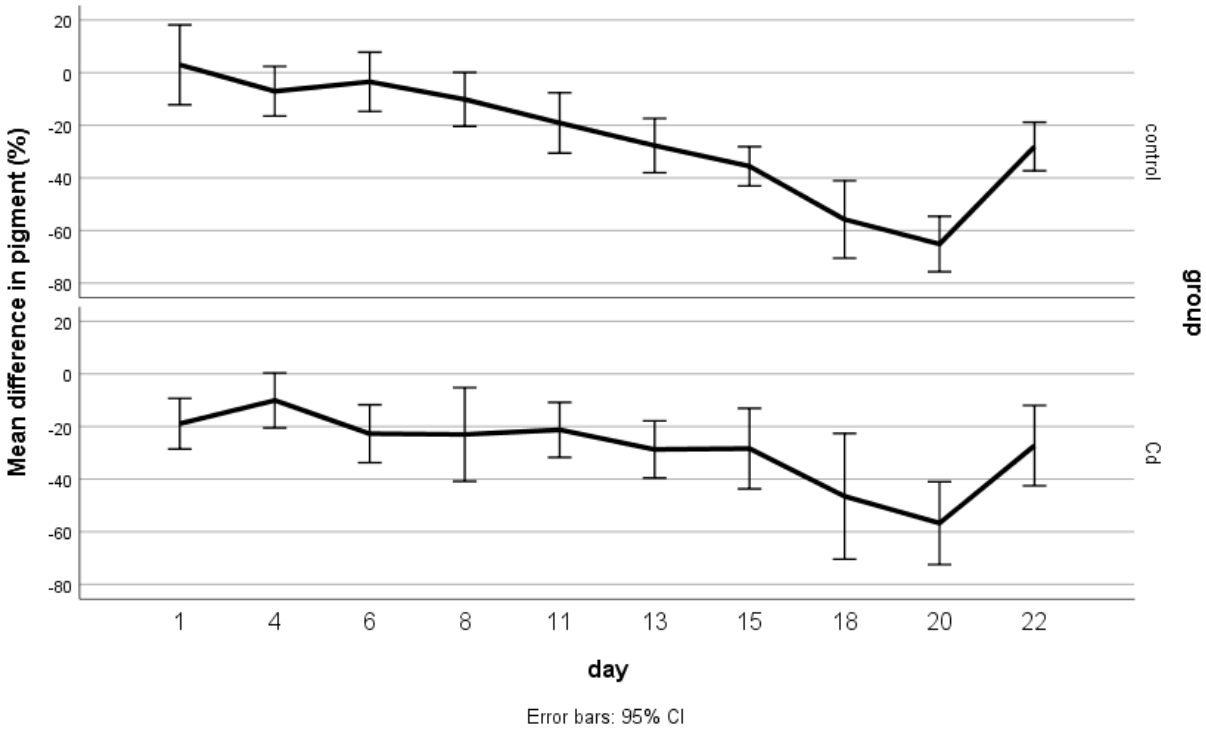

Figure 2.24 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment in control (top panel) and Cd (bottom panel) group in the Mersey experiment

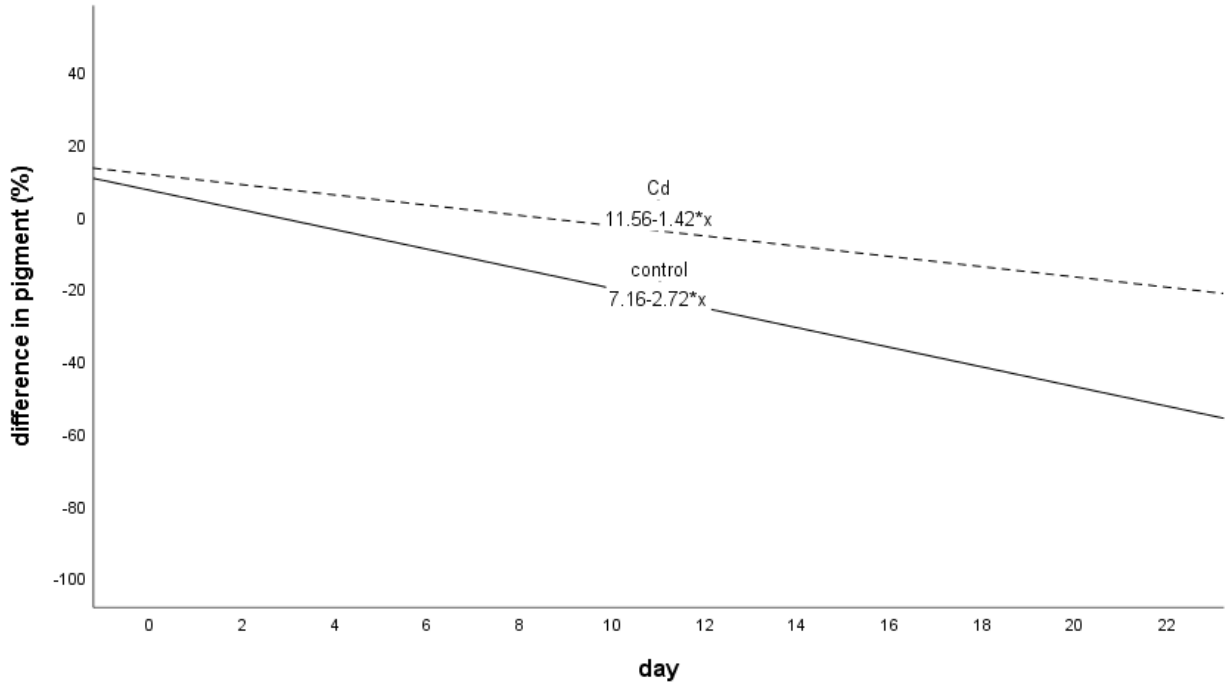

Figure 2.25 Regression slopes of colour change ability of the brown shrimp measured by the difference in dark pigment over time in both control and Cd group in the Mersey experiment, solid line represents control and dashed line represent Cd group

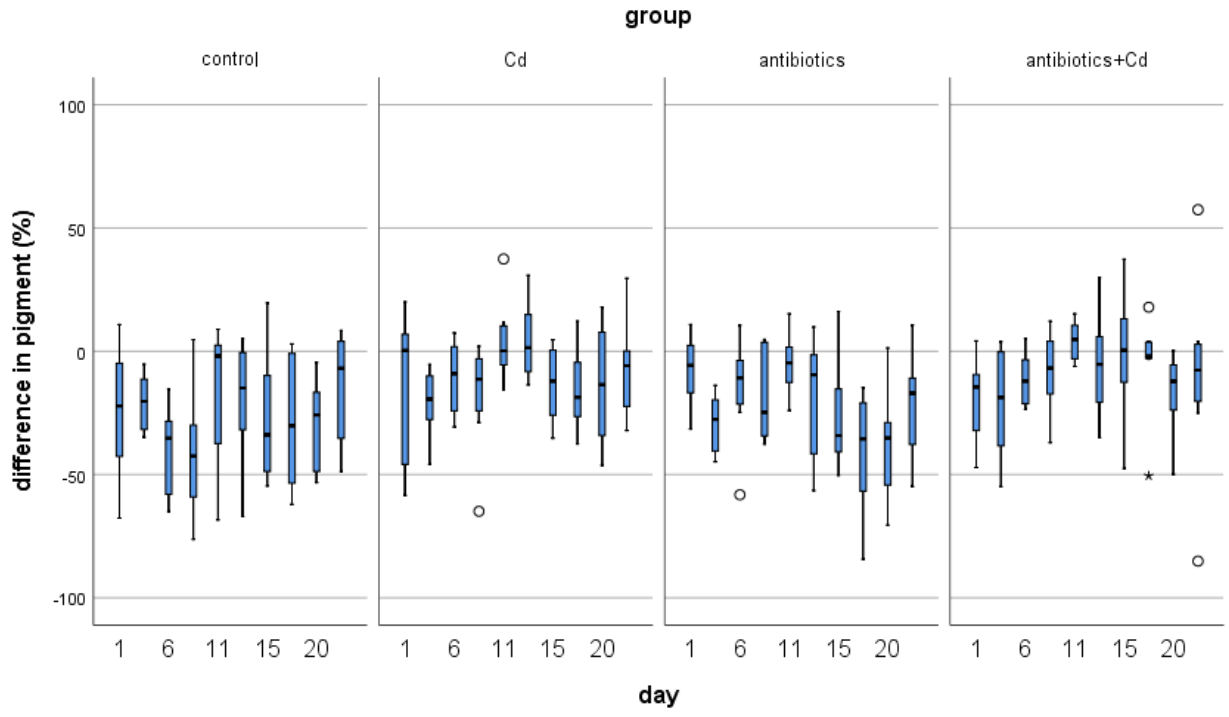

Figure 2.26 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment over time in all groups in the Dale experiment, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

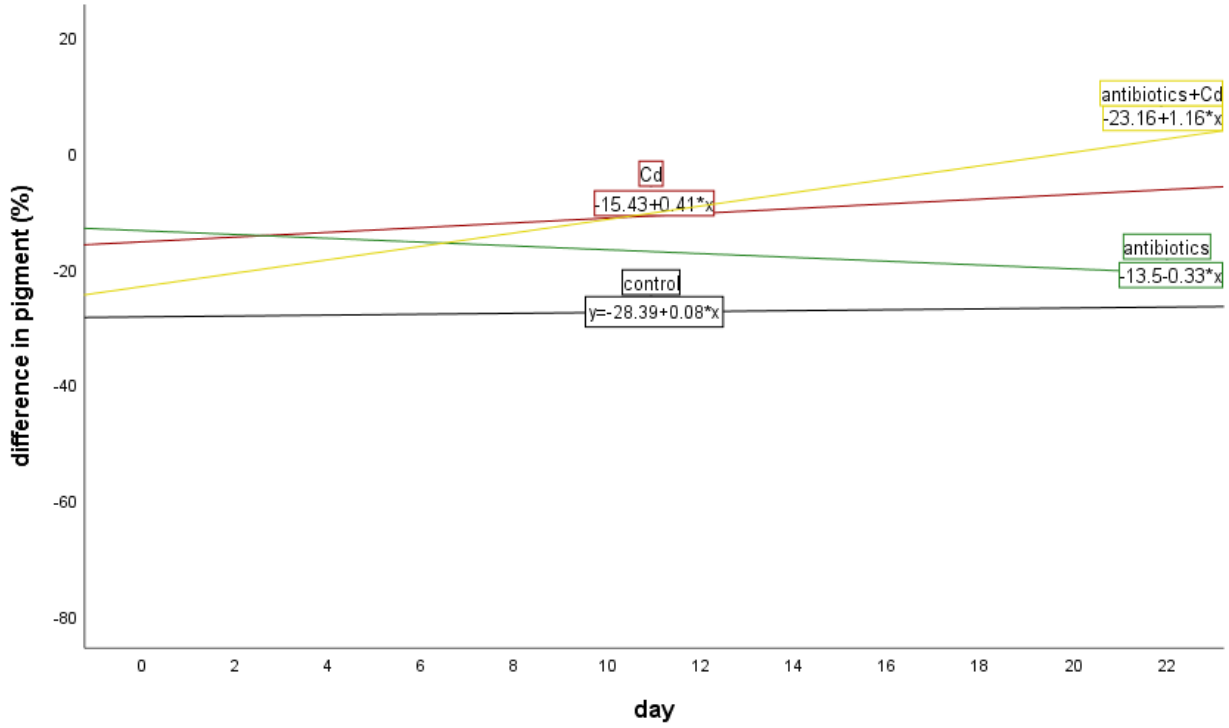

Figure 2.27 Regression slopes of Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment over time in all groups in the Dale experiment, black line represent control, red represent Cd, yellow represent antibiotics and green represent antibiotics+Cd group

With regard to between group analysis, no significant difference was found between control and Cd group in Mersey experiment in colour change ability measuring by the difference in pigment cover in black and white sediment (Table 2.8). This indicates that the shrimp in both groups were changing colour as it supposed to be when they were moved from black to white sediment Figure 2.28). In Dale experiment, there was significant difference (p-value<.001) between groups in the difference in pigment cover in black and white sediment (Table 2.8). Pairwise comparison showed that the difference in colour change ability of the shrimp in Dale experiment was between control group and both Cd group and antibiotics+Cd group (p-value<.001; Table 2.9) as well as between antibiotics and both groups treated with Cd either alone or with antibiotics. This indicates that the shrimp dosed with Cd, either alone or alongside antibiotics, were changing colour less when they were moved to different sediment (Figure 2.28).

Table 2.8 Descriptive statistics and ANOVA for colour change ability between groups in Mersey and Dale

group		Mean	Std. Error	F	p-value
Mersey	Control	-23.99	2.70	.951	.331
	Cd group	-27.47	2.36		
Dale	Control	-26.47	2.64	13.541	**.000**
	Cd group	-9.06	2.72		
	Antibiotics	-19.93	2.86		
	Antibiotics+Cd	-7.51	1.94		

- Bold values were significant (p-value<.05)

Table 2.9 Pairwise comparisons between the four groups in Dale

p-values using post-hoc test for groups

	Group	Control	Cd group	Antibiotics	Antibiotics+Cd
Dale	Control		**.000**	.075	**.000**
	Cd group			**.005**	.671
	Antibiotics				**.001**
	Antibiotics+Cd				

- Bold values were significant (p-value<.05)

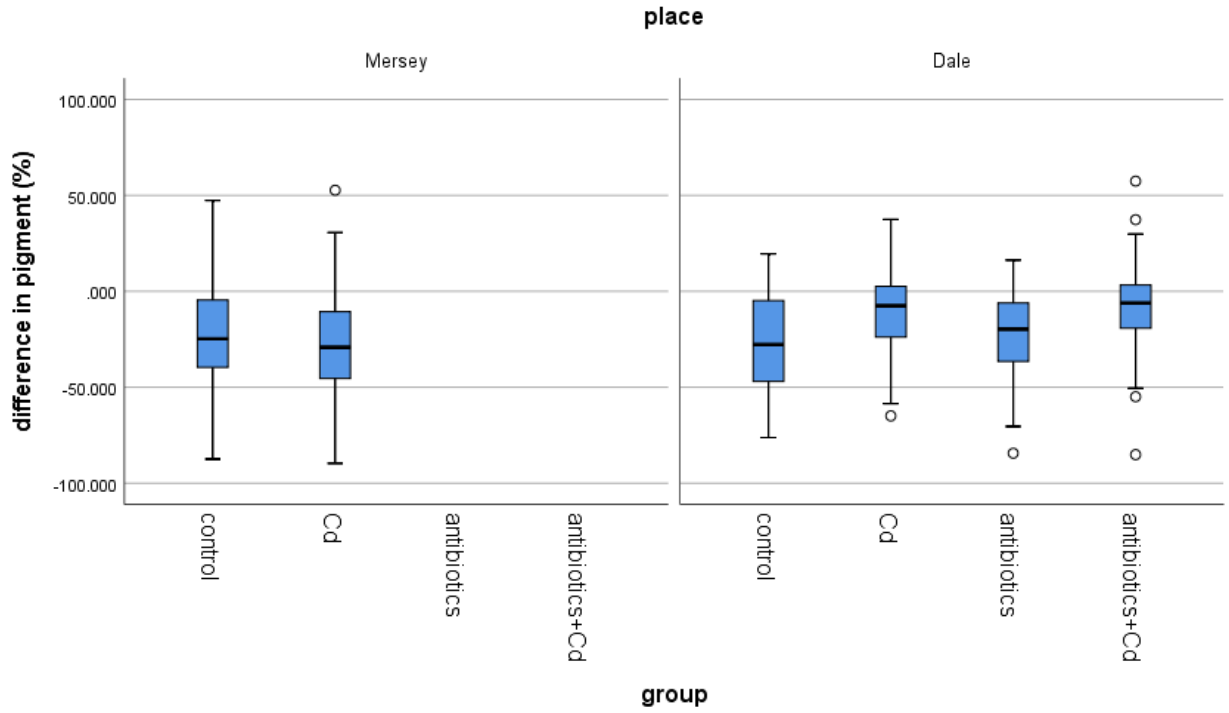

Figure 2.28 Colour change ability of the brown shrimp measured by the difference in pigment cover in black and white sediment between groups in Mersey and Dale, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

2.4.4 Heavy metals concentrations

2.4.4.1 Validation

Heavy metals measured in the CRM showed good recovery compared to the certified value (between 80-120%; Table 2.10).

Table 2.10 Recovery values for heavy metals

Heavy metals	**As**	**Ca**	**Cd**	**Cr**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**Pb**	**Zn**
Certified values (ppm)	6.7	1830	0.33	0.73	5.98	161	5370	1510	4.9	13900	2.18	71
Measured values (ppm)	5.5	1900	0.3	0.8	5.2	130	5000	1600	4.1	13000	1.9	72
Recovery (%)	82	104	92	110	86	81	93	105	85	94	87	101

2.4.4.2 Heavy metals concentrations in the environment and shrimp tissue

Due to the ICP protocol requirement of 0.2 g dry weight of each sample and the small size of the shrimp, only a few composite samples from Mersey in both control and Cd group were analysed. Water and sediment samples were analysed using the same protocol.

Most of heavy metals concentrations measured in water and sediment samples from Mersey and Dale were similar (Table 2.11). Iron concentration in water samples for both sites was below the limit of detection (0.04 ppm), as well as Ba and Pb (Table 2.11) in some water samples collected from both sites. Cadmium level was relatively low in both Mersey and Dale water samples (0.0006 and 0.0003 ppm respectively).

In terms of heavy metals found in shrimp tissue, Cd concentration in baseline shrimp (sacrificed immediately after sampling) was significantly high in Mersey shrimp at about 2088% compared to Dale. In control shrimp sacrificed after the experiment, Cd concentration was found to be slightly higher in both sites compared to their baseline (Table 2.12). On the contrary, baseline arsenic level in the shrimp was higher in Dale shrimp (18.91±3.83 ppm; Table 2.12). For the other metals, Mersey baseline shrimp seemed to have similar level of most of the heavy metals compared to Dale apart from Al, Ba and Mn.

Dale shrimp that were exposed to 0.04 ppm Cd for three weeks accumulated more Cd than Mersey shrimp (14.85 (n=13) and 7 (n=1) ppm respectively) but samples size was small. In respect of both groups that were treated with Cd alone or with antibiotics, the latter accumulated less Cd compared to the once that were treated with Cd alone (Table 2.13).

Table 2.11 ICP-MS results of heavy metals found in the environment (mean±SD ppm)

	Heavy metals	**Mersey (n=2)**	**Dale (n=3)**		**Heavy metals**	**Mersey (n=2)**	**Dale (n=3)**
Water	Al	0.024±0.01	0.007±0.01	**Sediment**	Al	15500±4950	2367±643
	As	0.057±0.0	0.074±0.01		As	55±4.24	2.5±0.4
	Ba	0.055±0.01	0.0045±0.0		Ba	530±283	3±1.3
	Ca	190±0.0	187±42		Ca	7350±3041	1200±0.0
	Cd	0.0006±0.0001	0.0003±0.0004		Cd	1.17±0.47	0.01±0.009
	Cr	0.005±0.00	0.006±0.001		Cr	130±14	4.8±1.6
	Cu	0.03±0.001	0.008±0.002		Cu	170±28	0.01±0.009
	Fe	0.02±0.0	0.02±0.0		Fe	33000±4243	4433±1012
	K	120±0.0	177±40		K	3400±1131	327±73.7
	Li	0.065±0.0	0.08±0.02		Li	44±11.31	7.3±1.3
	Mg	375±7.1	560±121		Mg	10800±1697	1967±379
	Mn	0.0041±0.002	0.014±0.01		Mn	795±49.5	69±4.7
	Na	3250±212	7200±755		Na	3150±212	2733±351
	Pb	0.00025±0.0	0.0005±0.0004		Pb	310±28.3	5.6±0.7
	Zn	0.02±0.014	0.087±0.006		Zn	775±120	22±5.5

Table 2.12 ICP-MS results of heavy metals found in baseline and control shrimp (mean±SD ppm)

	Heavy metals	**Mersey (n=2)**	**Dale (n=11)**		**Heavy metals**	**Mersey (n=2)**	**Dale (n=2)**
Baseline shrimp	Al*	101.5±19	48.64±18.2	**Control shrimp**	Al	19±11.3	6.4±3.25
	As*	3.8±0.0	18.91±3.83		As	18.6±21.8	31±16.97
	Ba*	24±0.0	3.52±2.2		Ba	6.5±1.1	3.65±0.92
	Ca	635000±91924	255636±246003		Ca	291500±337290	38500±106.7
	Cd*	3.55±3.04	0.17±0.1		Cd	4±0.3	0.4±0.2
	Cr	0.61±0.0	0.54±0.49		Cr	0.17±0.1	0.4±0.28
	Cu	46.5±23.3	63±22.95		Cu	115±7.1	41.5±12.02
	Fe	125±21.21	69.09±42.47		Fe	60.5±47.4	60.5±54.4
	K	103000±24042	40645±35502		K	103500±9192	7700±4667
	Li	0.24±0.02	0.4±0.16		Li	0.092±0.04	0.14±0.06
	Mg	23000±2828	18773±19065		Mg	26000±2828	3000±848.5
	Mn*	46.5±3.54	6.62±2.5		Mn	2.95±0.1	2.6±1.84
	Na	94500±21920	154455±151989		Na	170000±42426	24500±7778
	Pb	1.4±0.14	1.02±2.24		Pb	0.77±0.5	0.74±0.37
	Zn	985±21.21	374.6±377		Zn	1430±665	78.5±30.4

* showed significance at $p<0.05$

Table 2.13 ICP-MS results of heavy metals found in dosed shrimp (mean±SD ppm)

	Heavy metals	**Cd**		**Antibiotics**	**Antibiotics+Cd**
		Mersey (n=1)	**Dale (n=3)**	**Dale (n=5)**	**Dale (n=4)**
Dosed shrimp	Al	8.6	11.13±4.25	7.86±3.3	12.5±9.95
	As	2.6	28.5±10.34	43.8±17.95	24.5±9.4
	Ba	7.6	4.9±0.63	6.36±2.67	4.93±0.95
	Ca	550000	52500±8888	164800±226753	46250±12997
	Cd	7	14.85±3.66	0.322±0.29	8.30±3.85
	Cr	0.09	0.18±0.1	0.28±0.31	0.36±0.24
	Cu	110	79±30.63	65.2±17.7	73.5±26.04
	Fe	15	37±20.41	25.6±8.2	42.5±34.39
	K	110000	13000±1414	17840±16001	10200±2971
	Li	0.9	0.19±0.12	0.14±0.05	0.17±0.09
	Mg	27000	3000±356	3360±838.5	3300±1219
	Mn	2.4	3.7±0.94	3.5±1.01	4.25±2.86
	Na	190000	25250±8655	22400±5899	23750±7365
	Pb	0.42	0.41±0.09	1.46±2.26	0.66±0.52
	Zn	900	97.5±73.09	140±55.2	186±105.3

Based on all elements together, it was noticed that the suite of elements in Mersey shrimp was correlated to water (r=0.5760) and had stronger correlation with sediment (r=0.7793) (Table 2.14), but no relationship was found between water and sediment at the tested confidence level in Mersey (Table 2.14). Whereas, Dale shrimp were more correlated to water (r=0.8036) than to sediment, but Dale water and sediment was also correlated (Table 2.14).

Table 2.14 Spearman correlation between the whole suite of elements in water, sediment and shrimp in Mersey (all data presented in ppm)

	Matrix	**Water**	**Sediment**	**Shrimp**
Mersey	Water	1.0000	-	-
	Sediment	-	1.0000	-
	Shrimp	0.5760*	0.7793*	1.0000
Dale	Water	1.0000	-	-
	Sediment	0.5929*	1.0000	-
	Shrimp	0.8036*	0.6286*	1.0000

- Values were only present if significant at p <.01 and * showed significance at p<0.05

2.5 Discussion

2.5.1 Mortality

Crangon crangon was found to be sensitive to Cd (Jung & Zauke, 2008), and can accumulate high levels of Cd. Jung & Zauke (2008) reported 25 % of dead brown shrimp (15 out of 60) during 8 days of exposure to 0.005 ppm Cd. Similar results have been reported by (Pestana et al., 2007); they recorded a mortality rate of 30% in *Atyaephyra desmarestii* when they were exposed to 0.006 ppm Cd (calculated as LC_{10}) for 6 days, when the mortality was predicted in their experiment not to exceed by 10%. These results supported our findings regarding the mortality of the brown shrimp in Dale experiment (37 %).

2.5.2 Behaviour responses

The overall results showed no effect of Cd or antibiotics on body weight or daily food intake between the control and treatment group in the brown shrimp (at least not at the dosage used in this experiment), either in the Mersey or Dale population. Accumulation of Cd from water by the shrimp varied. This could be due to on the background profile of the heavy metals. Shrimp from Mersey had high baseline Cd concentration from the environment and accumulated less Cd than the shrimp from Dale (which had lower Cd in the tissue in the beginning but accumulated more Cd during the experiment compared to Mersey shrimp).

In terms of the colour of the shrimp, Mersey shrimp that were treated with Cd appeared to get darker in black sediment compared to the control. However, their ability to change colour from black to white was not significantly different between Cd and control group. In

the Dale population, the shrimp treated with Cd alone or alongside antibiotics were not able to become as pale as it was supposed to be when they were moved from black to white sediment. Hence, there was a sign that Cd could affect either the colour itself (by making them even darker in black sediment) or the colour change ability when they were moved from black to white sediment. Therefore, the experiment was performed again (see Chapter 3) with a slightly bigger sample size, in the attempt to overcome the high inter-individual variations.

2.5.2.1 Shrimp weight and food intake

Feeding behaviour has been reported to be one of the most important and useful bioindicators toward Cd in aquatic snails (Alonso & Valle-Torres, 2018). The sensitivity of feeding behaviour against some contaminants was described as sensitive as other physiological changes (Pestana et al., 2007). Hence, the effect of contaminants on populations and even on the ecosystem can be explained by the effect of them at the individual level (Forrow & Maltby, 2000; Pestana et al., 2007). In this study, no change was observed in body weight of the treated shrimp between days except for shrimp dosed with Cd in Mersey experiment. This could be because of the high inter and intra individual variations noticed among the shrimp, and/or the small sample size used in this experiment. Another explanation could be the low Cd concentration used in this study. Previous study has shown that the Cd has a negative effect on pond snails *Lymnaea luteola* L. by reducing the growth and feeding rate after exposure to 1 ppm Cd for 7 weeks (Das & Khangarot, 2010). However, they reported no effect of Cd on feeding when the dose was 0.001 ppm, and low effect was observed with 0.032 ppm Cd. Therefore, the lack of any change in body weight may be attributed to low Cd concentration used in the experiment and exposure time. Pestana et al. (2007) reported a reduction in feeding rate (measured by the difference between the dry weight of the leaf discs that used to feed the organisms before and after the experiment) in snails after 6 days of exposing to Cd (0.006 ppm).

On the other hand, no effect was observed in growth based on wet and dry weight mass of juvenile samples from channel catfish, fathead minnow and rainbow trout when they were fed dosed food with Cd for 30 days (Erickson et al., 2010). Therefore, Cd toxicity could depend on the type and time of dosage.

The shrimp in antibiotics+Cd group in Dale experiment showed higher average of food intake (p-value=.05, borderline maybe due to high individual variation) compared to the control group. This could be a sort of adaptation associated with the contamination in the environment. Blockwell, Taylor, Jones and Pascoe (1998) reported a significant increase of feeding rate in *Gammarus pulex* (L.) exposed to lindane. However, a very recent study conducted to evaluate the effect of antibiotic (sulfamethazine) on feeding and body weight (Yan et al., 2019) in zooplankton (*Brachionus calycifloru*) showed feeding inhibition, due to the effect of sulfamethazine on neurotransmission which influences the digestive enzymes.

2.5.2.1 Colour change ability

Chromatophores are specialized cells containing pigment that can be found in dermis, and they can change the colour of the animals by being aggregated or dispersed as a result of signals from the central nervous system and they have been used as a biomarker to assess the environmental pollution (Ahmad et al., 2018). The results recorded in this experiment showed that there were significant differences in dark pigment cover either in black sediment or white sediment between some days in Dale and Mersey populations. This could be due to the high individual variability in the brown shrimp (Siegenthaler et al., 2018).

Shrimp in Cd group and antibiotics+Cd group in Dale experiment appeared to change colour less than control group when they were moved from black to white sediment. This was due to the high percentage of dark pigment measured when the shrimp in these groups were placed in white sediment compared to control group. This might suggest that Cd and/or antibiotics may affect the contraction ability in the brown shrimp when they were exposed to them. The effect of Cd on colour change might be indirectly altering black pigment-dispersing hormone (BPDH) which control the pigment as it was reported before with different species, mainly fiddler crabs and fish. A previous study conducted by Reddy and Fingerman (1995) to evaluate the effect of Cd on fiddler crab *Uca pugilator*. Treated crabs, both *in vitro* and *in vivo,* were transferred from white to black substrate and failed to match the background colour. They have found that Cd has no direct effect on the melanophores, however it has an effect on the neuroendocrine regulatory process that play a major role in controlling the pigments. Also, Cd was found to inhabit BPDH synthesis in the eyestalk. Reddy and Fingerman (1995) have reported aggregation in the chromatophores in fiddler crab that were exposed to Cd. The crabs that were treated with Cd in the water (*in vitro*)

were found to be paler than the ones injected with Cd, despite the fact that the injected crabs (*in vivo*) reacted faster to the Cd (the chromatophores started to aggregate after 24 hours whereas the crabs that were in treated in water changed colour after 48 hours). Thus, the exposure route can play an important role in the toxicity of the Cd on colour change ability.

Another study was performed to determine the effect of polychlorinated biphenyl (PCB) Aroclor 1242 on *U. pugilator*. It was described that the reduction of the dispersion pigment in the treated crabs was due to the role of PCB in inhibiting the release of the BPDH from the sinus gland (Fingerman & Fingerman, 1978). It has been found that the expansion of the pigments in the chromatophores is regulated by the pigment-dispersing and concentrating neurohormones (Carlson, 1935; Fingerman, 1965; Sandeen, 1950).

Another study showed significant change in chromatophores of catfish, *Heteropneustes fossilis* by increasing in the number of the chromatophores, after one day of exposing to 392.92 ppm (subacute) and after 20 days of exposing to 98.23 ppm (acute) of Cd (Ahmad et al., 2018). However, by the end of the experiment (30 days for subacute and 96 hours for acute experiment), significant decrease in the number of the chromatophores compared to the control was noticed. This indicate that the fish became paler when they were treated with Cd. Meidivanto et al. (2018) published that the increase of cAMP or/and Ca^{2+} which induced by treated the fish with Cd cause aggregation in pigment of tilapia fish.

There is another external factor that can also influence the colour change ability of shrimp, fish and other animals besides contaminants such as temperature, salinity, light, and water quality (Ahmad et al., 2018). Meidivanto et al. (2018) reported negative impact in melanocyte-stimulating hormone and the number of melanophores of Tilapia *Oreochromis niloticus* with low water salinity in the presence of Cd.

From this study, it is not possible to definitely concluded the effect of Cd on the colour change ability in some aquatic animals by causing either darkness or paleness appearance. It has been known for some animals including fish and crustaceans that they change their colour to avoid predator in the environment and in some cases, they change colour to adapt to any changes in the environment happen over time (Duarte, Flores, & Stevens, 2018). Hence, being in contaminated environment could lead to lose this ability in aquatic organisms and then being hunted by predators and even being vulnerable to UV light (Meidivanto et al., 2018).

2.5.3 Heavy metals concentrations in the environment and shrimp tissue

Cadmium is one of most ecotoxic metals and it is accumulated easily by many aquatic animals (Sornom et al., 2012). Continued exposure of marine organisms to Cd even in small concentrations can result in transferring the toxic metal to the human through food chain (Untersteiner et al., 2005).

Culshaw et al. (2002) reported an average of 0.21 ppm Cd in sediment samples collected from nine sites in Severn Estuary and Bristol Channel which was lower than the Cd measured in Mersey sediment (1.17±0.47 ppm), whereas, Cd concentration in Dale sediment was less than the concentrations reported in some estuaries in the UK (Bryan & Langston, 1992; Culshaw et al., 2002). The highest Cd concentration in sediment that has been published was 2.17 ppm collected form the Tyne (Bryan & Langston, 1992).

In terms of baseline Cd concentration in water samples in both sites (Mersey=0.0006 and Dale=0.0003 ppm), it was slightly high compared to the average level reported in the UK costal water (0.00001 - 0.00017 ppm; Bryan & Langston, 1992), however, it was lower than Cd level found in Bristol Channel (above 0.001 ppm; Morris, 1984).

Cadmium level in the brown shrimp collected from Mersey was similar to the level found in the shrimp collected from Lydney (4.82±0.68 ppm) and Barry Island (4.49±0.65 ppm) which both were high compared to the shrimp collected from other sites in the Severn estuary and Bristol channel (ranging from 1.4 to 2.2 ppm; Culshaw et al., 2002). Regarding Cd level found in Dale shrimp (0.17±0.1 ppm), it was lower than the concentration measured in the brown shrimp (ranged from 15 to 1.4 ppm) reported by Culshaw et al. (2002). Cadmium concentration measured in Dale shrimp was in the same range of Cd concentration (between 0.23 to 0.5 ppm) reported by Bat et al. (2013) in the same species which were collected from Black Sea (Turkey). Considering the habitat of the brown shrimp, benthic near the sediment, they are more susceptible to metal pollution. Since the level of the heavy metals measured in the sediment of Mersey was high and the correlation between heavy metals in the shrimp and water and sediment was found, these could explain the high level of the heavy metal found in the shrimp collected from Mersey, besides being exposed to the toxic metals through water.

Control group that was sacrificed after the experiment showed similar Cd concentration in their body compared to the baseline, indicating that the depuration of Cd in the brown

shrimp could be slow. Szaniawska (1985) reported that the brown shrimp were accumulating Cd easily from the water, however, they seemed to have difficulties in excreting it during the depuration period.

The level of Cd accumulated by the brown shrimp throughout the experiment was different in Mersey and Dale. In our experiment Dale shrimp accumulated more than Mersey shrimp. This could be due to the concentration of Cd in the shrimp previously accumulated from the environment and/or inter-individual variations since the shrimp that sacrificed after the experiment were different from those sacrificed after sampling.

Accumulation of Cd by marine organisms is affected by different factors. One is the accumulation of Cd is associated with Ca level in the body (Rainbow, 1997). Wright (1977) reported that there was considerable inverse relationship between Cd and Ca in shore crab when they were dosed with Cd and this was attributed to the competition between Cd and Ca for deposition sites. This hypothesis seems to be somewhat supported by our results which also showed an increase in Cd and a decrease in Ca concentration in dosed shrimp compared to control/baseline. However, this is not conclusive as it cannot be verified since no significant negative correlation was found between Cd and Ca, which could be due to the small sample size and the high individual variations.

The other factor that could affect the accumulation of Cd in marine animals is salinity (Szaniawska, 1985; Wright, 1977; Wu & Chen, 2004). Low salinity causes high Cd accumulation. This is because the reduction in salinity causes a high number of toxic free ions in the water especially in the winter (Culshaw et al., 2002). In this study, the two populations were kept at different salinity levels, matching their original environment.

3. Chapter Three: Effects of cadmium on colour change in the brown shrimp (*Crangon crangon*) from two different populations

3.1 Introduction

Some animals use a wide range of techniques to hide from predators, from camouflage to colour change to match the background, and disruptive colouration. Some crustaceans are transparent, others change their colour to match the background (Siegenthaler et al., 2018). Thus, changing colour is one of the common techniques used by many crustacean species. It allows them to cope with the spatial and temporal changes in the environment. Colour change is a complex strategy and it links cells physiology, animal behaviour, vision perception and external factors. Understanding these factors and how they can participate in animal concealment is necessary to understand the development of the ability to change colour in animals. Colour change in animals involves two different mechanism: physiological changes (which take seconds to hours) and morphological changes (which take days to months), and it can be affected by some external factors such as temperature, contaminations, tide and circadian rhythm (see chapter 1 for more details). The effect of some of these factors has been studied mainly on colour change ability of crabs. However, studying the effect of heavy metals on the colour change ability in the brown shrimp has not been tested yet.

Following the previous chapter, there was some promising trends suggesting that Cd could have an effect on the colour change of the brown shrimp possibly interfering with their ability to contract the dark pigment in the chromatophores. However, there was no evidence that show an interaction between Cd and antibiotics. Hence, the aim of this chapter was to focus on testing the effect of Cd on colour change ability of the brown shrimp. In this chapter, Liverpool Bay was considered instead of Mersey pool due to the lack of shrimp in the pool.

The aim of this chapter was to determine the effect of Cd on two different populations and testing two different substrate directions (from black to white sediment and vice versa) for short term (7 days). Our hypotheses were that the shrimp from Liverpool would be affected

by Cd more than shrimp from Dale as we assumed that Liverpool is cleaner than Dale and the brown shrimp would still be able to expand and contract their pigment even if they were moved from white to black sediment instead of black to white sediment.

3.2 Methodology

3.2.1 Study sites

The first sampling site, Dale, is located in South West Wales, in the south west tip of Pembrokeshire (51.708284, -5.168316; Chapter 2 Figure 2.3), in the Pembrokeshire Coast National Park. The area faced a major ecological disaster in 1996 when the oil tanker "Sea Empress" spilled 73,000 tons of crude oil in the water. Hence, this site was assumed to have high contamination (Law & Kelly, 1998).

The second sampling site is located in the Liverpool Bay, Wallasey (53.437667, -3.035417; Figure 3.1), positioned at the north-eastern tip of the Wirral Peninsula, on the Irish Sea. This location was chosen instead of Mersey pool because of the lack of the shrimp in the latter.

3.2.2 Sampling

Crangon crangon specimens were collected from the sites and the first collection was performed in Dale, Pembrokeshire in April 2017. The second collection was from the estuary of the river Mersey on the Irish Sea in Liverpool Bay in May 2017. The samples were collected by using push nets (mesh size: 6mm). Water and sediment samples (at least two) were also gathered from each site, labelled and stored in the fridge for heavy metal analyses. Water salinity and temperature for each site were also measured. A specific code was given to each sample (e.g., D1: Dale 1; L1: Liverpool 1).

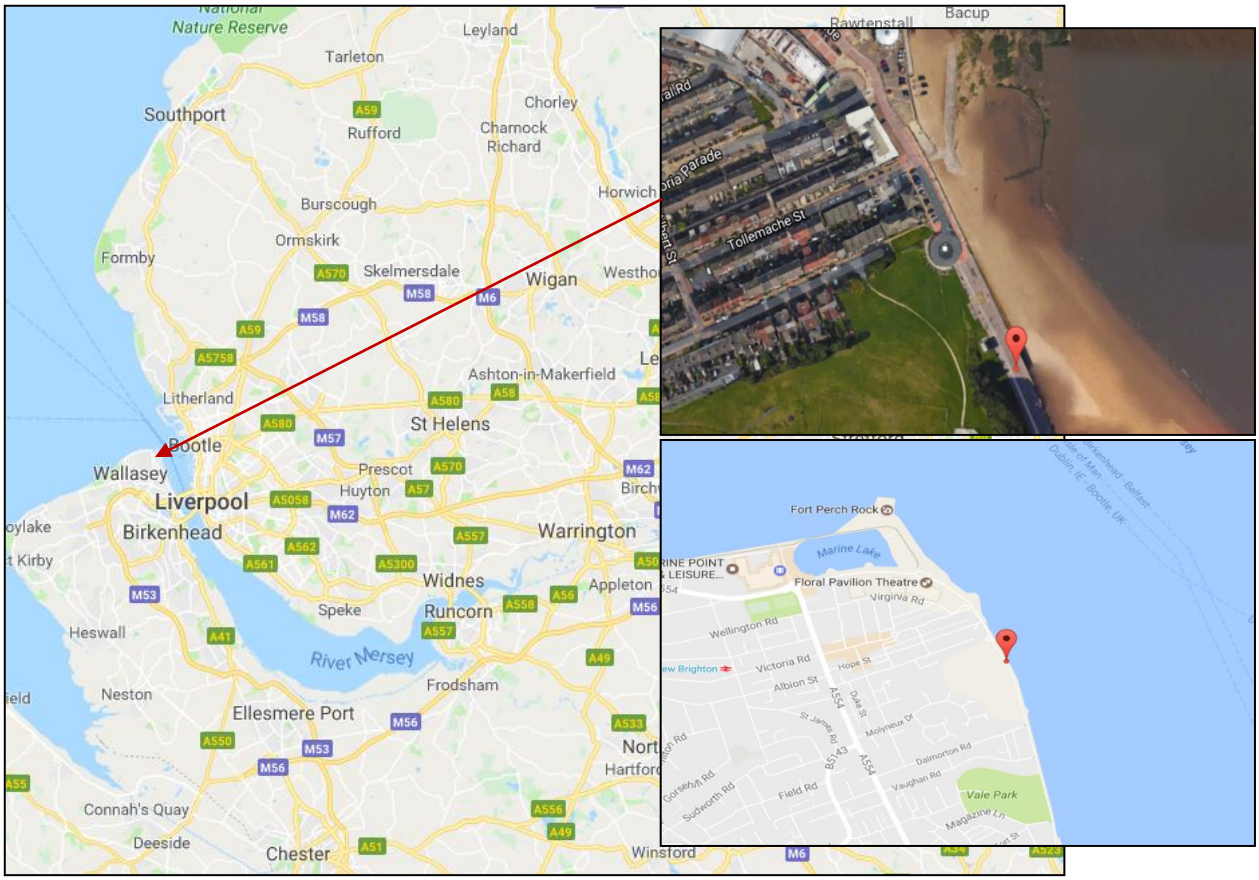

Figure 3.1 Sampling site to in the Liverpool Bay, England. A) Satellite map; B) General map (Google Maps, 2019)

3.2.3 Chemicals and equipment

To prepare the artificial seawater, 210 grams of Instant Ocean salt were added to 10 litres of deionised water (20 psu). Two tanks (commissioned to Aquaponics Labs) containing 24 chambers each were used, half of them were filled with a layer of black sediment, 1 cm thick, and the other half with a layer of white sediment, 1 cm thick. Air bubblers and artificial sea water were provided in each tank. The shrimp were maintained in the individual chambers to avoid cannibalism and to keep track of their ID (Figure 3.2).

A solution of 0.04 ppm of Cd was prepared dissolving 0.4 g of Cd chloride in 10 litres artificial seawater, following Tripathi and Pandey (2014), who have found that 0.04 ppm Cd affects colour changing and other behavioural aspects in prawns (*Macrobrachium dayanum*).

Figure 3.2 Set up of the experimental boxes (Aquaponics Labs)

3.2.4 Design of the main experiment

The shrimp were brought to the lab and since they were brought from two different areas, it was envisaged that heavy metal contamination profile in them including cadmium would be different. Hence, 8 animals were sacrificed to assess the baseline concentration of the heavy metals. In addition, water and sediment samples collected from the field were also analysed using ICP-MS and ICP-ES. This part was conducted by SOCOTEC group (see section 2.3.8 Heavy metal analysis, Chapter 2).

The shrimp were acclimatized for three days before starting the experiment which lasted one week. The reason behind reducing the time of the experiment compared to that in the last chapter we found the major changes in colour change was happened in the first week (Appendix Figure A.1). With regards to water evaporation, the level of the water in the aquaria was checked carefully each day during the experiment to ensure that the shrimp were exposed to the same level of the Cd every day. The shrimp were fed three times a

week after the colour change measurements. At the end of the experiment, all shrimp were sacrificed by freezing and placed individually in labelled bag.

3.2.4.1 Dale experiment

Forty-eight adult shrimp from Dale were divided randomly into 2 groups, control and treatment (n=24 each) and placed individually into labelled chamber (Figure 3.2). For the control group, 24 shrimp were kept in 10 litres of clean artificial seawater; 12 of them were kept in black sediment and the other 12 were in white sediment. In the treatment group (dosed with Cd) 24 shrimp were exposed to 0.04 ppm of Cd in 10 litres of artificial seawater; 12 of them were kept in black sediment and the other 12 were in white sediment.

Shrimp in each group were moved from a chamber with a certain colour substrate to another, of the opposite colour for two hours (Figure 3.2). The shrimp in direction 1 (B to W) were moved from black to white sediment and direction 2 (W to B) were moved from white to black sediment (Figure 3.3) to check how well they could change colour in both directions (from back to white and vice versa).

3.2.4.2 Liverpool experiment

The same setup described above repeated with shrimp from Liverpool Bay. Forty-eight adult shrimp were divided randomly into 2 groups, control and Cd, and placed individually into labelled chamber (Figure 3.2). To increase the sample size, a second batch of the same experiment was repeated again with 96 shrimp (48 in control and 48 in Cd group). Hence, the total sample size in this experiment was 72 (24+48) in each group. The same protocol described above was followed (see section 3.2.4.1 Dale experiment).

3.2.5 Colour change measurement

During the experimental period of 7 days, each day all the shrimp in each group were moved individually from their initial chamber into another with opposite coloured (the ones that were in black were transferred to white and vice versa) for two hours (Figure 3.3). Before transferring them, a photo of the right exopod in the telson (Figure 2.7, Chapter 2) for each individual shrimp was taken by using a dissecting microscope (Leica S6D). After two hours, a second photo of the right exopod in the telson was taken again and was used to assess colour change ability. All shrimp were then returned to the original sediment in the chamber. As mentioned above in section 3.2.4.1 Dale experiment, the colour change was

measured from B to W and from W to B (Siegenthaler et al., 2018), to control for the ability of the shrimp to contract and expand dark pigments in their chromatophores. All pictures were analysed by using ImageJ software and an established protocol (see section 2.3.7 for more details).

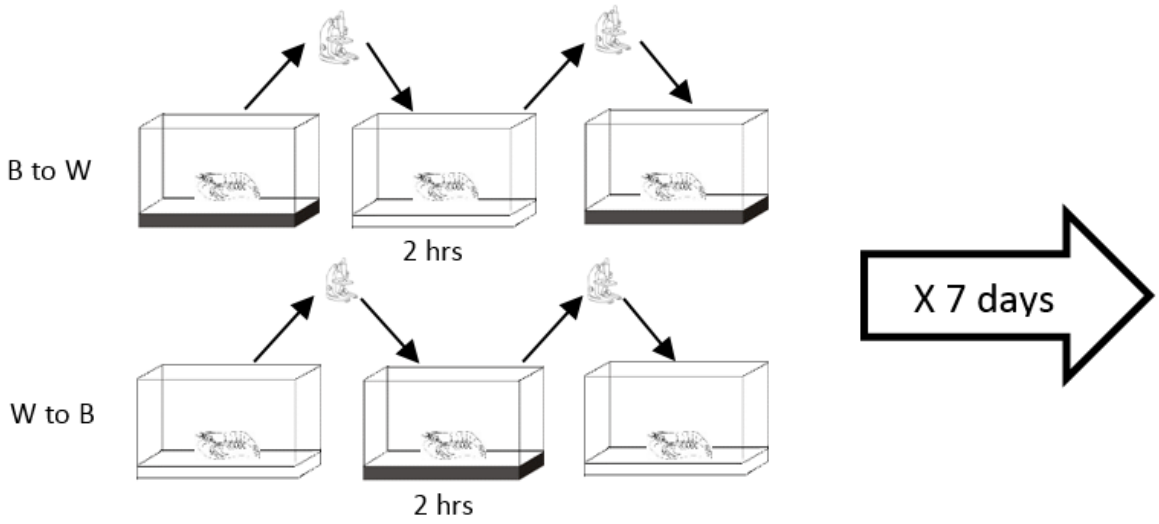

Figure 3.3 Schematic overview of the colour change protocol

3.2.6 Statistical Analysis

The aim of the experiment was to test for the effect of Cd on the ability of the brown shrimp to change colour by calculating the difference in dark pigment cover (surface of the 1mm^2 section photographed covered by dark pigments) in black and white sediment. This change was tested using two treatments (control and Cd) and two directions (from B to W and from W to B sediment) in two different populations (Dale & Liverpool; Figure 3.4). All dead shrimp were excluded from the analysis (Table 3.1).

The comparison within groups was performed using repeated measures ANOVA (Verma, 2015), followed by pairwise comparisons if the ANOVA was statistically significant. Pairwise comparisons based on multiple paired t-tests, with a Bonferroni correction to keep the type one error at 5% overall, was used to compare each pair of days. The comparison between groups (control and Cd in Dale and Liverpool) was performed using paired t-test between the two independent groups. For repeated measures ANOVA, the assumption of sphericity was checked using Mauchly's test (Field, 2009), which test equality of population variance at all possible changes (day 1, 2, 3, 4, 5, 6 and 7). This assumption was violated in all groups, hence, F test based on Greenhouse-Geisser (Epsilon<0.75) was used (O´Brien & Kaiser,

1985). Normality assumptions were checked using the K-S test, skewness and kurtosis for each group, and all data were found to be approximately normally distributed. Boxplots were used to display minimum, first quartile, median, third quartile and maximum of each data across time. Regression slopes were also performed to see the overall trend during the time of the experiment.

Spearman correlation and non-parametric t-test (Mann-Whitney) were used to check the relationship and the difference of the heavy metal elements among water, sediment and shrimp in both sites. All analyses were performed with IBM SPSS 24.

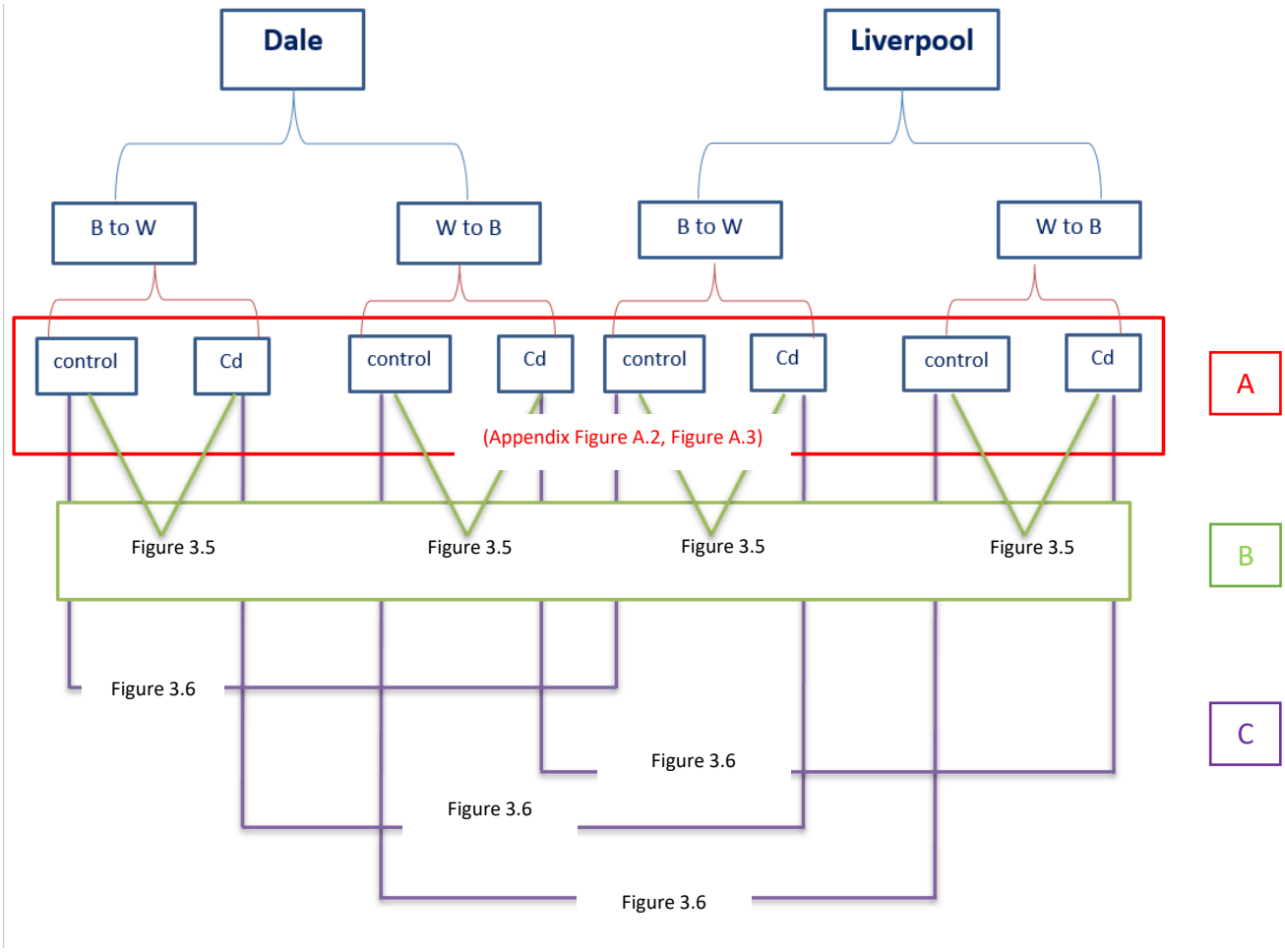

Figure 3.4 Schematic experimental analysis for colour change ability. A) temporal variation within each group (control and treatment); B) intrapopulation differences between groups; C) interpopulation differences within each group

3.3 Results

3.3.1 Mortality

In the Dale experiment, there was one dead shrimp in control (W to B direction) and three in the treatment group (2 from B to W direction and 1 was from W to B direction). In the Liverpool experiment, there were three dead shrimp in the control group (B to W direction) and 15 dead shrimp in the treatment group (four from B to W direction and 11 from W to B direction). Total sample size that was alive till the end of the experiment is summarised in Table 3.1.

Table 3.1 Actual sample size in Dale and Liverpool experiment that used in the analysis

Population	**Dale**		**Liverpool**	
Direction	B to W (n=12)	W to B (n=12)	B to W (n=36)	W to B (n=36)
Control	12	11	33	36
Cd	10	12	32	25

3.3.2 Colour change ability

Colour change ability was measured by the difference in dark pigment cover in black and white sediment either from B to W or from W to B direction. It was noticed high inter individual variability in colour change ability either in control or Cd group in both populations and both directions (Appendix Figure A.2, Figure A.3).

With regards to colour change ability between groups, t-test results showed no significant change between control and Cd group in both directions and for both populations (Table 3.2, Figure 3.5), which was not consistent with the results in Chapter 2 especially regarding Dale populations where a significant difference was noticed between Cd groups and the control.

Table 3.2 Descriptive statistics and t-test for difference in dark pigment cover in black and white sediment between groups in Dale and Liverpool for both directions

Population	Direction	Group	Mean	Std.Error	t-test	p-value
Dale	B to W	Control	-16.25	2.08	-1.423	.157
		Cd	-11.63	2.54		
	W to B	Control	16.67	2.49	1.136	.258
		Cd	12.95	2.13		
Liverpool	B to W	Control	-16.82	1.21	-.916	.360
		Cd	-15.24	1.23		
	W to B	Control	20.30	1.13	.300	.764
		Cd	19.77	1.36		

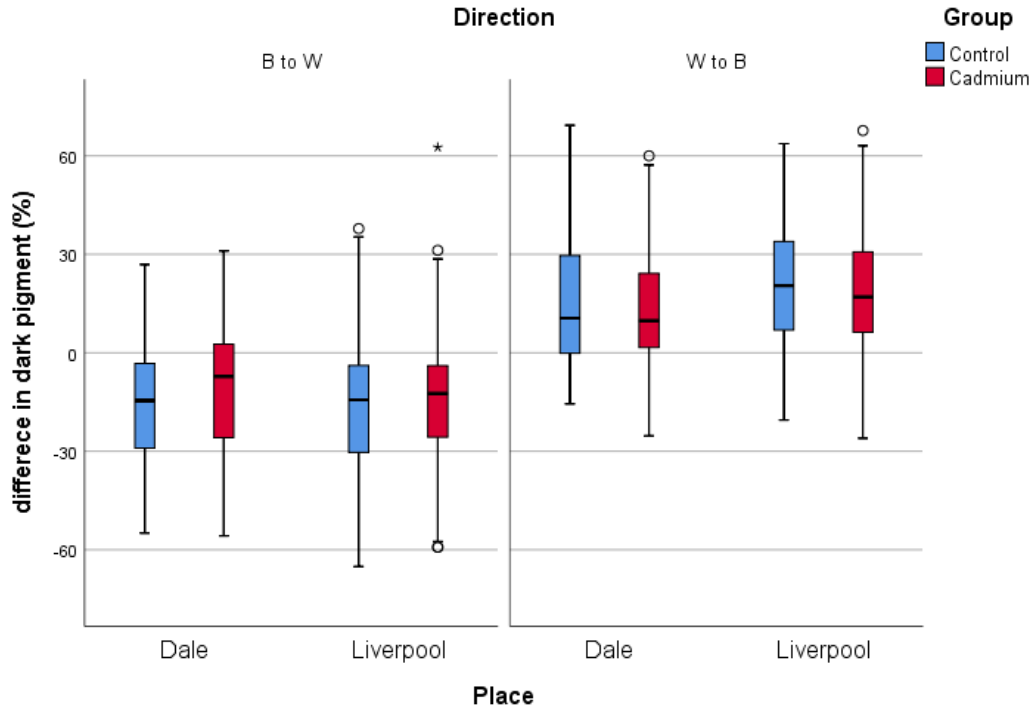

Figure 3.5 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment between groups in both populations (Dale and Liverpool) and in both directions, box plots display minimum, maximum, median and first and third quartile of each group; circles show outlier values and stars show extreme outliers

With regards to comparing two populations, there was no significant difference in colour change ability between control groups in Dale and Liverpool in both directions and between Cd groups in Dale and Liverpool in B to W direction (Table 3.3, Figure 3.6). The only significant difference was found between Dale and Liverpool in Cd group in W to B direction (Table 3.3, Figure 3.6 D), indicating that the shrimp that exposed to Cd in the water in Dale experiment changed colour less than the ones from Liverpool.

Table 3.3 Descriptive statistics and t-test for difference in dark pigment cover in black and white sediment between populations in control and Cd group for both direction

Direction	Group	population	Mean	Std.Error	t-test	p-value
B to W	Control	Dale	-16.25	2.08	.240	.810
		Liverpool	-16.82	1.21		
	Cd	Dale	-11.63	2.54	1.383	.168
		Liverpool	-15.24	1.23		
W to B	Control	Dale	16.67	2.49	-1.471	.142
		Liverpool	20.30	1.13		
	Cd	Dale	12.95	2.13	-2.736	**.007**
		Liverpool	19.77	1.36		

- Bold values were significant (p-value<.05)

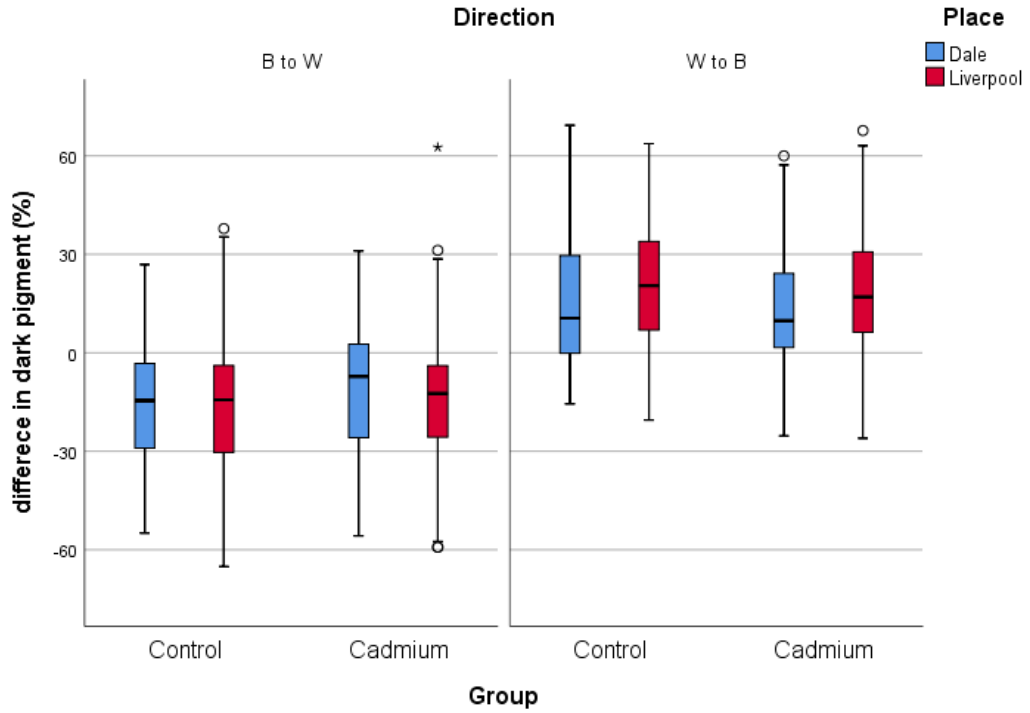

Figure 3.6 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment between groups in both populations (Dale and Liverpool) and in both directions, box plots display sent minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

Based on the results in Table 3.3, the only group that showed significant change in colour change ability was Cd group in W to B direction between Dale and Liverpool. Hence, regression slopes were drawn to see the overall trend over time. Both populations showed similar increasing trend in the dark pigment cover in black sediment in Cd group (Figure 3.7). However, when the shrimp in Cd group returned back to the original sediment colour (white in this case), they become darker with time in the Dale than in to the Liverpool one (Figure 3.8).

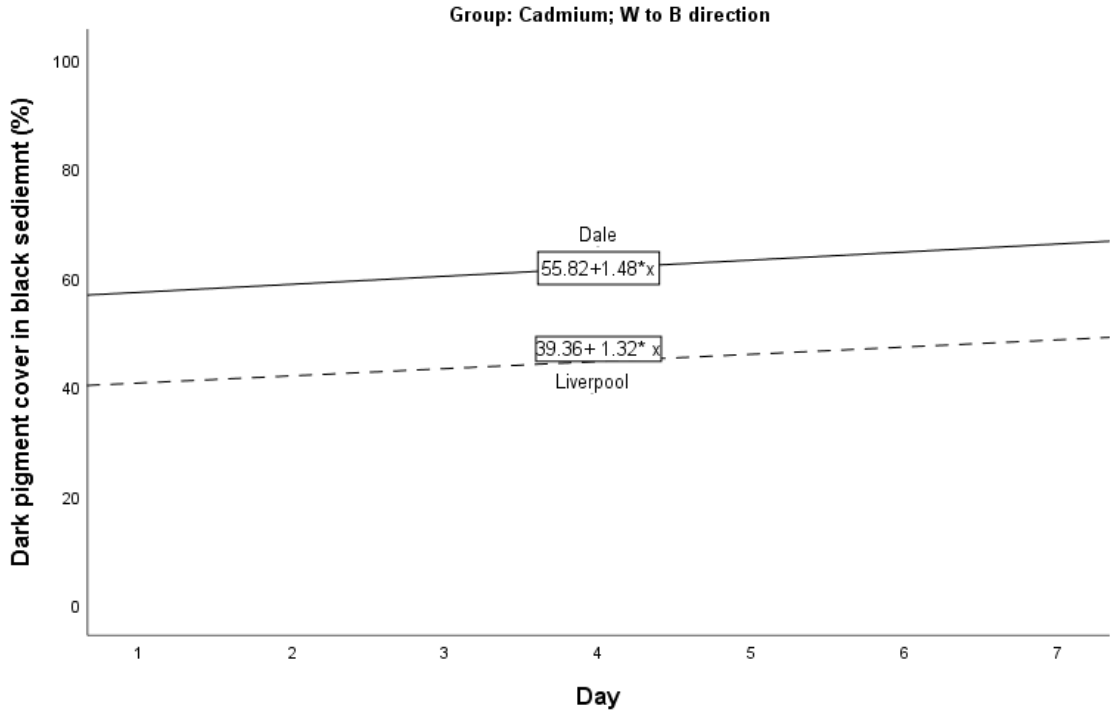

Figure 3.7 Regression slopes of dark pigment cover in black sediment in Cd group for W to B direction, solid line represent Dale population and dashed line represent Liverpool

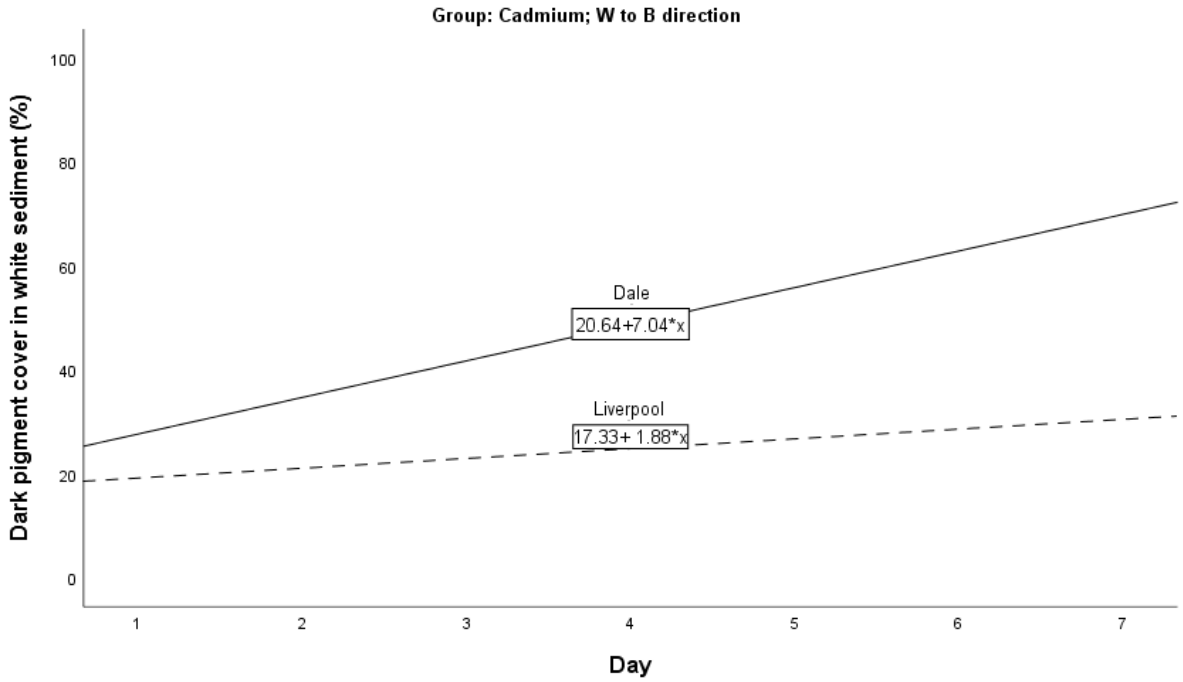

Figure 3.8 Regression slopes of dark pigment cover in white sediment in Cd group between Dale and Liverpool for W to B direction, solid line represent Dale population and dashed line represent Liverpool

Dale population in general have significantly high average of dark pigment either when they were place in black or white sediment and wither or not treated with Cd compared to Liverpool population (Table 3.4, Table 3.5, Figure 3.9, Figure 3.10). That is might indicate that the darkness appearance in Dale shrimp might not due to the effect of Cd.

Table 3.4 Descriptive statistics and t-test for dark pigment cover in black sediment between Dale and Liverpool in all groups and both directions

Direction	Group	Place	Mean	Std. Error	t-test	p-value
B to W	Control	Dale	63.78	1.37	9.381	**.000**
		Liverpool	44.17	1.16		
	Cd	Dale	59.79	1.50	4.925	**.000**
		Liverpool	47.89	1.27		
W to B	Control	Dale	64.98	1.36	8.844	**.000**
		Liverpool	46.92	1.05		
	Cd	Dale	61.74	1.55	6.655	**.000**
		Liverpool	44.62	1.56		

- Bold values were significant (p-value<.05)

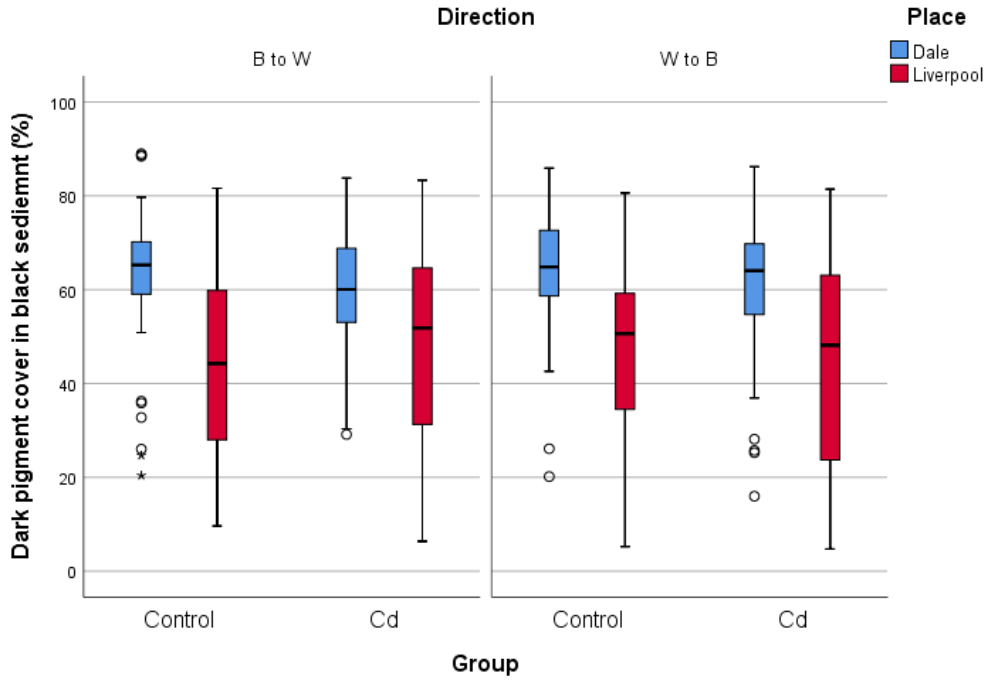

Figure 3.9 Dark pigment cover in black sediment between control and Cd group in Dale and Liverpool in both directions, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

Table 3.5 Descriptive statistics and t-test for dark pigment cover in white sediment between Dale and Liverpool in all groups and both directions

Direction	Group	Place	Mean	Std. Error	t-test	p-value
B to W	Control	Dale	47.53	2.13	9.603	**.000**
		Liverpool	27.35	1.00		
	Cd	Dale	48.15	2.36	6.340	**.000**
		Liverpool	32.39	1.18		
W to B	Control	Dale	48.31	2.09	9.159	**.000**
		Liverpool	26.61	1.14		
	Cd	Dale	48.79	2.43	9.143	**.000**
		Liverpool	24.85	1.37		

- Bold values were significant (p-value<.05)

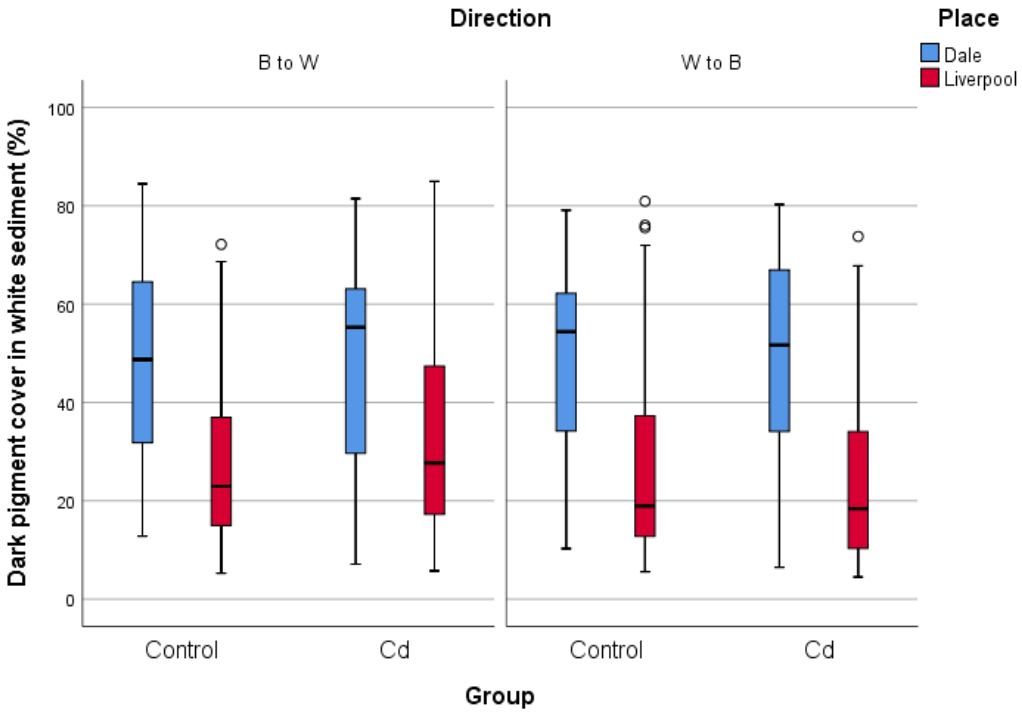

Figure 3.10 Dark pigment cover in black sediment between control and Cd group in Dale and Liverpool in both directions, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

3.3.3 Heavy metals analyses

Based on Table 3.6, heavy metals measured in water and sediment samples in both sites were similar apart from Zn in water samples between Dale and Liverpool. Shrimp from Dale and Liverpool showed low Cd concentration in both sites. With regard to baseline shrimp samples, the heavy metals measured in the tissue were not different between two sites with exception of Ba, Ca, K and Zn (Table 3.7). It was also noticed that Ca concentration was decreased when the shrimp were dosed with Cd compared to the baseline level (Table 3.7), similar to what was noticed in Mersey and Dale in Chapter 2, however, the variations between individuals were high and no relationship was found. Spearman correlation showed a similar positive correlation between water and sediment in Dale and Liverpool (r=.5929 and .5295 respectively; Table 3.8). The correlation between water and shrimp was higher in Dale than in Liverpool, whereas, the correlation between sediment and shrimp was higher in Liverpool (Table 3.8).

Table 3.6 ICP-MS results of heavy metals found in the environment. All data presented in (mean±SD ppm)

	Heavy metals	**Dale (n=3)**	**Liverpool (n=3)**		**Heavy metals**	**Dale (n=3)**	**Liverpool (n=2)**
Water	Al	0.007±0.01	0.002±0.002	**Sediment**	Al	2367±643	885±35
	As	0.074±0.01	0.07±0.01		As	2.5±0.4	5.2±0.21
	Ba	0.0045±0.0	0.0082±0.0032		Ba	3±1.3	3.6±0.28
	Ca	187±42	183±31		Ca	1200±0.0	16000±0.0
	Cd	0.0003±0.0004	0.00008±0.00004		Cd	0.01±0.009	0.02±0.007
	Cr	0.006±0.001	0.005±0.001		Cr	4.8±1.6	3.2±0.14
	Cu	0.008±0.002	0.006±0.002		Cu	0.01±0.009	0.02±0.0
	Fe	0.02±0.0	0.02±0.0		Fe	4433±1012	4000±141
	K	177±40	163±30.6		K	327±73.7	225±35.4
	Li	0.08±0.02	0.08±0.005		Li	7.3±1.3	2.3±0.0
	Mg	560±121	533±93		Mg	1967±379	1600±141.4
	Mn	0.014±0.01	0.002±0.001		Mn	69±4.7	135±7.1
	Na	7200±755	7000±700		Na	2733±351	1750±354
	Pb	0.0005±0.0004	0.00025±0.0		Pb	5.6±0.7	4.7±0.14
	Zn*	0.087±0.006	0.03±0.0		Zn	22±5.5	26±3.54

* showed significance at $p<0.05$

Table 3.7 ICP-MS results of heavy metals found in shrimp tissue. All data presented in (mean±SD ppm)

	Heavy metals	Dale (n=11)	Liverpool (n=4)		Heavy metals	Dale (n=12)	Liverpool (n=38)
Baseline shrimp	Al	48.64±18.2	106±82	**Cd dosed shrimp**	Al	20.92±8.21	10.73±16.04
	As	18.91±3.83	18.5±3.11		As	21.25±6.4	20.94±5.93
	Ba*	3.52±2.2	5.7±0.67		Ba	5.47±2.65	6.58±2.1
	Ca*	255636±246003	640000±102307		Ca	50167±9104	63474±11633
	Cd	0.17±0.1	0.11±0.06		Cd	3.97±1.69	3.41±2.1
	Cr	0.54±0.49	0.34±0.15		Cr	1.35±0.74	0.87±2.78
	Cu	63±22.95	44±12.4		Cu	87.58±29.41	60.66±15.31
	Fe	69.09±42.47	133±92		Fe	24.83±10.25	37.42±51.97
	K*	40645±35502	109750±22066		K	11225±1194	10632±1731
	Li	0.4±0.15	0.43±0.18		Li	0.089±0.04	0.14±0.05
	Mg	18773±19065	31500±6557		Mg	2567±267	3003±590
	Mn	6.62±2.5	9.13±4.05		Mn	4±1.24	2.53±1.28
	Na	154455±151989	190000±68313		Na	16250±2563	18471±4.77
	Pb	1.02±2.24	0.76±0.24		Pb	1.16±1.87	2.04±6.26
	Zn*	374.6±377	950±208		Zn	86.08±16.62	67.89±34.43

* showed significance at p<0.05

Table 3.8 Spearman correlation between the whole suite of elements in water, sediment and shrimp in Mersey

	Matrix	Water	Sediment	Shrimp
Dale	Water (ppm)	1.0000	-	-
	Sediment (ppm)	0.5929*	1.0000	-
	Shrimp (ppm)	0.8036*	0.6286*	1.0000
Liverpool	Water (ppm)	1.0000	-	-
	Sediment (ppm)	0.5295*	1.0000	-
	Shrimp (ppm)	0.7239*	0.8240*	1.0000

- Values were only present if significant at p<0.1, * showed significance at p<0.05

3.4 Discussion

In general, From the results in both Chapter 2 and 3, we cannot conclude that Cd cause a major effect in colour change ability in the brown shrimp since the results were not consistent, thus not providing strong support to the initial hypothesis. Thus, colour change ability in the brown shrimp may not be the right biomarker to be used to detect Cd in the environment, at least at the concentration used.

3.4.1 Colour change ability

Following the initial study in Chapter 2, a decision was made to mainly focus on Cd to cover short term (7 days) effect of Cd on colour change ability of the brown shrimp as well as to test any effect on different directions, i.e. from B to W and from W to B. The purpose was to investigate if the shrimp were capable of contracting or expanding its pigment depending on the substrate colour.

The results in this study suggested that the shrimp from different populations may react differently in response to Cd. Dale population was changing less in colour compared to Liverpool and the difference was significant in W to B direction, suggesting that Dale shrimp were getting darker and they were not able to contract their pigment. This might be caused by the effect of Cd inhibiting the contraction of the pigment in paler background. The difference in behaviour of the two populations might attributed to the background level of heavy metals in general and Cd in particular found in the shrimp. However, Cd level found in the shrimp collected from both sites was similar as well as in water and sediment samples. Hence, these differences between population could be due to interindividual variability.

From the results of the previous study (Chapter 2) and these results, it can be concluded that Cd may affect the colour change ability of the brown shrimp, at least in part, as it has been reported for some fish and crab as well (Table 1.2). As discussed in chapter 2, there was an observed effect of Cd on pigment in other animals even though the effect of Cd was not similar to our results. For the fiddler crab that were exposed to Cd, they got paler unlike our brown shrimp, which became darker when exposed to Cd (Reddy & Fingerman, 1995). The same paleness in appearance has been reported using fish dosed with Cd (Ahmad et al., 2018).

The reason could be due to the difference in circadian rhythm, fiddler crab showed darkness in appearance during the day and paleness appearance during the night (Caro, 2018).

However, the opposite has been reported in the brown shrimp, which were getting darker in the night and paler during the day (Siegenthaler et al., 2018) and some other crustaceans such as horned ghost crab *Ocypode ceratophthalmus* (Stevens et al., 2013). Also, it was interesting to notice the high interindividual variation in response in the shrimp (highlighted by high confidence intervals). Such high variation could be interpreted as behavioural "noise", but more probably could represent high plasticity, which might play a role on the background matching abilities of these shrimp (Duarte et al., 2016, 2018; Appendix Figure A.2, Figure A.3). Since there were no published studies regarding the effect of Cd on the brown shrimp neither in one population nor in different populations, more studies are needed to clarify the results.

3.4.2 Heavy metals in the environment and shrimp tissue

Heavy metals concentration results showed similar level in water and sediment samples in Dale and Liverpool. Regarding Cd level found in water samples from Dale and Liverpool (0.0003 and 0.00008 ppm respectively), it was similar to what has been reported by Andres, Ribeyre, Tourencq, and Boudou (2000) & Roast, Widdows, and Jones, (2001) for natural Cd concentrations in rivers and estuaries (0.00025 to 0.0003 ppm). That means both sites were cleaner than we assumed. Especially for Dale, it was assumed to be less in clean than Liverpool considering the spill oil disaster back in 1996. There was a positive correlation in heavy metals between water and shrimp and sediment and shrimp in Dale and Liverpool, suggesting that if the level of the heavy metals increased in water or sediment, it would increase in the shrimp too. This may indicate that the heavy metals that was found in the shrimp tissue were coming from either water of sediment which can be ingested by the brown shrimp.

The results showed that after dosing the shrimp with 0.04 ppm Cd for one week, both shrimp from Dale and Liverpool accumulated similar concentration of Cd (an increase of 23335 % and 3100 % respectively compared to the baseline level in both populations). *Crangon crangon* like some decapods, including crayfish, prawn *Palaemon serratus* and crab *Carcinus maenas,* cannot regulate Cd in their body, which make it harmful, unlike essential metals such as Zn and Cu (Culshaw et al., 2002). It was also noticed, in this chapter, the same relationship that found in shrimp samples from Chapter 2 between Cd and Ca, the higher Cd level in the body, the lower Ca will be found in the same samples. However, the

inter individual variations were high and the sample size was not equal, thus, more investigations are needed.

In conclusion, colour change ability in the brown shrimp is not a sensitive biomarker to detect Cd in the environment, thus, we considered in the next chapter another heavy metal, inorganic As, to assess the colour change ability in our shrimp.

4. Chapter Four: Effects of arsenic on the brown shrimp, *Crangon crangon*

4.1 Introduction

Researchers have recently begun focusing on aquatic organisms as bioindicators to assess environmental contamination and have found that, among aquatic animals, fish and shrimp are both considered good biomarkers (Copat et al., 2013; Fakhri et al., 2018; Herreros, Iñigo-Nuñez, Sanchez-Perez, Encinas, & Gonzalez-Bulnes, 2008; Mol, Karakulak, & Ulusoy, 2017; Yi, Yang, & Zhang, 2011). The accumulation of heavy metals in marine organisms varies depending on their concentrations in the water and sediment, salinity of the water, chemical form of the contaminants, as well as the species, sex and age of the animal (Fakhri et al., 2018; Smedley & Kinniburgh, 2002). High levels of environmental contamination can cause an accumulation of contaminants within aquatic organisms, which can be then transferred to humans through the food chain as seafood is a valuable source of vitamins and protein (Husain et al., 2017). Contaminants in general, and heavy metals in particular, can lead to significant health issues in humans, such as cancers caused by arsenic (As) and cadmium (Cd) (Koedrith & Seo, 2011). Hence, it is essential to monitor the levels of heavy metals in the aquatic environment and their accumulation in marine animals. Equally important is the study of single or multi-biomarkers in animals to understand the health of aquatic environments (Dalzochio, Rodrigues, Petry, Gehlen, & da Silva, 2016). Recent studies (Kohler, Parker, & Ford, 2018; Scott & Sloman, 2004) have used behavioural responses in marine animals to understand and to link the biological and biochemical effects of contamination at the ecosystem level. On the contrary to LC_{50} studies, organisms' behavioural changes can provide an early warning of contamination, when contaminants are present at low concentrations (Sharma, 2019). For some marine animals, colour change is a sensitive bioassay to determine the presence of toxicants and has been used in previous studies (Chapter 1, Table 1.2), but no attempt has been made to investigate the activity of chromatophores in shrimp due to arsenic (As).

Within aquatic environments, As is one of the more prevalent heavy metals found in organisms, water and sediment, irrespective of its chemical form (Chapter 1, Figure 1.5).

The inorganic forms of As (iAs) are primarily found in water and sediment, while the organic forms, along with small quantities of iAs, are found in organisms (Francesconi et al., 1999). Aquatic animals usually have higher levels of As in their bodies compared to terrestrial animals (up to 100 ppm vs. less than 1 ppm, respectively; Phillips, 1990). Arsenic is known to be toxic, although its toxicity to marine animals has not been fully investigated (Madsen, 1992).

Allen et al. (2004) have illustrated changes in chromatophores' activity in the fresh water fish, *Channa punctatus*, treated with 1 ppm of As trioxide for 90 days. A similar study was conducted by Akarte and Agnihotri (2013) using the same fish species, but by exposing them to a higher dose of As trioxide (6 ppm) for 30 days. Both studies found that the fish became paler after 30 days of exposure to iAs. Since toxicity of As is highly dependent on its chemical form, there is a need to investigate the As species in marine animals (Zhang & Wang, 2018) both under natural conditions and during laboratory experiments. There are very few studies that have examined how iAs is metabolised, for example in bivalves and fish (Zhang et al., 2016; Zhang, Guo, Song, et al., 2018; Zhang, Wang, & Zhang, 2016). Thus, considering the risk for humans to intake As from seafood consumption, it is important to distinguish the As species present in marine organisms and understand how those forms could have been accumulated or produced by the organism due to metabolism. For these two reasons, As was selected in this study to illustrate its effect on colour change and its bio-transformation in the brown shrimp.

The aim of this chapter was to investigate if different iAs concentrations interfere with the ability to change colour in *Crangon crangon* and further determines the accumulation and the possible transformation of iAs to orgAs species. Hence As speciation (the differentiation and quantification of the various As species; Nearing, Koch, & Reimer, 2014) was used to indicate metabolic processes in the shrimp. Although the levels of As used in this study were much higher that the levels found in the environment, this study serves as a compromise between actual environmental exposure and the effective experimental dosage that leads to a measurable increase of heavy metals (in this case iAs and its metabolites) in the organism.

4.2 Methodology

4.2.1 Study sites and sampling

Crangon crangon specimens were collected in June 2018 from the estuary of the river Mersey in Liverpool Bay, Irish Sea (53.437667, -3.035417; Figure 3.1). All the sampling procedures used in this experiment have been described in more detail in chapter 3. Ethical approval number ST1617-64 for this study was obtained by the University of Salford (appendix Figure A.8).

4.2.2 Toxicity test

Following Madsen (1992), 25 ppm As(V) were used for a toxicity experiment to test the survival of 5 adult specimens of brown shrimp, held in a small glass aquarium with 2 cm layer of black sediment and 2 L of aerated artificial dosed seawater (20 psu).

The stock solution [100 ppm As(V)] was prepared using Sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$, Alfa Aesar; molecular weight 312.01 g/mol). The molecular weight of As is 74.92 g/mol, thus, 0.415 g were added to 1 L of artificial seawater to prepare the stock solution. A 2 L solution of 25 ppm As(V) was prepared adding 500 ml of 100 ppm stock solution to 1500 ml of artificial seawater. The experiment was planned for three weeks, however, after one week all the shrimp were dead. Hence, a decision was made to reduce the As(V) dose to 20, 10 and 5 ppm instead of 25 ppm as used by Madsen (1992). Although the concentrations used in this experiment were higher than the level found in the natural environment (see section 1.3 in Chapter 1), the goal was to observe a response in a short period of time.

4.2.3 Experiment 1 - Effect of arsenic on colour change

All the shrimp were brought from the field and acclimatised to lab conditions (temperature: ~17˚C; salinity: 20 psu) for three days before starting the experiment. A total of 92 adult shrimp were used in this experiment. The specimens were divided randomly into 4 groups: three treatments groups (5 ppm, 10ppm and 20 ppm As(V) with 24 shrimp each) and a control group of 20 shrimp (Figure 4.1). The 24 shrimp in each treatment groups were kept in 1 cm layer of black sediment and then moved to white sediment for two hours (n=12; B to

W) or in white sediment and then moved to black sediment for two hours (n=12; W to B) for dark pigment measurements, before being returned to the initial substrate.

Shrimp were kept in individual chambers (to avoid cannibalism) and fed every other day with a piece of raw chicken instead of fish to minimise the arsenic that could come from seafood (Francesconi et al., 1999). The level of the water, temperature and salinity were monitored regularly to ensure that conditions remained the same throughout the experiment (for the overall duration of 21 days). Dark pigment cover in black or white sediment was measured every other day, alternating control and 5ppm in a day and 10 ppm and 20 ppm the following day, due to the time required to process the large sample size; Figure 4.1). Colour change measurements and analyses of pictures have been described in detail in Chapter 2 (2.3.6.2 and 2.3.7). All the shrimp were sacrificed at the end of the experiment and kept frozen for further analyses.

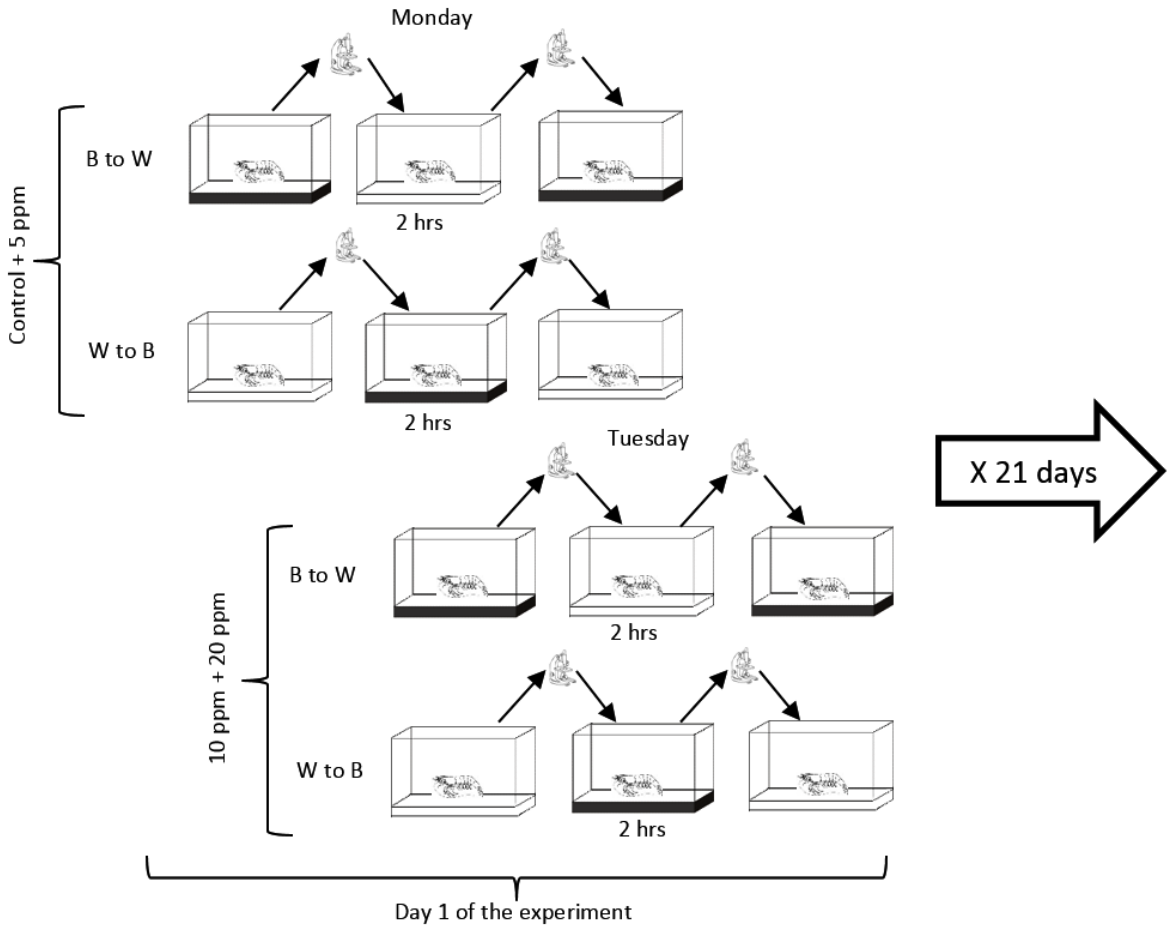

Figure 4.1 Experimental design. B to W direction= shrimp moved from black to white substrate; W to B direction= shrimp moved from white to black substrate. Sample size: n= 12 in all treatments, except for control (n= 10)

4.2.4 Experiment 2 - As accumulation and metabolism over time

The same set up as was duplicated with 45 adult shrimp for experiment 2, run simultaneously with experiment 1. The shrimp were divided randomly into three treatment groups (5, 10 and 20 ppm As(V) in 10 L of artificial aerated seawater; n= 15 in each group) and kept in individual chambers with 1 cm layer of black sediment. The same feeding regime was applied as experiment 1. In day 7 and 14, between seven to eight shrimp of each group were sacrificed to measure the amount of As bioaccumulated in the shrimp and test for potential bio-transformation. In day 21, shrimp were sacrificed from experiment 1 (Figure 4.1). The samples from the control group were also obtained from experiment 1. All shrimp were labelled and frozen for speciation analyses.

4.2.5 Experiment 3 – Measuring As species and total As in shrimp samples

Arsenic speciation analyses were performed in collaboration with Prof Jörg Feldmann, the director of trace element speciation laboratory (TESLA) and his team at the University of Aberdeen, Scotland following their protocol (Marschner et al., 2019).

Once the shrimp were sacrificed, they were freeze dried for two days (Heto, PowerDry LL3000). The wet weight of all samples was recorded before freeze drying. The dry weight was measured twice to ensure that the samples were completely dry. The samples were ground into a fine powder using mortar and pestle. If individuals were too small, more than one from the same day was used (combined sample) to reach the minimum amount required for the analysis (0.05 g dry weight). A total of 21 samples were used for the arsenic speciation study, sacrificed from experiment 1 and 2. The CRM BCR-627 (obtained from dried, homogenised tuna fish muscle tissue) was used.

4.2.5.1 Extraction of total arsenic

Empty vials (50 ml) were weighted and filled with 50 mg of the fine powder from each sample, to which 1 ml of nitric acid (HNO_3) was added. The homogenate was left overnight to allow the HNO_3 to digest the samples and to release the bound materials. Then, 2 ml of hydrogen peroxide (H_2O_2) was added to the mixture. The full vial weight was recorded after every step to calculate the dilution factor. All samples were extracted using microwave technique (MARS) with open vial system. The microwave power, temperature and exposure time used were: 800w, 50 °C for 5 mins, 800w, 75 °C for 5 mins and 1600w, 95 °C for 30 mins. After cooling, 10 ml of deionised water was added to dilute the digest and the final vial weight was recorded again. All digests were transferred to 15 ml plastic vial to fit perfectly in the Inductively coupled plasma mass spectrometry (ICP-MS) tray (Figure 4.2). The blank and three replicates of CRM samples were prepared in the same way as the rest of the samples.

The standard solutions concentrations used in this experiment for calibration were 1, 5, 10, 25, 50, 100 and 500 ppb As(V). This wide range of concentrations was used to cover all the As(V) concentrations that could be present in the samples. Two stock solutions (10 ppm and 100 ppm; AccuStandard) were used to prepare these concentrations. All the standards were diluted with 15 ml of 1% HNO_3. Rhodium (5 ppb) was used as an internal standard solution.

ICP-MS (8800 ICP-MS Triple Quad, Agilent Technologies) was used to measure the total arsenic in the samples.

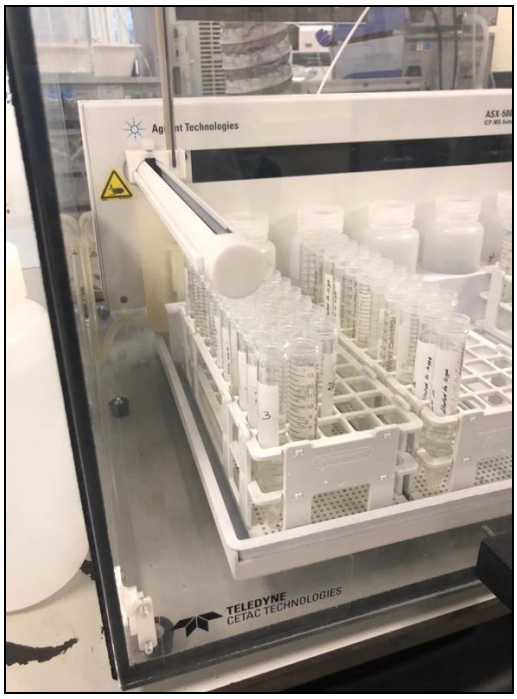

Figure 4.2 Digests ready for analysis in the inductively coupled plasma mass spectrometers (ICP-MS)

4.2.5.2 Extraction for arsenic speciation

To extract and measure the arsenic species in the shrimp, 50 mg of each fine powdered samples was placed into weighted 15 ml vial. For digestion, 5 ml of Milli-Q water was added to the samples and it was mixed in a vortex mixer for two minutes. All the samples were left overnight. Then, they were transferred to the centrifuge (VWR, Mega star 1.6R) to separate the solution from the small particles. The centrifuge was set for 3000 revolutions per minute (rpm), at 18°C for 10 minutes. After, 1 ml of the supernatant was taken and added to a new 15 ml vial (empty weight was measured before). Hydrogen peroxide was added (100 µl) to the solution. All samples were mixed again. A sample of 300 µl was taken from the extracted solution and the remaining aliquot was kept for total As analysis for each of the samples (performed by Dr Andrea Raab, University of Aberdeen). The whole batch of samples involved blank and three replicates of CRM along with the shrimp samples. After each step, the weight of the vial was recorded. One litre of ammonium carbonate ($(NH_4)_2CO_3$), pH= 9.0, was used as a mobile phase, prepared on the same day of the analysis by adding 19.218 g of $(NH_4)_2CO_3$ into 500 ml Milli-Q2 water. The solution was mixed to allow the salt to dissolve in the water (heating should not be used to dissolve ($(NH_4)_2CO_3$) otherwise, it will become

ammonia gas, carbon dioxide gas, and water vapour). An extra 500 ml of the water was added to reach 1 L of the solution and pH was checked. Dimethylarsinic Acid Sodium Salt was used as a standard solution. The concentrations of the standard solution used were 0.1, 0.5, 1, 5, 10, 25, 50, 100 and 250 ppb of DMA diluted with 1% HNO_3. All these concentrations were made using two stock solutions (10 ppm and 100 ppm) diluted with Milli-Q water.

All samples were transferred to the high-performance liquid chromatography-inductively coupled plasma mass spectrometer (HPLC-ICP-MS; Agilent technologies 1290 infinity) as the arsenic-specific detector. This instrument has a Hamilton PRP x100 anion exchange column (dimensions 250 x 4.1 mm, particle size= 10 µl; Figure 4.3). PEEL tubing was used to connect the HPLC system with the nebulizer of ICP-MS (Figure 4.4). The software program Origin 6.1 was used to convert and integrate the chromatograms (Figure 4.5). Missing values that were below LOD was replaced by 50 % of LOD for each element (Płotka-Wasylka et al., 2018; Shrivastava & Gupta, 2011).

Figure 4.3 Anion exchange column used in As speciation analysis

Figure 4.4 High-performance liquid chromatography-inductively coupled plasma mass spectrometer (HPLC-ICP-MS) used to analyse arsenic species in the shrimp

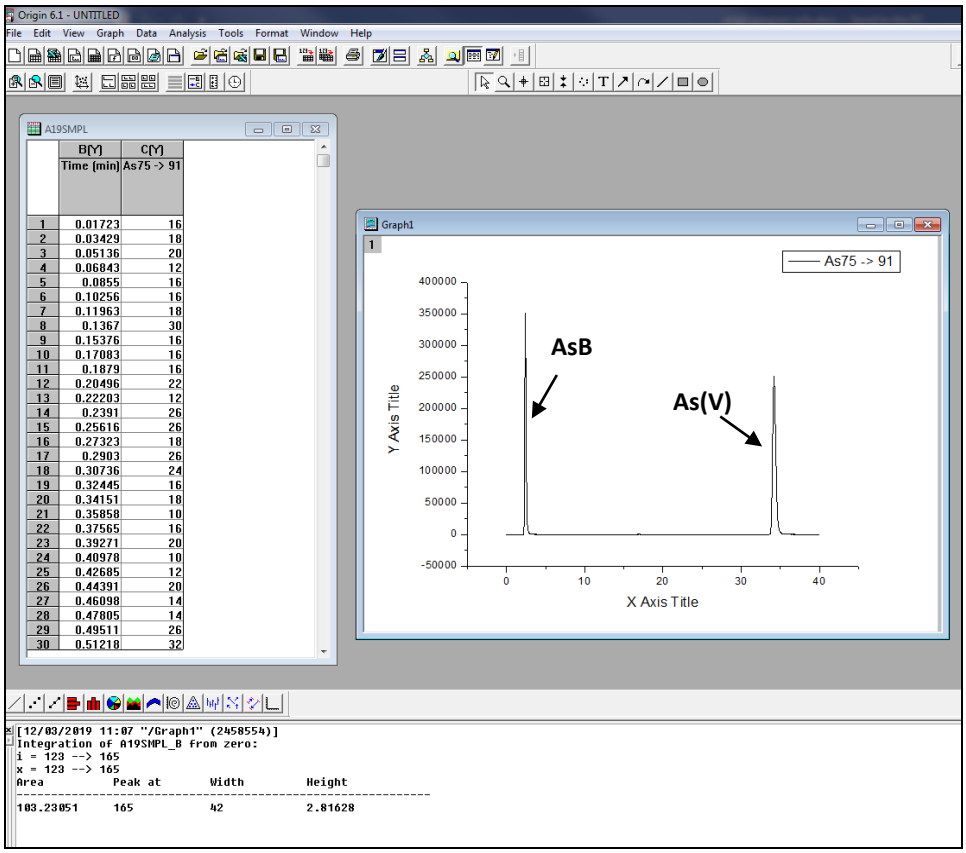

Figure 4.5 Chromatogram showing the separation of As species using Origin 6.1 software

4.2.6 Statistical Analysis

Experiment 1 was based on examining changing in behaviour of the brown shrimp at different concentrates of As(V) (5ppm, 10ppm and 20ppm) and control group. The change in colour (dark pigment cover in white and black sediment, final -initial) for the two directions (B to W, from black to white sediment and W to B, from white to black sediment for each group were measured for three weeks (day 1, 4, 6, 8, 11, 13, 15, 17 and 21). For repeated measures ANOVA, the assumption of sphericity was checked using Mauchly's test (Field, 2009), which test that the population variances of all possible difference changes (day 1, 4, 6, 8, 11, 13, 15, 17 and 21) are equal. The second assumption was violated, hence F test based on Greenhouse-Geisser (Epsilon<0.75) was used (O´Brien & Kaiser, 1985). The comparison within treated group was performed using repeated measures ANOVA (Verma, 2015) for three outcomes (colour change, dark pigment cover in black sediment and dark pigment cover in white sediment) followed by pairwise comparisons if the ANOVA was statistically significant. The pairwise comparisons based on multiple paired t-tests, with a Bonferroni correction to keep the type 1 error at 5% overall, was used to compare each pair of days. The comparison between groups (control, 5, 10 and 20ppm), was performed using one-way ANOVA between independent groups for each outcome. Normality assumption was checked using the K-S test, skewness and kurtosis for each group and all data were found to be approximately normally distributed. Boxplots were used to display minimum, first quartile, median, third quartile and maximum of each data across time. Regression slopes were also performed to see the overall trend during the time of the experiment, together with survival analysis.

Missing values were frequently encountered in the dataset. These can undermine the reliability of the data and cause a bias in the results. Missing values can be random (such as death) without being influenced by other specific factors. This was tested using a chi-square test (Roderick, 2010). As missing data were indeed random (since chi-square was not significant) imputation analysis was used to replace missing values with estimated values, computed using an expectation maximization algorithm of the likelihood distribution (Enders, 2010). With regard to total arsenic and arsenic speciation analysis, no advance statistical analyses were provided because all the data were obtained from a single sample, thus, only a descriptive analysis is reported. All analyses were performed with IBM SPSS 24.

4.3 Results

4.3.1 Mortality

In the control group, only 8 shrimp out of 20 were still alive at the end of the experiment. However, the highest mortality rate was observed in the group treated with 5 ppm As(V), where only two out of 24 shrimp survived till the end of the experiment (one housed in black sand and one in white (Table 4.1). Whereas, in 10 and 20 ppm As(V), there were 5 and 7 shrimp alive out of 24 respectively on day 21 (end of experiment). Most of the mortalities happened between day 13 and day 21 (Figure 4.6).

Table 4.1 Survival time of the shrimp in each group during 21 days

group	Total sample	Number of dead shrimp	Censored		Median survival time (Day)
			Number of alive shrimp	Percent	
control	20	12	8	40.0%	17
5 ppm As(V)	24	22	2	8.3%	13
10 ppm As(V)	24	19	5	20.8%	17
20 ppm As(V)	24	17	7	29.2%	10
Overall	92	70	22	23.9%	15

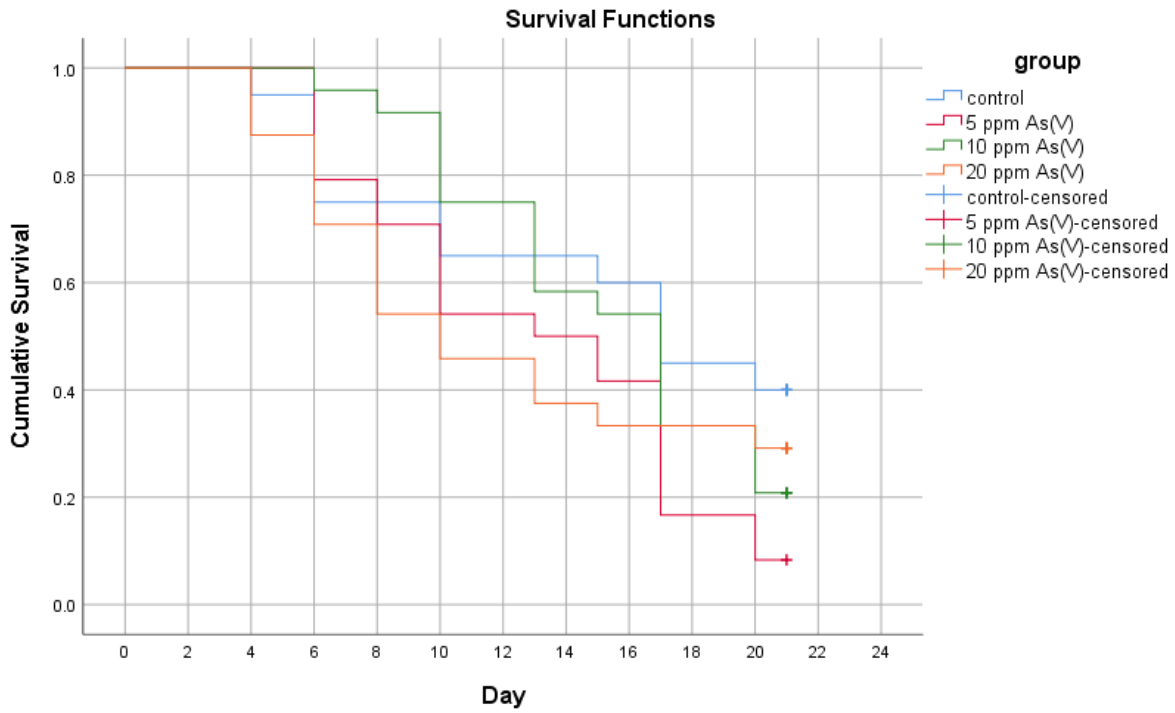

Figure 4.6 Survival time of the shrimp in each group during 21 days of the experiment, blue line control group; red 5 ppm As(V); green 10 ppm As(V) and orange 20 ppm As(V)

4.3.2 Colour change ability

4.3.2.1 Comparison of colour change ability over time in individual groups

In each treatment, there was significant difference between days in the performance of the same group of shrimp in their colour changing ability (measured by the difference between dark pigment cover in black and white sediment) for both B to W and W to B directions (Table 4.2; Appendix Table A.6-9). This indicates there was high inter individual variation. A general trend for all groups, including control, was a decreased ability in matching the background as the experiment progressed (Figure 4.7, Figure 4.9, Figure 4.9, Figure 4.10)

Table 4.2 Repeated measures ANOVA for difference in pigment between days in all groups and both directions

Direction	Group	F (Greenhouse-Geisser)	p-value
B to W	Control	6.119	**.001**
	5ppm As(V)	5.845	**.002**
	10 ppm As(V)	5.126	**.004**
	20 ppm As(V)	4.573	**.005**
W to B	Control	6.292	**.002**
	5 ppm As(V)	5.632	**.002**
	10 ppm As(V)	8.291	**.000**
	20 ppm As(V)	3.963	**.019**

- Bold values were significant (p-value<.05)

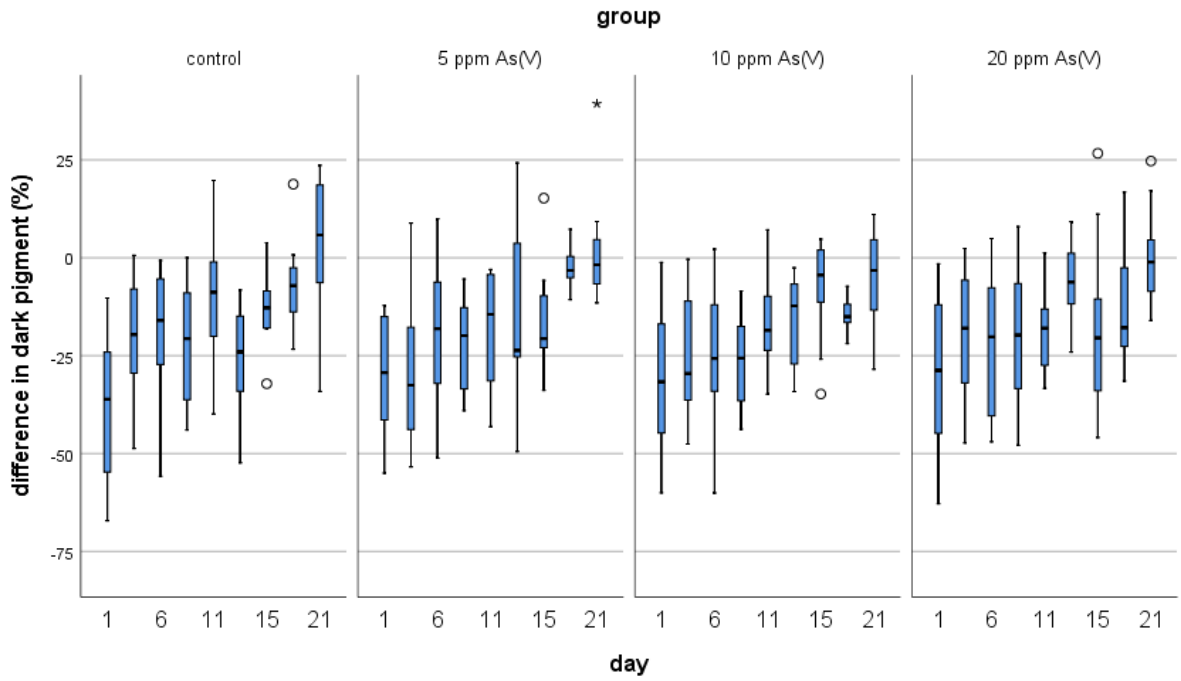

Figure 4.7 Colour change ability of the shrimp measured by the difference in dark pigment cover in black and white sediment over time when they were moved from black to white sediment (B to W) in all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

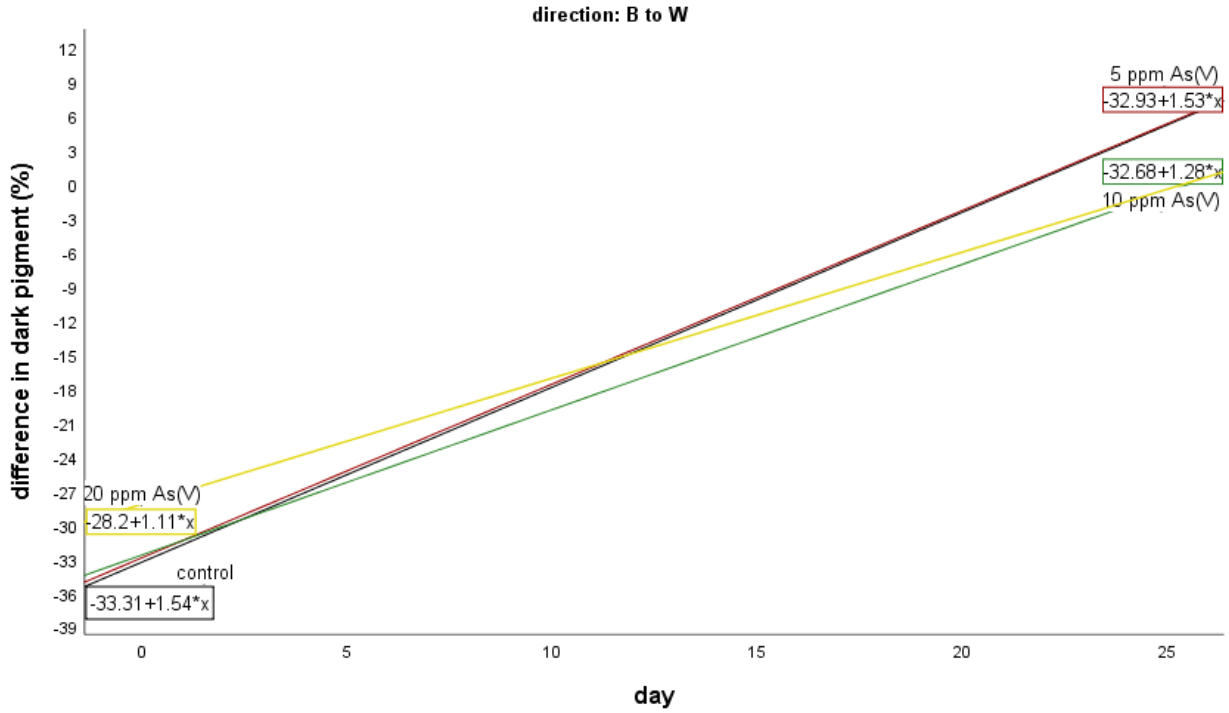

Figure 4.8 Regression slopes of colour change ability of the brown shrimp measured by the difference in the dark pigment cover in black and white sediment over time in all groups for B to W direction, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

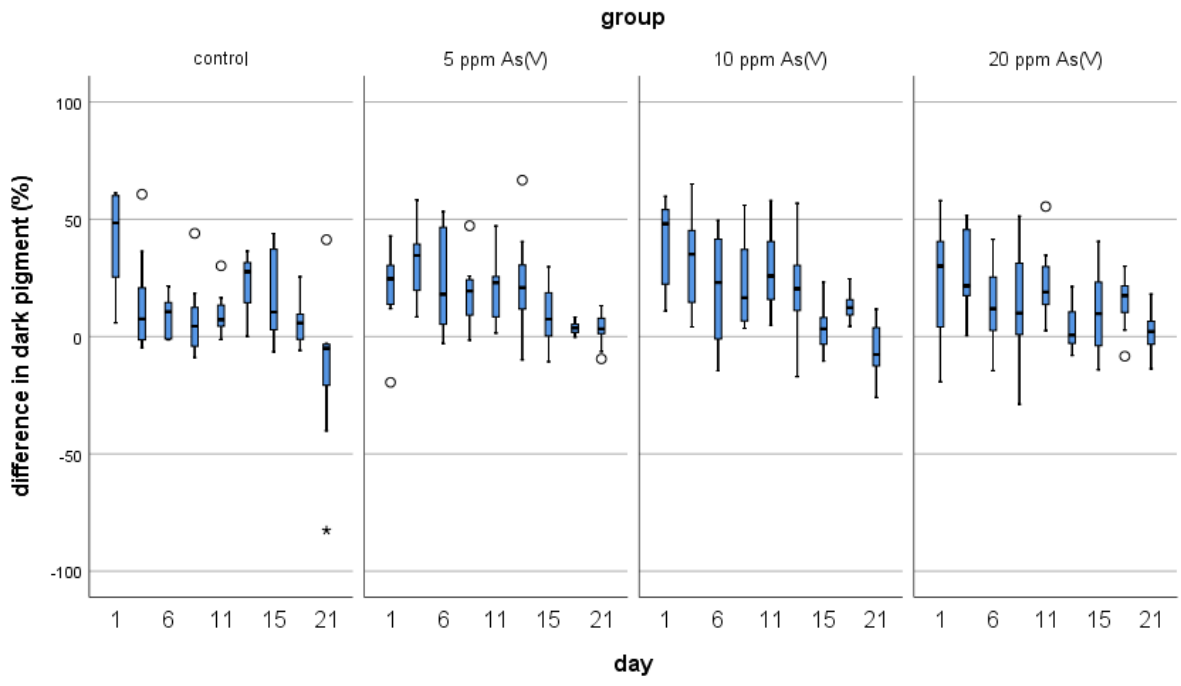

Figure 4.9 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment over time when they were moved from white to black sediment (W to B) in all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

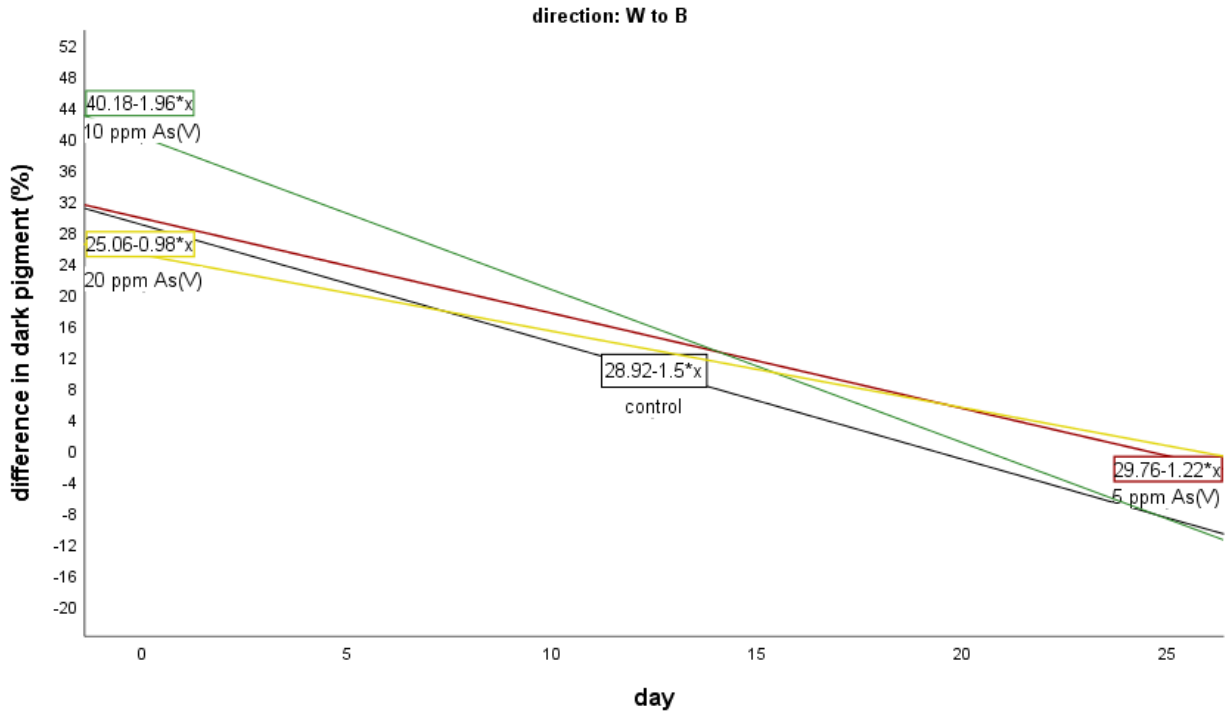

Figure 4.10 Regression slopes of colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediment over time in all groups for W to B direction, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

4.3.2.2 Comparison of colour change ability between group

When shrimp were moved from black to white sediment or vice versa, the average of colour change ability in all treatments in both directions were close to the average in control group (Table 4.3). Based on the ANOVA test, there was no significant difference between groups (F=.083, p-value=.969; Table 4.3), in colour change ability and this was clearly shown in box plot (Figure 4.11).

Table 4.3 Descriptive Statistics and ANOVA for colour change in all groups for both directions

Direction	group	Mean	Std. Error	F	p-value
B to W	control	-16.9	2.0	.325	.808
	5 ppm	-16.6	1.7		
	10 ppm	-18.4	1.5		
	20 ppm	-16.4	1.7		
W to B	control	12.9	2.3	2.094	.100
	5 ppm	16.8	1.6		
	10 ppm	19.3	2.0		
	20 ppm	14.6	1.7		

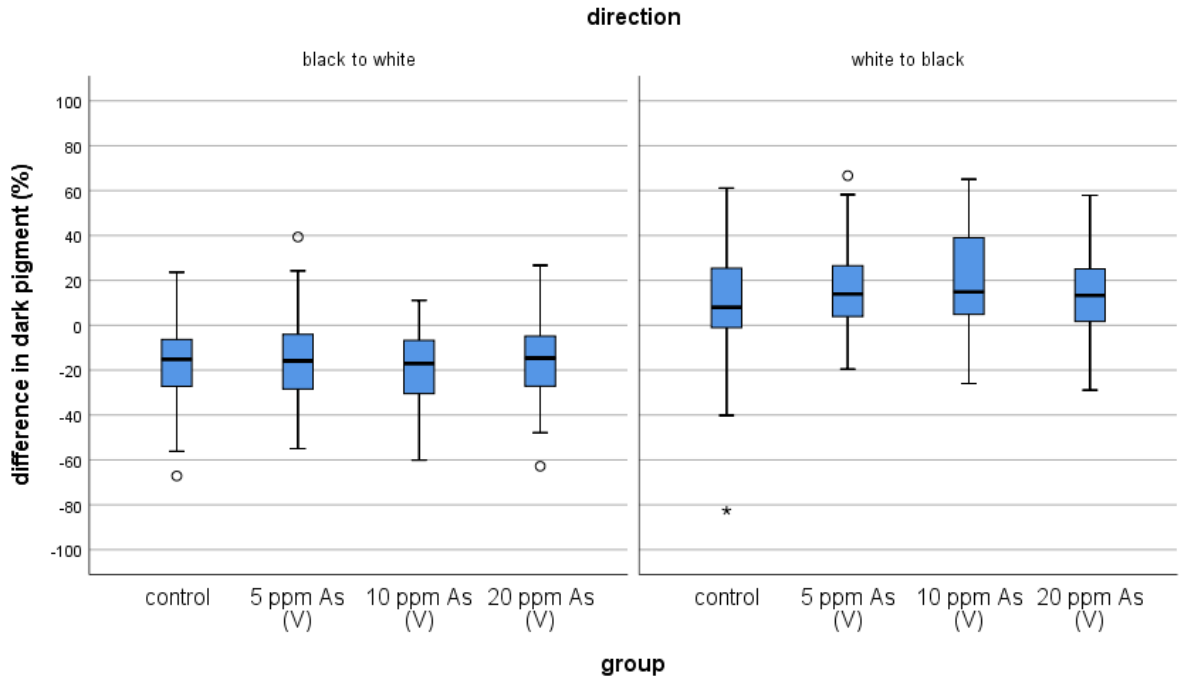

Figure 4.11 Colour change ability of the brown shrimp measured by the difference in dark pigment cover in black and white sediemnt (final – initial in %) between groups in both directions, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

4.3.3 Dark pigment cover in black and white sediment

4.3.3.1 Comparison of dark pigment cover in black or white sediment over time

The previous measures were useful to test how well the shrimp would match their background in each and among treatments when moved from one type of substrate (black or white) to the opposite (ability to change colour). Another measurement relevant to see any potential effect of As is how dark animals are in black sediment (dark pigment cover in

black sediment) and how pale they are in white sediment (dark pigment cover in white sediment) across days. Based on Table 4.4, dark pigment cover in black sediment was significantly different in the control group between days in B to W direction (p-value=.030). In addition, dark pigment cover in white sediment was significantly different between days when control shrimp were placed in white sediment in both directions (Table 4.4). With regard to 5 ppm As(V) group, only dark pigment cover in white sediment in W to B direction was significant between days (Table 4.4). In 10 ppm group, the mean in dark pigment cover in white sediment was significant in B to W direction (p-value=.001) and W to B direction (p-value<.001). Shrimp in 20 ppm group appeared to have significant difference in dark pigment cover in black sediment when the shrimp were moved from white to black sediment (Table 4.4). Pairwise comparison showed high inter individual variability between days in all groups in both directions (Appendix Table A.10-14).

Table 4.4 Repeated measures ANOVA for dark pigment cover in black and white sediment within group between days for both directions in all groups

Direction	**Group**		**F (Greenhouse-Geisser)**	**p-value**
B to W	Dark pigment cover in black sediment	control	4.185	**.030**
		5 ppm	2.101	.155
		10 ppm	.758	.533
		20 ppm	.103	.103
	Dark pigment cover in white sediment	control	3.183	**.041**
		5 ppm	3.619	.051
		10 ppm	5.557	**.001**
		20 ppm	2.570	.075
W to B	Dark pigment cover in white sediment	control	4.369	**.014**
		5 ppm	5.109	**.003**
		10 ppm	6.102	**.000**
		20 ppm	2.193	.126
	Dark pigment cover in black sediment	control	2.573	.086
		5 ppm	1.669	.202
		10 ppm	2.756	.051
		20 ppm	2.984	**.044**

- Bold values were significant (p-value<.05)

In general, the dark pigment measured in the shrimp either in black or white sediment fluctuated during 21 days (Figure 4.12, Figure 4.14, Figure 4.16, Figure 4.18). The overview trend of dark pigment cover in black sediment in both directions slightly decreased over time compare to day 1 in all groups (Figure 4.13, Figure 4.17) apart from 5 ppm As(V) group in B to W direction (Figure 4.13), indicating that the shrimp were not as dark as they were at the beginning of the experiment. However, regression slopes showed an increase in the dark pigment cover in white sediment in all groups and both directions (Figure 4.15, Figure 4.19), compared to day 1, suggesting that the shrimp were slightly darker in white sediment.

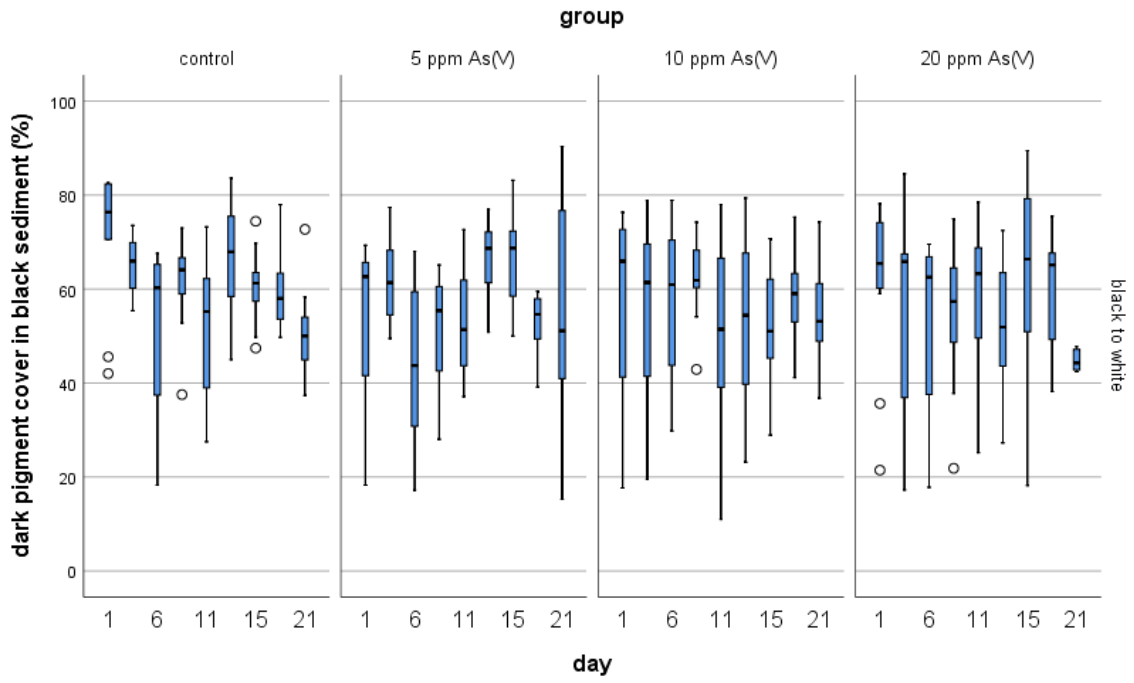

Figure 4.12 Dark pigment cover in black sediment in B to W direction for all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

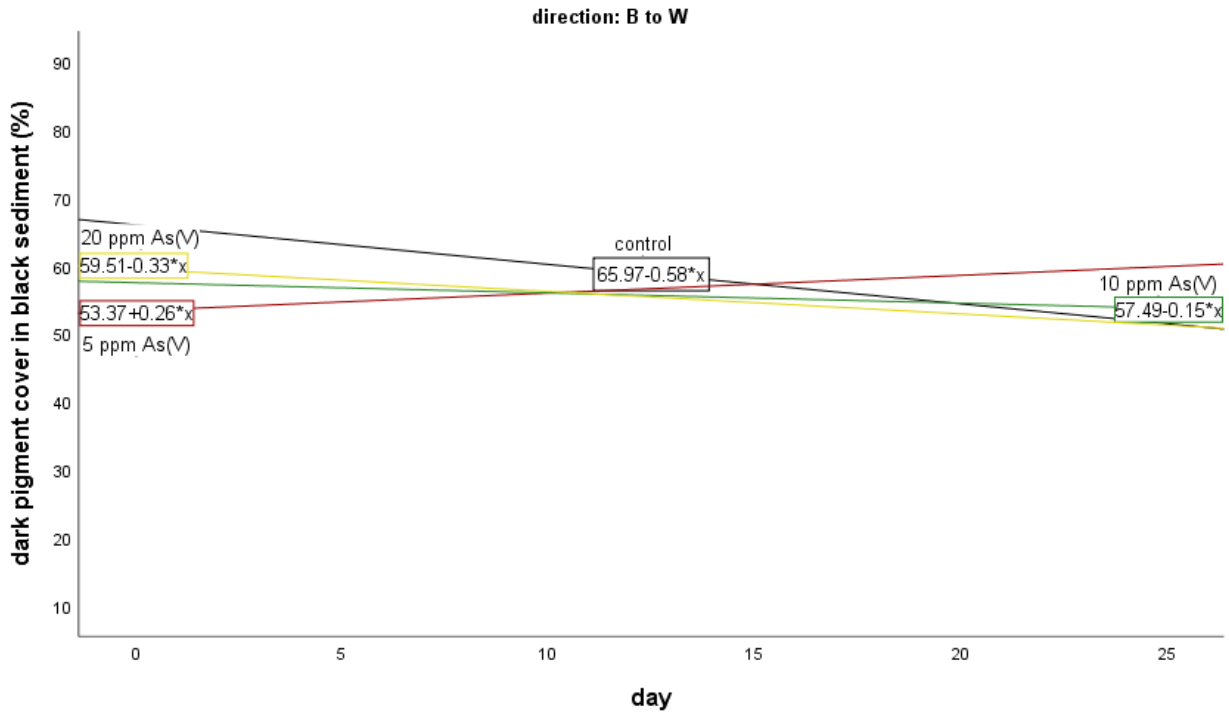

Figure 4.13 Regression slopes of dark pigment cover in black sediment in B to W direction for all groups, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

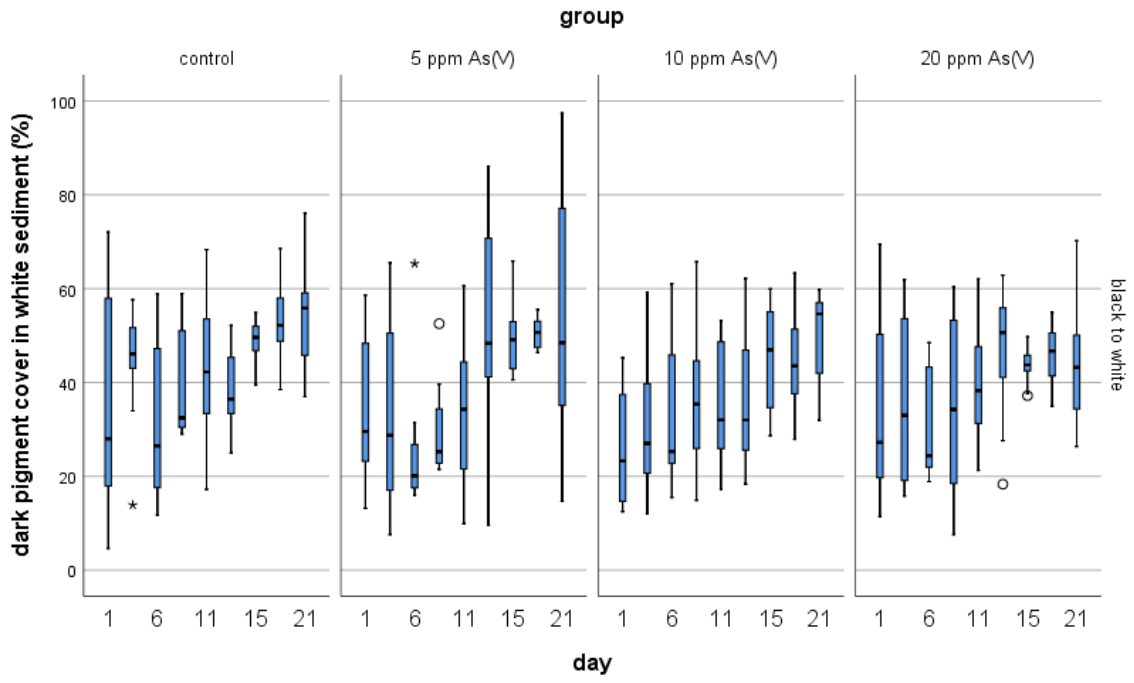

Figure 4.14 Dark pigment cover in white sediment in B to W direction for all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values and stars show extreme outliers

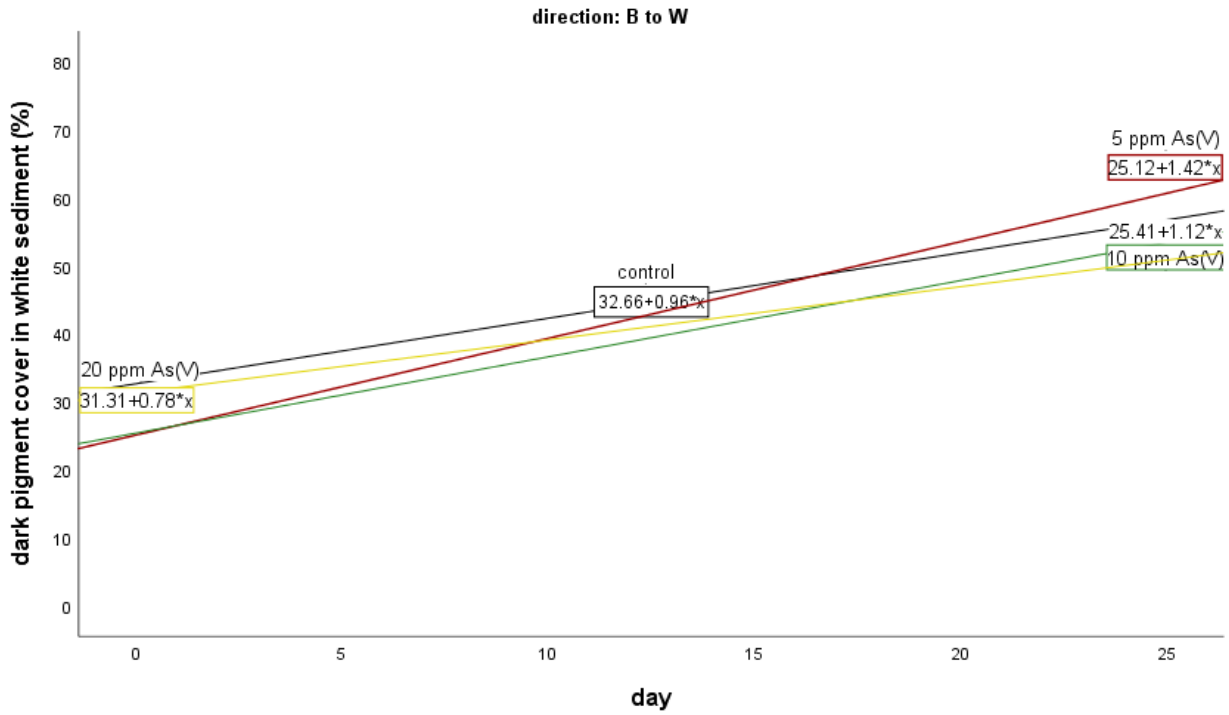

Figure 4.15 Regression slopes of dark pigment cover in white sediment in B to W direction for all groups, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

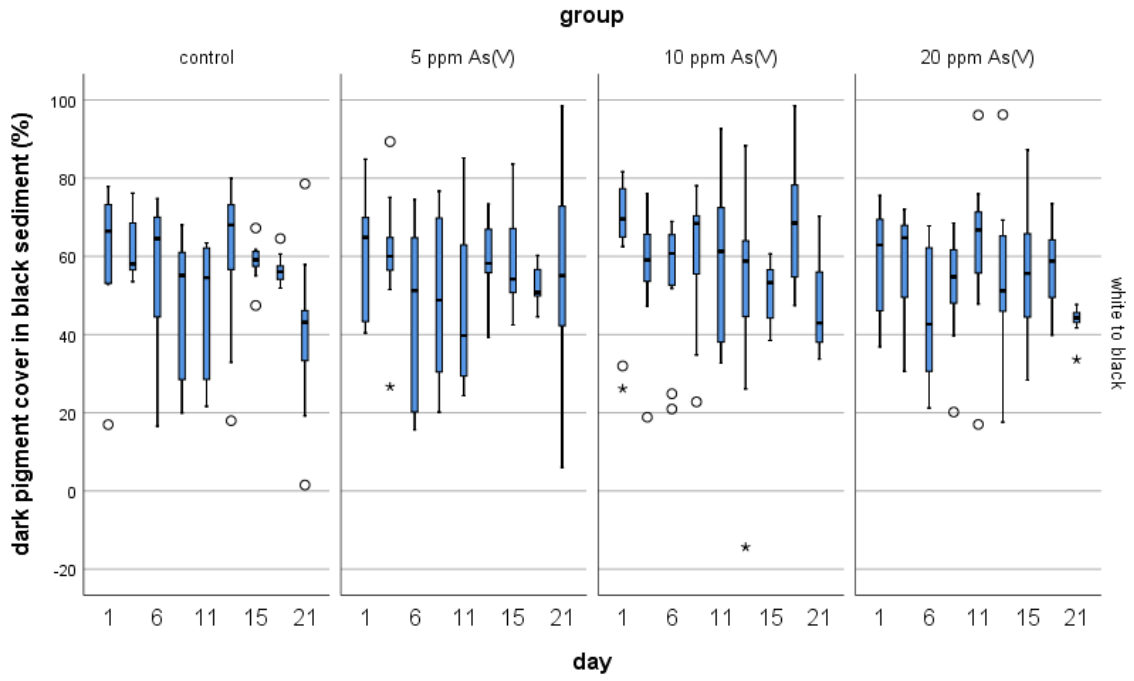

Figure 4.16 Dark pigment cover in black sediment in W to B direction for all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

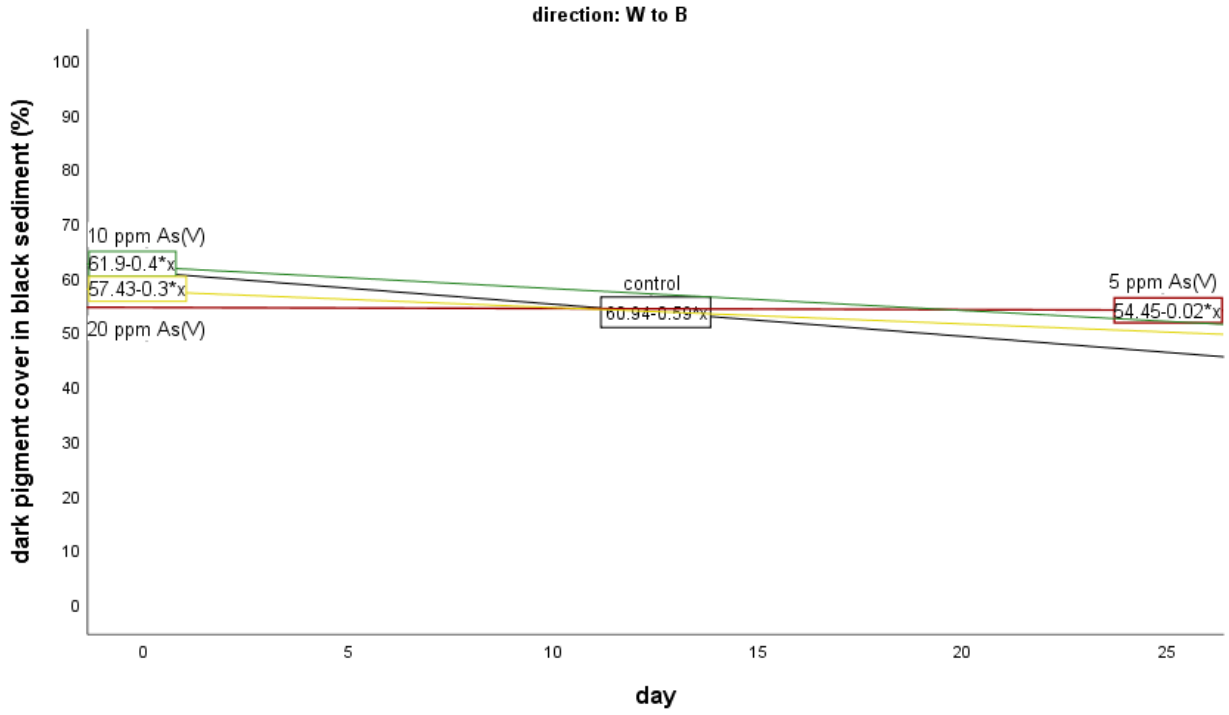

Figure 4.17 Regression slopes of dark pigment cover in black sediment in W to B direction for all groups, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

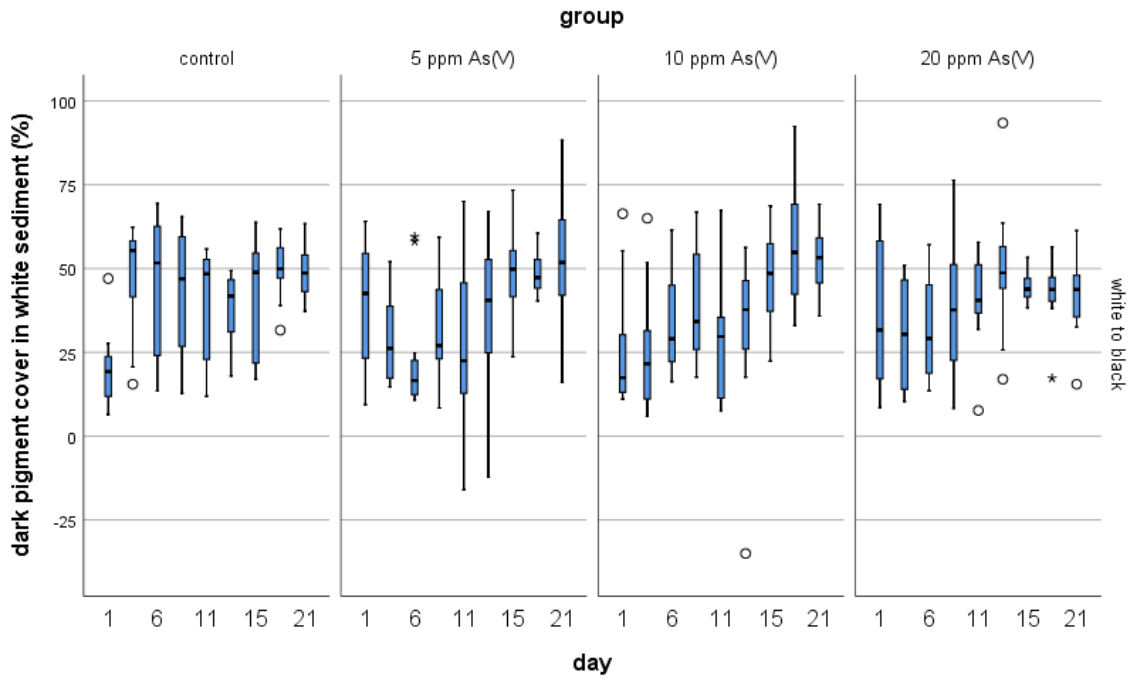

Figure 4.18 Dark pigment cover in white sediment in W to B direction for all groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

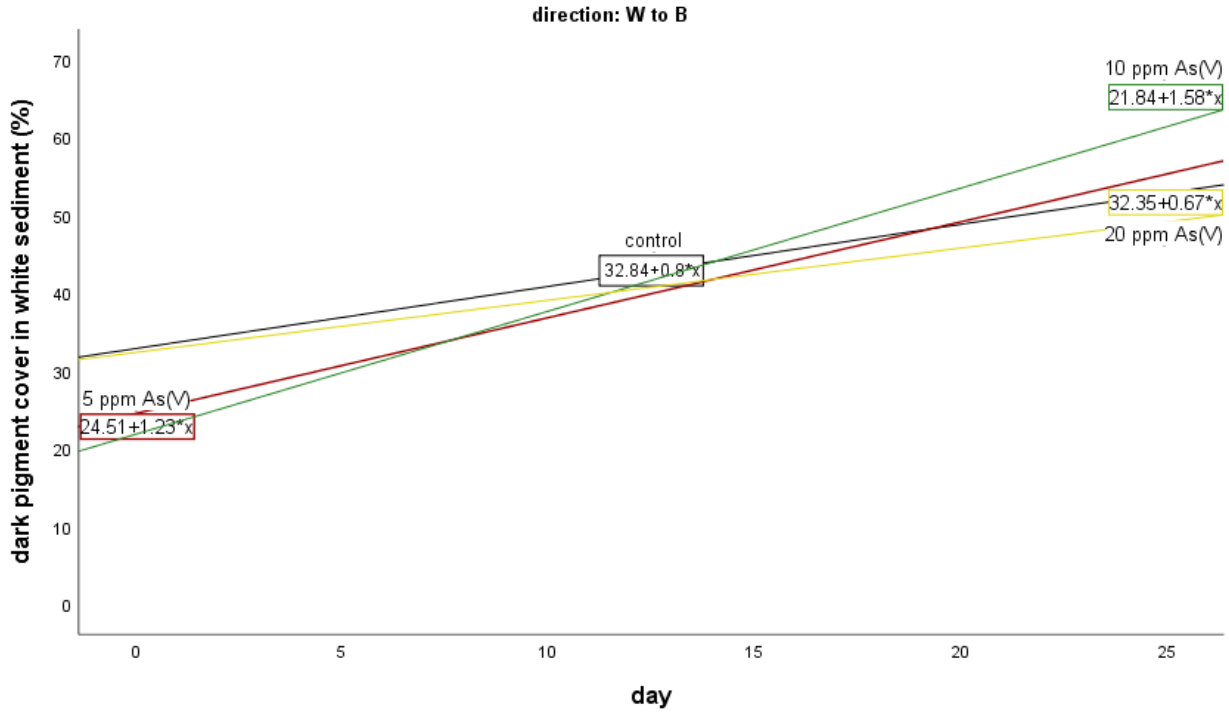

Figure 4.19 Regression slopes of dark pigment cover in white sediment in W to B direction for all groups, black line represent control, red represent 5 pp As(V), green represent 10 ppm As(V) and yellow represent 20 ppm As(V)

4.3.3.2 Comparison of dark pigment cover in black or white sediment between groups

The average of dark pigment cover in black or white sediment in both directions were similar between control and treatment groups (Table 4.5). Although the median of dark pigment cover in black or white sediment in some treatment groups appeared to be slightly lower than the control group, there was high variation between individuals (Figure 4.20, Figure 4.21). One-way ANOVA test showed no significant difference in dark pigment cover either in black or white sediment between control and three treatment groups (Table 4.5), indicating that As(V) did not affect the colour of the shrimp.

Table 4.5 Descriptive Statistics and ANOVA for dark pigment cover in black and white sediment for both directions in all groups

Direction		Group	Mean	Std. Error	F	p-value
B to W	Dark pigment cover in black sediment	control	59.79	1.40	1.153	.327
		5 ppm	57.44	1.96		
		10 ppm	55.85	1.47		
		20 ppm	55.97	1.61		
	Dark pigment cover in white sediment	control	42.92	42.92	1.679	.171
		5 ppm	40.85	40.85		
		10 ppm	37.40	37.40		
		20 ppm	39.61	39.61		
W to B	Dark pigment cover in white sediment	control	40.08	40.08	.842	.471
		5 ppm	41.35	41.35		
		10 ppm	37.38	37.38		
		20 ppm	38.06	38.06		
	Dark pigment cover in black sediment	control	54.23	1.92	.810	.489
		5 ppm	54.15	1.99		
		10 ppm	57.37	1.69		
		20 ppm	54.26	1.50		

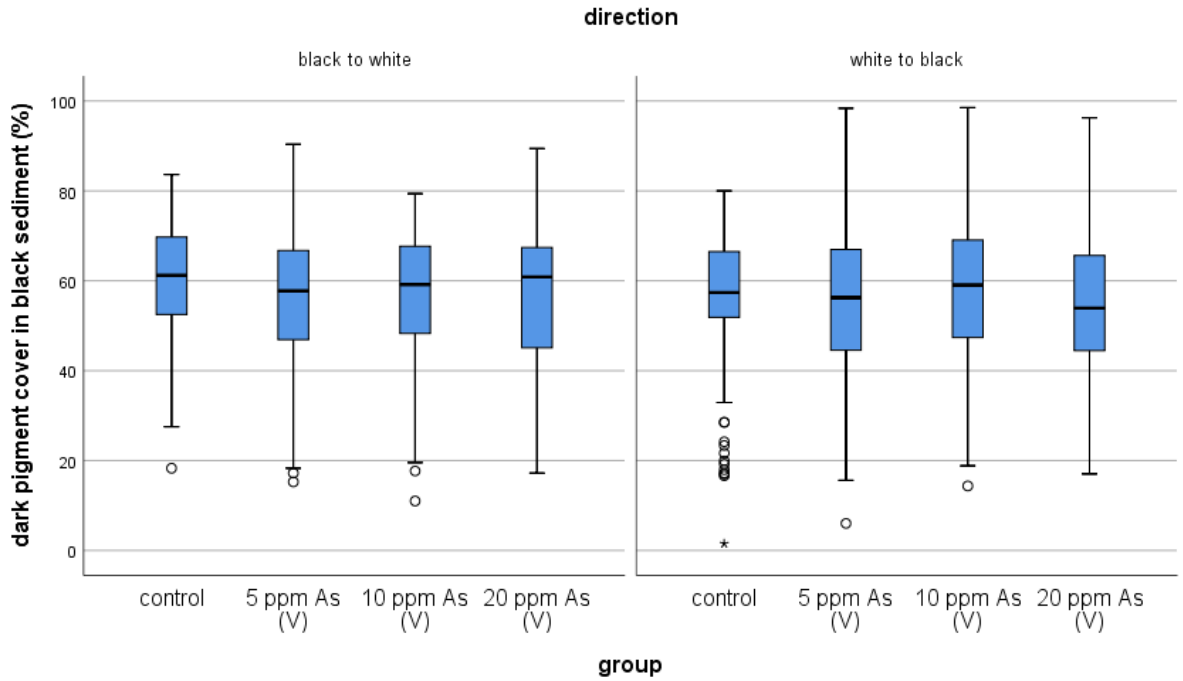

Figure 4.20 Dark pigment cover in black sediment in both directions between groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

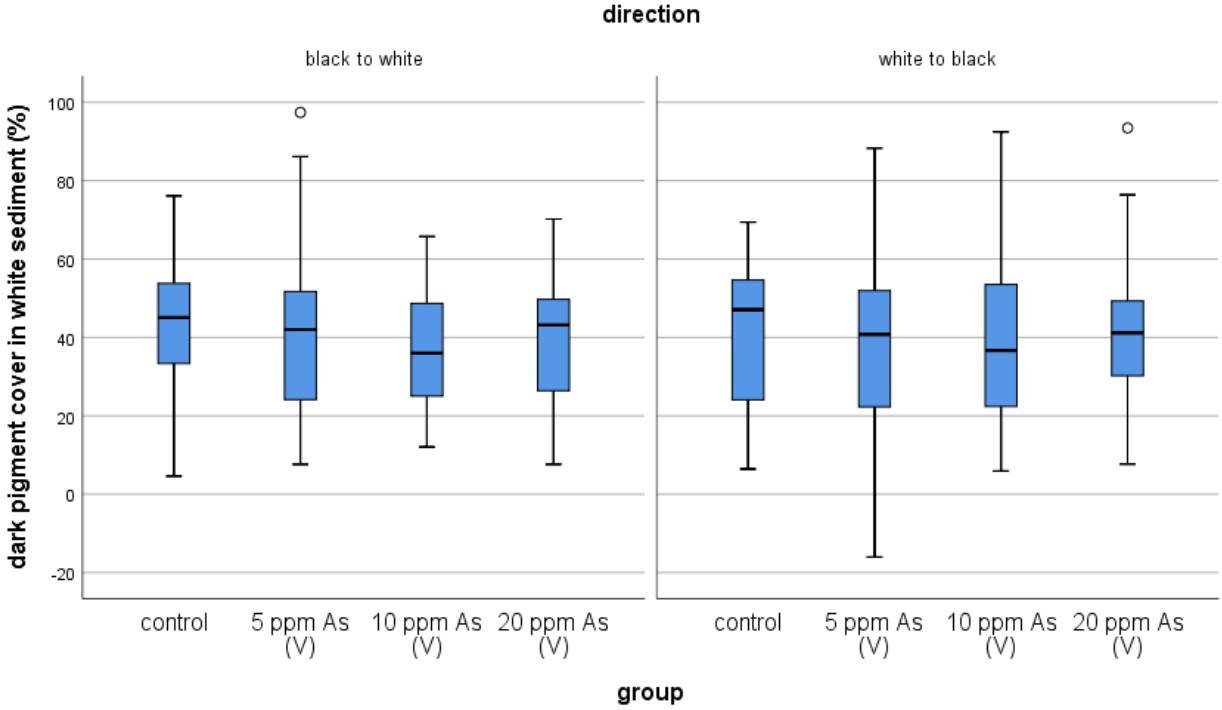

Figure 4.21 Dark pigment cover in white sediment in both directions between groups, box plots display minimum, maximum, median and first and third quartile; circles show outlier values

4.3.4 Arsenic estimation

Three different dilution concentrations of CRM (BCR-627) were prepared following the same preparations of the samples using both HNO_3 and water as a solvent. The CRM results (n=3) obtained from acid digestion showed an average of 4.27±0.15 ppm for total As with hence recovery of 89.02 % (corresponding to the certified value 4.8±0.3 ppm As). The total As measured in the CRM digested with water (4.43± 0.04) showed a recovery of 92.29 %. Based on these results, it appeared that the recovery of total As was higher in the water digestion compared to acid digestion. The extraction efficiencies in the CRM samples of all As species compared to the total As digested with water were ranged from 54 to 67 % (Table 4.6).

Table 4.6 As species measured in CRM and extraction efficiencies

Samples	AsB ppm	DMA ppm	As(V) ppm	Sum of As species	Total As ppm (acid digestion)	Total As ppm (water digestion)	Water Extraction efficiency (%)
	Extracted with water						
CRM 1	2.46	0.05	0.34	2.85	4.29	4.48	63.62
CRM 2	1.90	0.25	0.24	2.39	4.41	4.41	54.20
CRM 3	2.06	0.42	0.50	2.98	4.12	4.42	67.42

*Total As in CRM=4.8±0.3 ppm

4.3.5 Total arsenic

Due to time limitation, most of these results, except for the control and baseline (n=3), were obtained from single sample in each time point. All treatment groups showed an increase in total arsenic after one week of being exposed to different concentrations of As(V) in both types of digestion compared to the baseline (Figure 4.22). However, over three weeks, only the lowest concentration group (5 ppm in both types of digestion) seemed to continue accumulating further As. The other two groups stopped accumulating and started to lose (possibly depurate) the internal As, after two weeks (10 ppm group) and after one week (20 ppm group; Figure 4.22). Whereas, in water digestion, the total internal As increased during three weeks in 10 ppm but decreased after a week in 20 ppm (Figure 4.22). The extraction efficiency of As species in shrimp samples was calculated using the ratio between the sum of all As species that were found in water-extracted samples and the total As measured in the same samples. The results showed that the extraction efficiencies for CRM samples calculated in all groups ranged between 17 to 54 % compared to total arsenic found in the same extraction solution (Table 4.7).

Table 4.7 HPLC data including total As and As speciation

Samples	**AsB ppm**	**DMA ppm**	**As(V) ppm**	**Sum of As species**	**Total As ppm (acid digestion)**	**Total As ppm (water digestion)**	**Water Extraction efficiency (%)**
	Extracted with water						
Baseline	1.75	0.37	2.98	3.35	8.51	10.48	31.96
Baseline	4.82	0.02	0.45	5.22	9.08	9.72	53.70
Baseline	4.85	0.02	0.32	5.12	9.30	9.87	51.91
Control	4.73	0.02	0.12	4.89	8.38	9.89	49.47
Control	4.56	0.02	0.12	4.61	9.37	9.65	47.72
Control	4.62	0.02	0.03	4.65	9.22	9.56	48.69
5 ppm As(V)*	4.49	0.02	0.30	4.78	17.71	15.73	30.41
5 ppm As(V)**	3.50	0.02	2.75	6.25	22.10	18.08	34.59
5 ppm As(V)***	5.41	0.04	3.57	9.02	24.48	21.49	42.71
10 ppm As(V)*	7.85	0.24	5.31	13.40	31.26	25.51	52.55
10 ppm As(V)**	5.66	0.16	5.80	11.62	50.53	36.03	32.25
10 ppm As(V)***	4.72	0.16	3.87	8.75	27.41	38.07	30.51
20 ppm As(V)*	7.82	0.02	12.69	20.51	71.06	66.78	30.72
20 ppm As(V)**	4.56	0.17	8.38	13.10	48.20	38.06	34.42
20 ppm As(V) ***	4.75	0.15	6.90	11.80	45.15	31.69	37.23
Chicken (food)	0.14	0.14	0.23	0.52	0.16	0.04	1457.90

LOD for DMA<0.04, *day 7, **day 14 and ***day 21

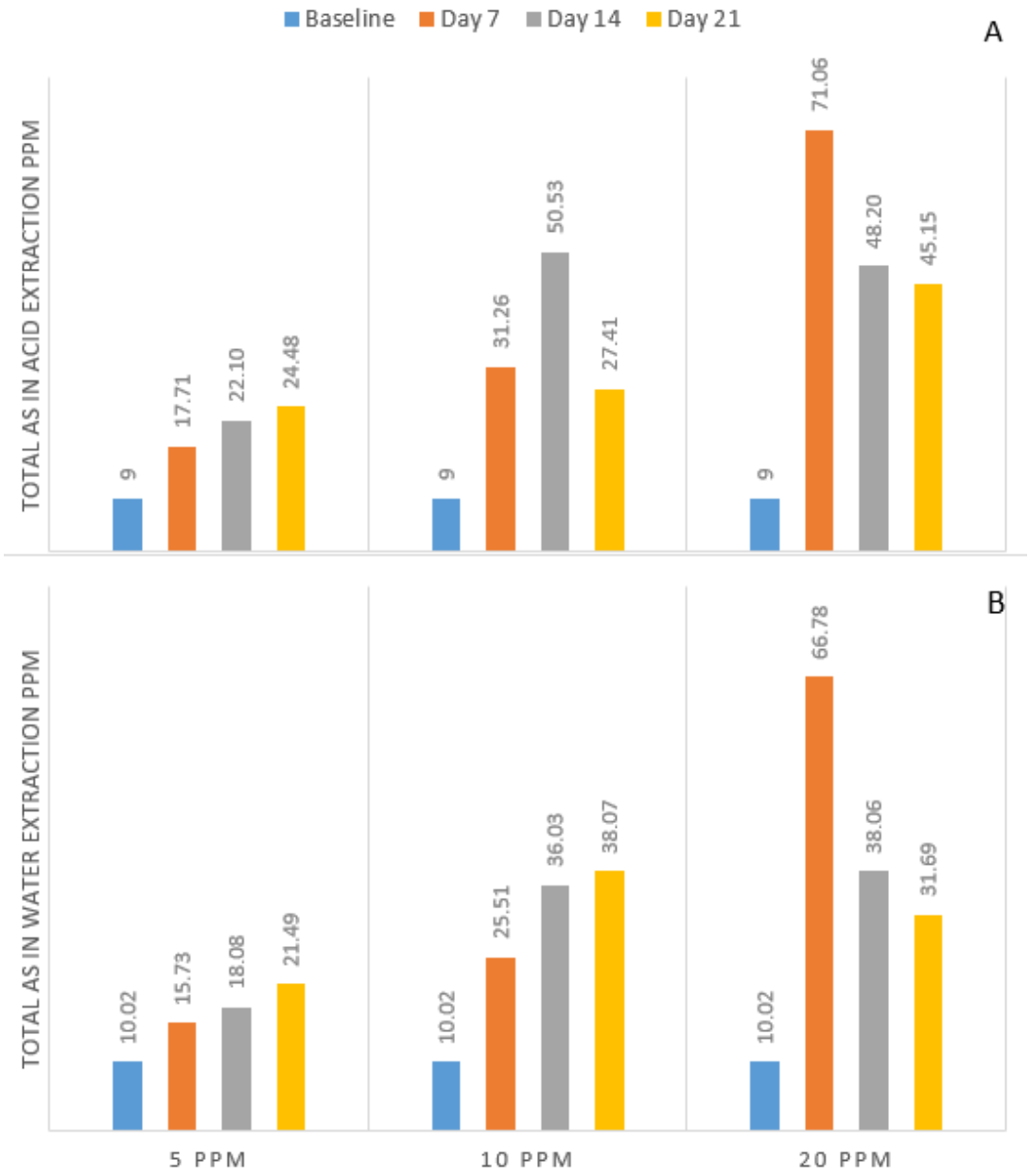

Figure 4.22 Total As in: A) acid digestion; B) water digestion

4.3.6 Arsenic speciation

The shrimp in 5 ppm showed a decrease in the level of As(V) in the first week of exposure and then they started to accumulate iAs in their body between day 7 and 21 (Figure 4.23). However, the samples in 10 ppm group accumulated As(V) in day 7 and day 14, the increase was about 425 % between day 7 and the baseline. After two weeks of exposure to 10 ppm As(V) in the water, the shrimp started to lose the internal As(V). The results in Figure 4.23 showed a reduction in As(V) concentration after a week of increasing the internal As(V) in the shrimp in 20 ppm group.

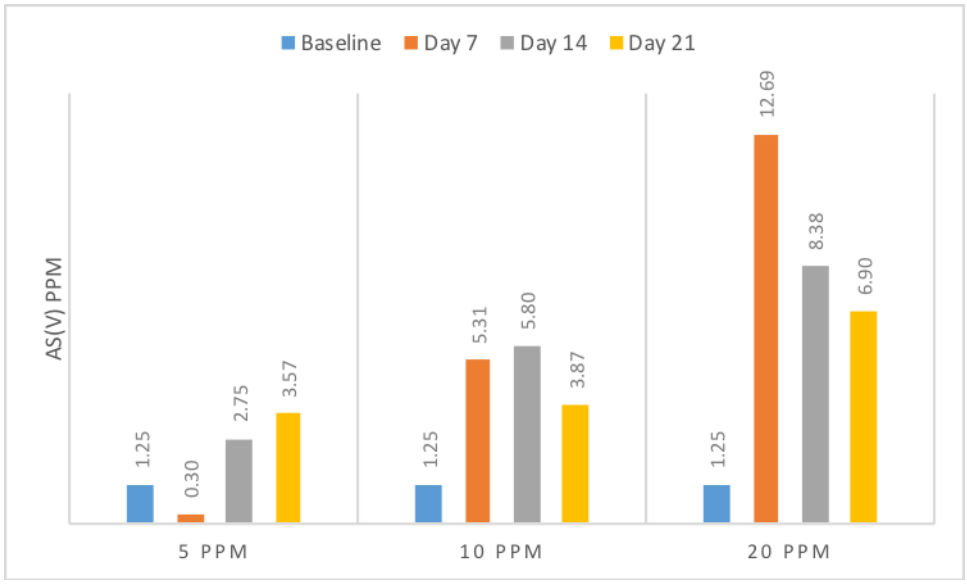

Figure 4.23 Arsenate [As(V)] concentrations in all groups

With regard to DMA, it was not detected in 5 ppm group in day 7 and day 14, however, it was detected in day 21 but in a level less than the baseline (Figure 4.24). In 10 ppm group, DMA was recorded throughout the experiment and the highest concentration was found in day 7 (0.24 ppm). However, in the 20 ppm group, DMA was not detected in day 7, but it was detected in day 14 and 21. DMA was the less dominant species in the brown shrimp compared to AsB and As(V).

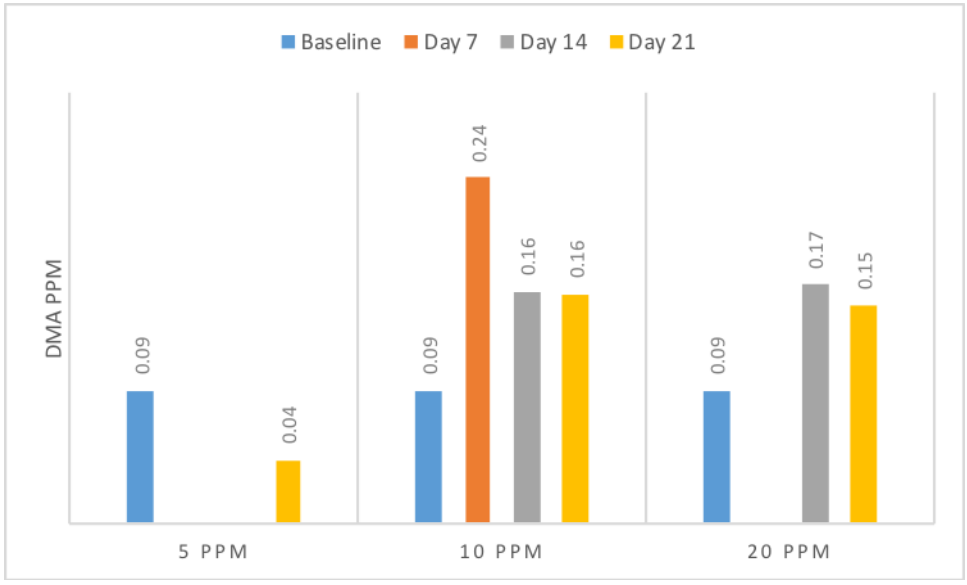

Figure 4.24 Dimethylarsinic acid (DMA) concentrations in all groups

Arsenobetaine in 5 ppm group that measured in the shrimp decreased during two weeks of treatment compared to the baseline and then increased in the last week of the experiment to be higher than the baseline level (Figure 4.25). Nevertheless, AsB seemed to elevate in the first week in 10 and 20 ppm groups, then it decreased again to almost the same level as the baseline (Figure 4.25).

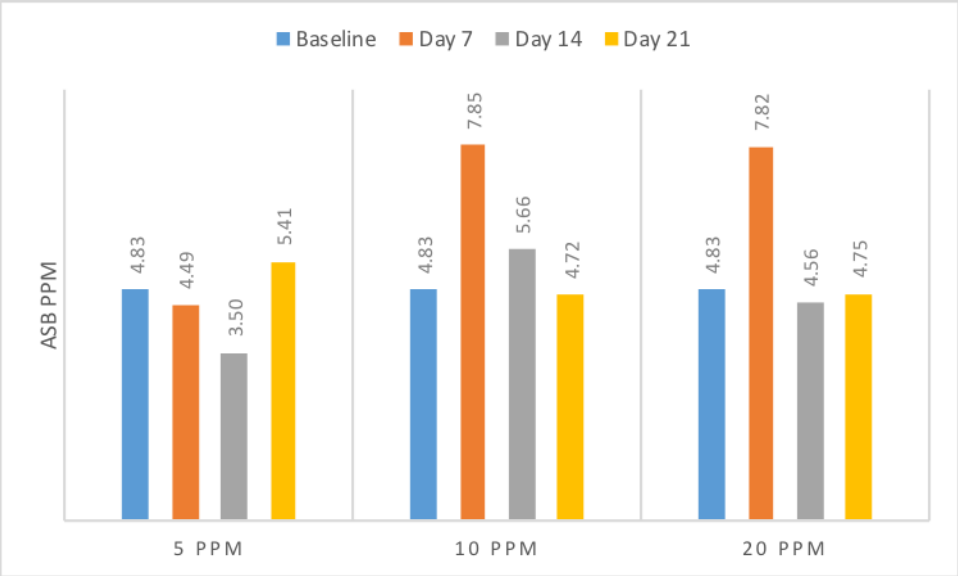

Figure 4.25 Arsenobetaine (AsB) concentrations in all groups

The ratio between As(V) and AsB in the treatment groups decreased in between day 7 and 14 compared to the day 0 due to the high level of As(V) accumulated by the shrimp (Figure 4.26). There was an increase in the ratio between day 14 and 21 in all group except the highest As group. This may strongly suggest that the brown shrimp were able to transform As(V) to AsB and this was efficient when the external As was low (Figure 4.26).

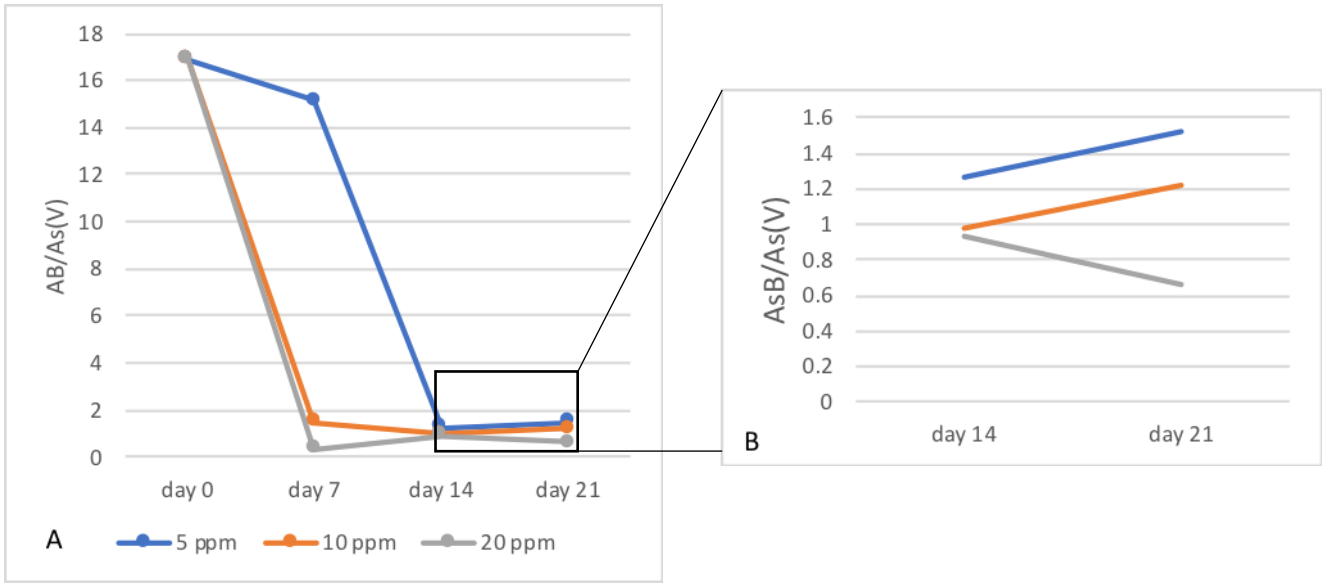

Figure 4.26 AsB/As(V) ratio in the treatment groups; A) all days; B) between day 14 and 21 only

4.4 Discussion

4.4.1 Mortality

In this study, highest mortality was found in the lowest treatment group (As (V)=5 ppm). It was also noticed that in the control group there were some casualties during the experiment although not as many as the treatment groups. This indicates that the mortality may not just be caused by As and could be due to the fact that shrimp were at the end of their life cycle when they were collected, or they reached the end of their life cycle faster under the experimental conditions (Brix et al., 2003). It has been reported that brine shrimp died faster when they were exposed to contaminants (Brix et al., 2003; Gillespie & Stephens, 1977). Mortality rates were similar between a treated As(V) group and a control group in the amphipod *Gammarus pulex* exposed to different As(V) concentrations and temperature degrees (Vellinger et al., 2012). Also, 100% mortality rate has been reported in the marine clam *Asaphis violascens* exposed to 10 ppm and 20 ppm As(V) after 8 and 4 days of exposure (Zhang, Guo, Wu, Qiao, & Zhang, 2018a).

On the other hand, Madsen (1992) reported that the survival of the *Crangon crangon* was dose dependent. Thus, the mortality was due to external As, when the brown shrimp were exposed to concentrations of As(V) higher than 25 ppm.

4.4.2 Colour change and dark pigment cover in black and white sediment

The mechanism of colour changes of brown shrimp has been studied extensively for a long time (Czerpak & Czeczuga, 1969; Pautsch, 1953; Skorkowski, 1971, 1973) and is connected to a black pigment dispersing hormone.

In this study, we focused on the effect of As(V) on the ability to change colour in the brown shrimp, which have not been investigated yet. The results showed no significant difference in colour change (final – initial dark pigment cover) between control and treatment groups. In addition, there was no significant difference in the average of dark pigment measured in black or white sediment between control and treatment groups. Thus, it appeared that As(V) did not affect the ability to change colour in the brown shrimp if they were placed in different substrates or even if they were kept in the same sediment colour. The reason behind this could be due to the ability of some marine invertebrates and fish to metabolise As(V) to orgAs. Zhang and Wang (2018) published work showing that clams and polychaetes transformed iAs to orgAs when they were fed with As-spiked sediments (30 ppm AsV). Similar results were reported when rabbitfish and seabass were fed spiked fresh prey diets contained different concentrations of iAs (Zhang et al., 2016). Therefore, As could be similarly bio-transformed in *C. crangon.*

With regards to dark pigment cover in black or white sediment during the duration of the experiment (three weeks), significant differences were noticed between some days especially between day 1 and day 21 even in the control group. Hence, the decrease of mean of difference in pigment cover in black and white sediment may not due to As since control group was performing similarly to the treated group. However, this could be due to also to inter-individual variations (Siegenthaler et al., 2018; Stevens & Ruxton, 2019). Possibly, a bigger sample size could help to reduce these variations, but plasticity in colour change might be a strategy (Siegenthaler et al., 2018).

The only few studies that can be compared with this study, consider the effect of As on the colour change of the fish *Channa punctatus.* Allen et al. (2004) have studied the colour of *C. punctatus* scales when treated with a sublethal dose of As trioxide (1 ppm) every other day for 7, 14, 30, 60 and 90 days. The melanophores in the scale showed reticulated shapes, however, they decreased after 7 days of exposure to be all punctuated shapes in day 14. These results suggest that the pigments were contracted and the fish looked paler. After 90

days of exposure, the melanophores shape was similar to the control group and the fish stopped accumulating As in their scale. Thus, iAs affect fish to some extent (between 7 to 60 days) by causing paleness of the fish before they develop mechanisms to adapt against its toxicity. A similar study has been conducted by (Akarte & Agnihotri, 2013) using the same fish species with higher As dose (6 ppm) for only 30 days. The authors have reported that As trioxide cause paleness in the fish due to aggregation of the pigment. These results contrast with our results: we have found that As(V) does not affect the colour of the brown shrimp. One explanation could be that *C. punctatus* is not able to bio-transform iAs to the less toxic organic form (at least for 30 days of exposure). As none of these studies (Akarte & Agnihotri, 2013; Allen et al., 2004) or other studies have investigated the metabolism of As in *C. punctatus*, this hypothesis cannot be verified. The other reason could be due to high toxicity of the species of As used in these studies. Jiang, Chen, Zhao and Zhang (2013) have been reported a sever DNA damage in the cells treated with arsenic trioxide compared to those treated with sodium arsenite.

4.4.3 Quality assurance

The total arsenic recovery for CRM samples using water for digestion was 92% and extraction efficiencies were ranged from 54.2 to 67 % for speciation which is in good agreement with CRM values. Extraction efficiency in our samples was relatively low. Hirata et al. (2006) reported that the low extraction efficiencies found in some marine organisms was because of the difficulties of completing the extraction in organisms. Using acid digestion was noticed to work less in extracting total As compared to water digestion. Besides, water as an extractor has been reported to extract AsB effectively from marine animals (Jia, Wang, Ma, & Yang, 2018). As we interested in AsB thus water digestion has been used in this study to extract As species. However, some of the orgAs such as DMA and arsenocholine (AsC) are more extractable with methanol (Jia, Wang, Ma, & Yang, 2018). This could explain the differences found in total As between water and acid digestion.

4.4.4 Total arsenic

Based on total As results, *C. crangon* started to accumulate As slightly in their body over time when they were exposed to low concentration of external As(V). In contrast, when the external As(V) was high (10 and 20 ppm), As was accumulated very rapidly in the first week

then it stopped, as it reached saturation or possibly was depurated by the shrimp. This may indicate that the accumulation of As in the brown shrimp was proportional to the external As(v) concentrations but also dependent on the time needed to reach a saturation point (this was not reached with 5 ppm).

It has been noted that the As level in the shrimp *Lysmata seticaudata* increased when the animals were exposed to high level of external As(V) (Fowler & Yaşar Ünlü, 1978). Also, benthic shrimp who ingested As(V) from food had more retention period than the ones exposed to it in the water (Fowler & Yaşar Ünlü, 1978), indicating that the shrimp could not keep As in their body for a long time when they were exposed to iAS in the water. This was comparable to the result in this study where the shrimp in 10 and 20 ppm lost As after a certain time. One possible mechanism that could also allow the shrimp to get rid of iAs is moulting. (Fowler & Yaşar Ünlü, 1978) reported 50 % reduction in accumulated As in *Lysmata seticaudata* because of the moult.

Kuroiwa, Yoshihiko, Ohki, Naka and Maeda (1995) reported that the total As accumulated by *Macrobrachium resenbergii* increased with the increase of external As(V). However, at concentration of 30 and 100 μg As cm^{-3}, the total As decreased slightly (note that these concentrations correspond to LC_{50}).

In contrary, Hunter et al. (1998) have published that no As was accumulated by the brown shrimp when they were exposed animals to As(V) in seawater. This could be due to the low As(V) concentration that were used (0.1 ppm) compared to the concentrations (5, 10 and 20 ppm) used in this study. Other factors that could affect As accumulation in crustaceans are temperature and body size: in *L. seticaudata* there is a relationship between high As accumulation and larger body size (Fowler & Yaşar Ünlü, 1978). In addition, salinity is another factor that can affect the As accumulation: crustaceans tend to accumulate more when the salinity is low (Fowler & Yaşar Ünlü, 1978).

4.4.5 Arsenic speciation

The brown shrimp accumulation of As(V) from water in their body corresponded to the external As(V) concentration. However, reaching a saturating point was dependent on the time and external As(V) concentration. The brown shrimp appeared to accumulate As(V), but the retention time of As(V) decreased with the increase of time and external As(V) dose. A similar study that has investigated the uptake of As(V) in the brown shrimp has reported

that accumulation of ingested As(V) decreased after 16 days to reach similar level of the control group after 24 days (Hunter et al., 1998). Subsequently, the brown shrimp seemed not to deal with high concentrations of As(V) after a certain time or after they reached the saturation limit.

On the other hand, Hunter et al., (1998), have found that exposing the brown shrimp to As(V) or trimethylarsine oxide (TMAO) in the water result in no accumulation of these species in any tissue of the shrimp i.e. tail, midgut, gills, exoskeleton and the rest of the body. The low dose of As(V) used in their experiment (0.1 ppm), compared to this study where 5, 10 and 20 ppm of As(V) were used, could be the reason why the shrimp could not accumulate it in their body. Previous study has been reported that the fish *T. mossambica* can accumulate As(V) in their body when they were exposed to it in the water and the accumulation increased with the increase of the external dose (Suhendrayatna, Ohki, Nakajima, & Maeda, 2002).

With regard to DMA, it was the less abundant species in term of its abundance in the brown shrimp compared to AsB and As(V). It was not detected in the 5 ppm group in the first two weeks of the experiment. Nevertheless, it was recorded in the other two groups in this experiment apart from day 7 in 20 ppm group. This suggest that the bio-transformation of As(V) to DMA in the brown shrimp might not be sufficient when the external iAs is low or converted to other species of iAs.

The same observation has been published by (Suhendrayatna et al., 2002). They have reported that DMA was detected in trace amounts (not measurable) in *T. mossambica* exposed to a low concentration of As(V) in the water (0.1 ppm) for one week. However, with the high external As(V), *T. mossambica* biomethylated the iAs to DMA. This indicated that external iAs species could play a role in transforming iAs to DMA as it was reported with clams (Zhang, Guo, Wu, et al., 2018a). The latter could biosynthesise As(III) to DMA more than when they were exposed to As(V). They have also suggested that DMA perform as a precursor of AsB. Therefore, the clam might be accumulating As(V), which is then reduced to As(III), methylated to MMA and DMA, and then transformed to AsB.

Regarding the AsB, it has reported as major As species found in fish and crustaceans (Hirata et al., 2006; Zhang, Guo, Wu, et al., 2018). However, it is often not detected in seawater, sediment and algae. Some studies have been carried out to establish the origin of AsB present in aquatic animals. It seems that AsB can be transformed within the animal

(Goessler et al., 1997). This was tested by a field experiment with molluscs fed on algae. They found that molluscs contain AsB as a major As compounds, although the AsB was not detected in the algae (Goessler et al., 1997). Another laboratory study reported that the brown shrimp converted part of ingested trimethylated arsenosugar to AsB (Francesconi et al., 1999). Therefore, some marine organisms can bio-transformed and generate AsB in their body. More studies need to be conducted with bigger samples size to understand the mechanisms of transforming iAs to AsB.

In this study, *C. Crangon* appeared to metabolise part of the iAs to AsB. This was noticed when the shrimp were treated with low concentration of As(V) in the water (5 ppm) after two weeks of exposure, indicating that the brown shrimp can be more efficient in bio-transforming the iAs to organic form at lower external As(V). This result was comparable to former research (Zhang, Guo, Wu, et al., 2018a), where AsB concentration decreased proportionally with the increase of external iAs in clams.

In another study, brown shrimp was noticed to transform the trimethylated arsenosugar into AsB efficiently at low doses (Francesconi et al., 1999).

The other possible explanation of increasing the level of AsB, if not from converting iAs, was from the food (Francesconi et al., 1999): food need to be the main source of AsB found in the brown shrimp. Since the shrimp in this experiment was fed with chicken and HPLC result in showed a total of 0.14 ppm AsB in the chicken which was very low compared to the AsB found in the shrimp, this hypothesis was rejected. Thus, the brown shrimp can be consumed by human even if grown in a polluted area.

5. Chapter Five: General discussion

5.1 Main findings

Crangon crangon has great ecological value in European estuaries and is one of the most commercially important shrimp for fisheries in northern European waters (Tulp et al., 2016). For a great part of the year, the brown shrimp inhabits estuaries and shallow waters, which are often subject to environmental or anthropogenic pollution. These contaminants, in particular heavy metals produced by human activities, can have a detrimental effect on the aquatic ecosystems. Cadmium and As, for instance, are toxic metals and found ubiquitously in the aquatic systems. In previous studies, both were tested for their effects on some behavioural change in aquatic organisms and on biomarkers of such changes and were found to have some effect/s, therefore they have been used in this project for eco-toxicological assessment using *C. crangon* as the study organism.

In order to asses environmental contamination, the use of behavioural markers (behavioural ecotoxicology) has been proposed, as organisms' behaviour can be very sensitive even to small-non-lethal concentrations of contaminants (Pestana et al., 2007, Stevens & Ruxton, 2011). Hence, after considerable literature research (as elaborated in chapter2), two behaviour biomarkers have been chosen in this work: feeding rate and colour change ability. Colour change for background matching for instance increases survival as it helps animals to hide from predators (Duarte et al., 2017; Smithers, Rooney, Wilson, & Stevens, 2018). It is an important ecological biomarker among animals that have the ability to change colour and could be affected by pollutants. This project thus focused on one of the main behavioural traits that characterize *C. crangon*: colour change ability besides feeding rate.

Feeding rate has been studied using different organisms and has been found to be sensitive biomarker to detect even small concentration of contaminants including heavy metals like Cd (Chapter 1; table 1.1). In the literature, only a few studies have considered colour change of aquatic animals to detect environmental stressors, such as contaminants, but none have been conducted on the brown shrimp. Since the brown shrimp show a high ability to match their background (Siegenthaler et al., 2018) and they use it often to avoid predators, colour change ability was used to test the effect of Cd and As. The toxicity of As depends on its chemical form and it has been found that the majority of the As in aquatic organisms is

organic in nature which is less toxic than iAs (Du, Wang, Yang, Liu, & Li, 2019). However, there is a lack of knowledge whether aquatic animals bio-transform iAs into orgAs or they absorb mainly orgAs from the environment. Another concerning issue that could affect the health of aquatic organisms is the presence of antibiotics in the water (Kang et al., 2018). The excessive use of antibiotics, including their use as growth promoters in animal husbandry, has raised concern for the spread of antibiotic resistance in the marine environment, since this can be exacerbated in the presence of heavy metals. For example, the combined effect of selection and co-selection of antibiotic-resistant bacteria may occur when antibiotics from animal production and agriculture are released into the aquatic environment alongside heavy metals (Seiler & Berendonk, 2012).

The aim of this project was to illustrate the effects of heavy metals on the behavioural responses in the brown shrimp. To achieve that, three experiments were conducted. The first experiment was to assess individual and combined effect of Cd and antibiotics on feeding rate and colour change. Second experiment was performed to assess the effect of Cd on colour change ability of the brown shrimp collected from different populations and if that could influence their behaviour, as different sites could have different contamination. The hypothesis was that the Cd, As and/or antibiotics would affect colour change ability and feeding rate.

In this study when brown shrimp were exposed to 0.04 ppm of Cd and two antibiotics (10 ppm FZD and 2 ppm Gt) in the water for 21 days to test colour change ability and feeding rate (**Chapter 2**), the results showed no change in body weight or food intake (measured by the difference in body weight before and after feeding), suggesting either the concentration of Cd and antibiotics used did not affect the feeding rate in the brown shrimp or the concentration of Cd used was not sensitive enough to produce any measurable change. With regard to colour change, there were some trends that suggested some changes in the ability of the brown shrimp to match the background when the shrimp were exposed to Cd alone or in combination with antibiotics; however, the trends reported in the treatment group were not significantly different from the control group. Said that this experiment included multiple factors (Cd, antibiotics and combination of both) and hence too variables were analysed at one point in time making it difficult to interpret the results. Hence, the following experiment was focused one only effect of Cd on colour change ability. The same experimental protocol as in **Chapter 2** was used again in **Chapter 3** with a larger sample size

and two different populations but for a shorter period of time (7 days). The results showed no consistent change in the ability of the brown shrimp to change colour across two populations when they were exposed to 0.04 ppm Cd (see **Chapter 2** and **3**). In addition, there was a high intra- and inter- individual variability in the ability of the brown shrimp to change colour. These variabilities are important to many aquatic animals that can change colour to be concealed by matching different and unpredictable habitats (Hughes, Liggins, & Stevens, 2019). The different populations used in this experiment showed significant differences in their baseline colour (Dale was always darker than Liverpool), however, more investigations are needed to confirm any such appreciable difference. When As was used to test the colour change ability of the brown shrimp (**Chapter 4**), no effect of As was noted. In this experiment, brown shrimp were exposed to various concentrations of As (5, 10 and 20 ppm AsV) for 21 days and along with total As the concentration of different As species were measured in the tissue of the shrimp to evaluate the As bio-transformation ability in the shrimp. In both experiment with Cd and As, the baseline concentrations of Cd and As in the shrimp tissue was analysed before dosing (**Chapter 2 and 3**) to determine any difference between the different populations (Mersey vs Dale vs Liverpool). The results showed that the concentrations of some elements found in shrimp tissue were significantly different including Cd between Mersey and Dale. These differences in the baseline concentrations of heavy metals could be one reason for the differences found between populations in behaviours (the darkness in appearance in Mersey shrimp could be caused by prolonged exposure to high concentration of Cd and other heavy metals in the sediment). However, concentration of different elements found in water and sediment samples collected from Mersey, Dale and Liverpool were mostly similar except for Zn which was found to be significantly different in water samples between Dale and Liverpool.

In terms of As speciation results, dosed shrimp showed evidence of bio-transformation of iAs (AsV) into orgAs in form of AsB as suggested by the increase of AsB in return of decrease of AsV. The results in **Chapter 2, 3** and **4** showed overall high concentration of total As in the shrimp. However, this does not necessary reflect the toxicity of the brown shrimp: the majority of the total As accumulated in the shrimp was in organic form (AsB). Indeed, shrimp dosed with different concentrations of iAs, bio-transformed it into the less toxic organic form, AsB (**Chapter 4**), which makes them more tolerant to As compared to other species. This has relevant implications for aquaculture and fisheries of crustaceans in areas

contaminated by inorganic As, as they can be consumed by human even if grown in contaminated area.

To summarise, based on the three experiments described in Chapter 2,3 and 4, it is not possible to definitely conclude that Cd, and/or As administered at the concentrations specified, had any significant effect on the colour change ability and feeding rate in the study species. However, certain trends observed might be further investigated after careful consideration of the limitations discussed below.

5.2 Limitations

Colour change abilities in the brown shrimp was not consistent and presented high individual variation also within the control group; such variability cannot be easily reduced, even increasing the sample size of the experiment (Dalzochio et al., 2016). Behavioural ecotoxicology relies on the consistent response to non-toxic concentration of pollutants. In the case of high individual variations (Dalzochio, Rodrigues, Petry, Gehlen, & da Silva, 2016), which naturally occur in many crustaceans including the brown shrimp (Siegenthaler et al., 2018), this response is not an effective indicator of pollutants (Blockwell et al., 1998).

Another limitation in this study was the difficulties to maintain a big sample size, lacking a proper rearing system. We started with very basic rearing tanks, which could hold a small sample size (8 to 12 shrimp), then we developed (in collaboration with Aquaponic Lab) a new recirculating system, able to hold a bigger sample size (24 chambers per compartment). Studying behaviour toxicology responses of aquatic organisms in the laboratory is challenging (Nagelkerken & Munday, 2016). Most of the work that have been done in related to colour change ability was conducted to address the background matching ability and substrate choice as a camouflage tool (for example: Siegenthaler et al., 2018; Smithers et al., 2018; Stevens, Lown, & Wood, 2014), but little has been done to assess the effect of environment contaminations on the colour change ability (Ahmad et al., 2018; Akarte & Agnihotri, 2013; Allen et al., 2004; Fingerman & Fingerman, 1978; Hanumante et al., 1981; Reddy & Fingerman, 1995). Hence, measuring colour change and food intake of the brown shrimp in lab condition was challenging.

5.3 Implication for ecosystem management

This thesis has focused on the brown shrimp, which is exploited as a valued commercial species and represent one of the main components in the aquatic ecosystems of many countries, including the UK (Temming & Damm, 2002). However, due to the level of anthropogenic pollution in European waters and following the European Water Framework Directive (Baattrup-Pedersen, Larsen, Rasmussen, & Riis, 2019), there is a serious need to develop effective biomarker to allow a quick and efficient assessment to evaluate water quality in the marine ecosystem (Pestana et al., 2007). We tested the efficiency of colour change ability in the brown shrimp as a sensitive biomarker, as it has been reported for some fish and crabs (Ahmad et al., 2018; Allen, Singhal, & Rana, 2004; Fingerman & Fingerman, 1978; Reddy & Fingerman, 1995). The results obtained do not provide strong evidence on the effect of Cd on the colour change ability of the brown shrimp. Therefore, the investigated behaviours of the brown shrimp in this work (colour change and food intake) may not be the right tools to effectively assess the effects of environmental pollution on marine organisms. However, colour change ability could be used to investigate other environmental stressors such as global warming. A pilot project has tested the effect of high-water temperature (25 ± 0.5°C) on the ability of the brown shrimp to match its background. The results have shown that, in the high temperature treatment group, the shrimp were significantly paler when they were moved to different sediment colour compared to the control group, regardless the direction of change (from black to white or vice versa). Hence, colour change could still be used as a biomarker for other environmental stressors (Dalzochio et al., 2016), and could potentially provide information on whether these changes could be applied in polluted environments not only at the individual level, but also at the community level (Blockwell et al., 1998).

5.4 Future directions

Future studies could use other environmental stressors such as temperature or change in salinity to address the colour change ability in the brown shrimp or other species. Using natural substrate and water from the original environment to study the effect of different heavy metals (other than Cd and As) in the lab could be considered in a future study as well. In addition, studying the effect of contaminants on colour change in the brown shrimp could be applied in naturally contaminated environment.

More research can also be done with different species that inhabits naturally contaminated environments, to investigate the ability to bio-transform iAs to orgAs, as they might be able to detoxify the environment. The mechanisms of transforming the iAs should be investigated.

The use of antibiotics in **Chapter 2** was a pilot study to address the potential role of these substances, often found in increasing concentrations in the environment. A follow up project stemming from the pilot is currently looking at microbiome changes (using molecular metabarcoding) in shrimp dosed with different concentrations of As.

5.5 Final conclusion

Colour change ability in the brown shrimp is not the most effective biomarker to detect effect of environmental pollution (at least for Cd and As). It could be tested for other environmental stressors such as high temperature. By studying As speciation, this work provides fundamental information on the potential ability of *C. crangon* to bio-transform iAs to orgAs: thus this shrimp could be consumed by humans even if grown in contaminated areas.

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A. Appendix

1. Supplementary material for chapter 2

1.1 Initial body weight

Based on the results shown in Table A.1, the significant difference in initial body weight was found to be of the shrimp between day 1 and day 13, 15, 18, and 20. This indicated that the shrimp lost weight slightly in some days when they were exposed to 0.04 ppm Cd.

Table A.1 Pairwise comparisons for initial body weight before feeding between days in Cd group in Mersey

				p-values using post-hoc test for days								
	day	Mean	Std. Error	4	6	8	11	13	15	18	20	22
Cd group	1	.418	.052	.679	.239	.596	.109	**.007**	**.009**	**.013**	**.029**	.233
	4	.412	.046		.206	.879	.212	**.000**	**.010**	**.011**	**.015**	.178
	6	.390	.036			.274	.596	.636	.832	.313	.397	.822
	8	.410	.044				.204	**.005**	**.013**	**.012**	**.015**	.362
	11	.399	.045					**.017**	.074	**.004**	.107	.741
	13	.383	.045						.461	.313	.740	.407
	15	.386	.046							.260	.473	.537
	18	.371	.043								.603	.252
	20	.380	.042									.245
	22	.394	.045									

- Bold values were significant (p-value<.05)

1.2 Dark pigment cover in black sediment

Pairwise comparison test (Table A.2) showed that the significant change in dark pigment cover in black sediment was between day 1 and the other days starting from day 11 in control group of Mersey experiment. With regard to Cd group in the same experiment, the lowest mean of dark pigment cover in black sediment was recorded in day 1 (Table A.2), and it was significantly different from all other days apart from day 15 and day 22.

Table A.2 Pairwise comparisons between days for dark pigment cover in black sediment for Mersey experiment

				p-values using post-hoc test for days								
	day	Mean	Std. Error	4	6	8	11	13	15	18	20	22
Control	1	36.74	5.78	.065	.048	.192	**.027**	**.005**	**.008**	**.002**	**.000**	**.019**
	4	50.75	4.19		.424	.749	.626	.169	**.029**	**.000**	**.000**	.394
	6	55.90	7.51			.356	.792	.782	.839	**.035**	**.000**	.966
	8	48.57	6.97				.487	.196	.150	**.006**	**.000**	.407
	11	53.58	4.76					.370	.413	**.004**	**.000**	.678
	13	58.17	2.87						.884	**.036**	**.000**	.646
	15	57.48	4.15							**.004**	**.000**	.850
	18	76.74	6.61								**.035**	**.038**
	20	93.14	1.91									**.000**
	22	56.30	4.49									
Cd group	1	47.91	4.91	**.001**	**.001**	**.011**	**.003**	**.000**	.054	**.001**	**.000**	.182
	4	69.17	4.86		.877	.949	.257	.664	.050	.047	**.000**	**.012**
	6	68.36	5.31			.860	.161	.851	.118	.078	**.000**	.067
	8	69.60	3.00				.170	.701	.051	.115	**.001**	**.009**
	11	63.21	2.55					.209	.365	.007	**.000**	.176
	13	67.58	3.43						**.016**	.064	**.000**	.016
	15	59.42	3.67							**.008**	**.000**	.403
	18	81.86	5.43								.054	**.002**
	20	92.38	2.67									**.000**
	22	56.99	3.41									

- Bold values were significant (p-value<.05)

In Dale experiment, pairwise test for Cd group showed that the changes in dark pigment cover in black sediment was between day 1 and day 8, 11 and 13 (Table A.3), this suggested that the shrimp were less darker in the black sediment in those days compared to day 1. However, the significant decrease in dark pigment cover in black sediment was found between day 1 and day 22 of the experiment in shrimp dosed with Cd alongside antibiotics (Table A.3).

Table A.3 Pairwise comparisons between days for dark pigment cover in black sediment in Dale experiment for Cd and antibiotics+Cd group

				p-values using post-hoc test for days								
Group	day	Mean	Std. Error	4	6	8	11	13	15	18	20	22
Cd group	1	74.26	2.12	.596	.457	**.005**	**.001**	**.004**	.066	.525	.064	.073
	4	76.88	3.62		.931	.265	**.000**	**.008**	**.031**	**.039**	.078	**.002**
	6	77.06	2.50			.172	**.000**	**.001**	**.032**	.121	.049	**.005**
	8	83.21	3.59				**.000**	**.001**	**.007**	.118	**.016**	**.013**
	11	49.00	2.76					.080	**.008**	**.005**	**.012**	.059
	13	57.33	3.95						.109	.100	.057	.502
	15	66.51	3.70							.533	.785	.301
	18	69.90	5.06								.517	.022
	20	65.63	4.09									.474
	22	61.23	5.30									
Antibiotics+Cd	1	75.37	3.66	.923	.173	.052	**.008**	**.031**	.006	**.002**	.061	**.006**
	4	75.71	4.31		.068	.007	**.007**	.033	**.011**	**.012**	**.039**	**.003**
	6	70.24	4.15			.026	**.012**	.143	.051	.061	.063	**.005**
	8	64.05	4.57				.109	.647	.261	.777	.423	**.015**
	11	55.71	3.96					.228	.988	.286	.624	.564
	13	61.07	3.87						.395	.813	.838	.234
	15	55.79	5.79							.358	.732	.629
	18	62.46	4.26								.617	.231
	20	59.40	6.31									.397
	22	53.02	5.24									

- Bold values were significant (p-value<.05)

1.3 Dark pigment cover in white sediment

The results presented in Table A.4 showed that the average of dark pigment cover in white sediment in control shrimp of Mersey reduced significantly in day 15 and 18 compared to day 1. Whereas, shrimp in Cd group were much darker in white sediment in day 4, 6, 8 and 11 compared to their colour in day 1 (Table A.4).

Table A.4 Pairwise comparisons between days of dark pigment cover in white sediment for both groups in Mersey experiment

				p-values using post-hoc test for days								
Group	day	Mean	Std. Error	4	6	8	11	13	15	18	20	22
Control	1	39.71	4.64	.469	**.043**	.859	.496	.209	**.007**	**.006**	.150	.172
	4	43.69	5.31		.088	.272	.197	.040	**.003**	**.002**	.052	.096
	6	52.44	4.63			**.010**	**.028**	**.006**	**.000**	**.000**	**.002**	**.013**
	8	38.41	5.84				.522	.111	**.010**	**.006**	.103	.267
	11	34.46	4.03					.374	**.004**	**.005**	.169	.380
	13	30.46	4.95						**.040**	**.015**	.566	.738
	15	21.90	2.17							.474	.075	.284
	18	20.93	2.43								.016	.172
	20	28.01	4.42									.97
	22	29.74	6.09									
Cd group	1	28.97	5.32	**.000**	**.022**	**.015**	**.030**	.194	.577	.483	.400	.914
	4	59.10	5.67		**.004**	.070	**.008**	**.004**	**.000**	**.010**	**.004**	**.001**
	6	45.62	4.96			.893	.545	.240	.060	.291	.225	.068
	8	46.58	7.33				.494	.359	**.041**	.301	**.033**	**.024**
	11	41.94	5.21					.602	.071	.451	.371	.061
	13	38.86	5.78						.234	.692	.685	.211
	15	31.03	5.45							.581	.537	.824
	18	35.33	7.78								.971	.374
	20	35.66	7.50									.159
	22	29.74	6.09									

- Bold values were significant (p-value<.05)

1.4 Colour change ability

Based on Table A.5, it was noticed that shrimp in day 18 and 20 were performing better in terms of changing colour from black to white sediment compared to other days in both control and Cd group in Mersey experiment.

Table A.5 Pairwise comparisons between days for difference in pigment cover in black and white sediment in both group for Mersey experiment

				p-values using post-hoc test for days								
	day	Mean	Std. Error	4	6	8	11	13	15	18	20	22
Control	1	2.971	6.902	.260	.438	.099	.049	**.012**	**.001**	**.001**	**.000**	**.002**
	4	-7.057	4.284		.660	.628	.077	**.010**	**.000**	**.000**	**.000**	**.019**
	6	-3.462	5.116			.386	.065	**.018**	**.000**	**.000**	**.000**	**.001**
	8	-10.155	4.649				.189	**.015**	**.000**	**.000**	**.000**	**.012**
	11	-19.127	5.211					.150	.004	**.000**	**.000**	.136
	13	-27.710	4.685						.220	**.003**	**.000**	.950
	15	-35.582	3.388							**.003**	**.000**	**.111**
	18	-55.805	6.690								.217	**.002**
	20	-65.135	4.779									**.000**
	22	-28.090	4.189									
Cd group	1	-18.93	4.37	.216	.623	.652	.677	.185	.140	**.039**	**.000**	.285
	4	-10.07	4.74		.059	.098	.121	**.003**	**.044**	**.003**	**.000**	**.014**
	6	-22.74	5.00			.978	.841	.389	.582	.084	**.003**	.657
	8	-23.02	8.10				.845	.536	.517	.130	**.000**	.624
	11	-21.28	4.74					.282	.361	.060	**.001**	.481
	13	-28.72	4.94						.965	.177	**.001**	.823
	15	-28.38	6.94							.188	**.004**	.841
	18	-46.53	10.86								.390	.101
	20	-56.72	7.15									**.000**
	22	-27.25	6.93									

- Bold values were significant (p-value<.05)

2. Supplementary material for chapter 3

Figure A.1 Mean of pigment cover in black and white sediment

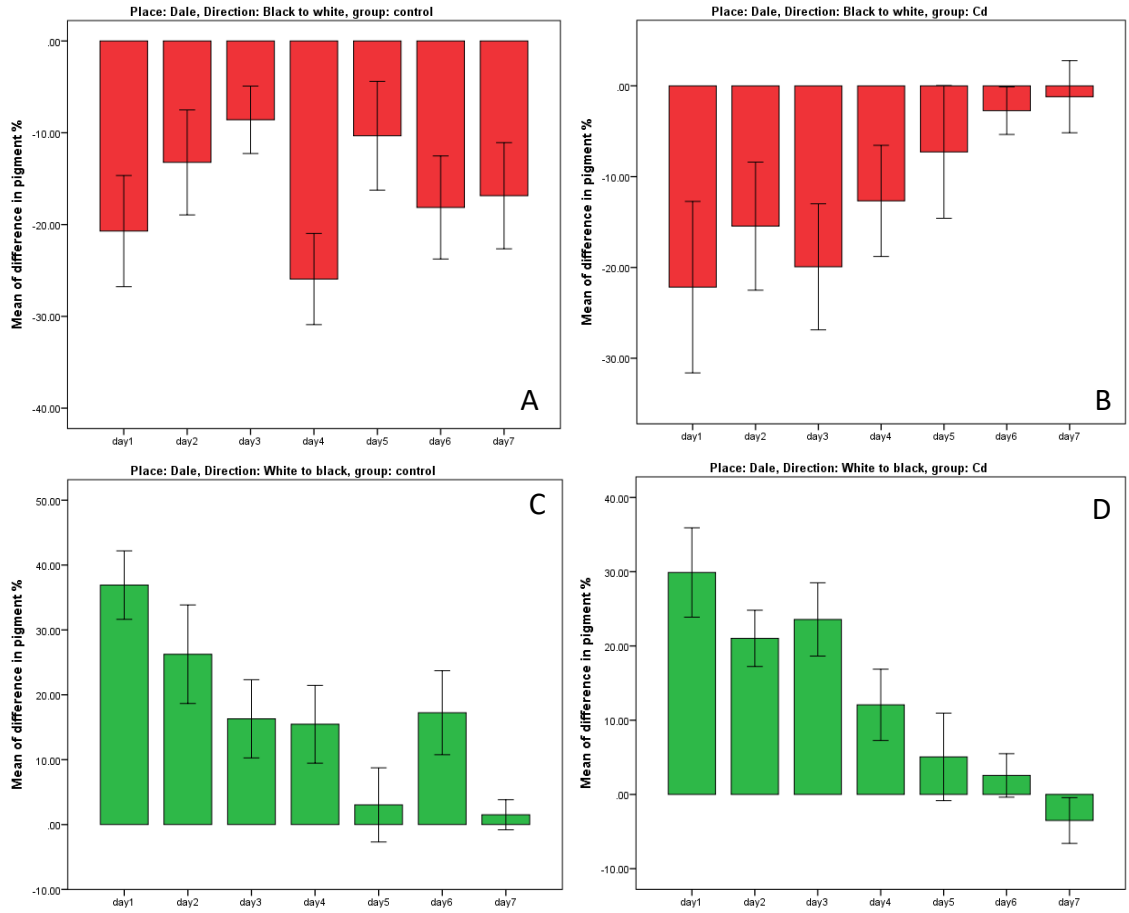

Figure A.2 Difference in pigment cover in black and white sediment between days in Dale in B to W direction (A= control, B= Cd) and W to B direction (C= control, D= Cd)

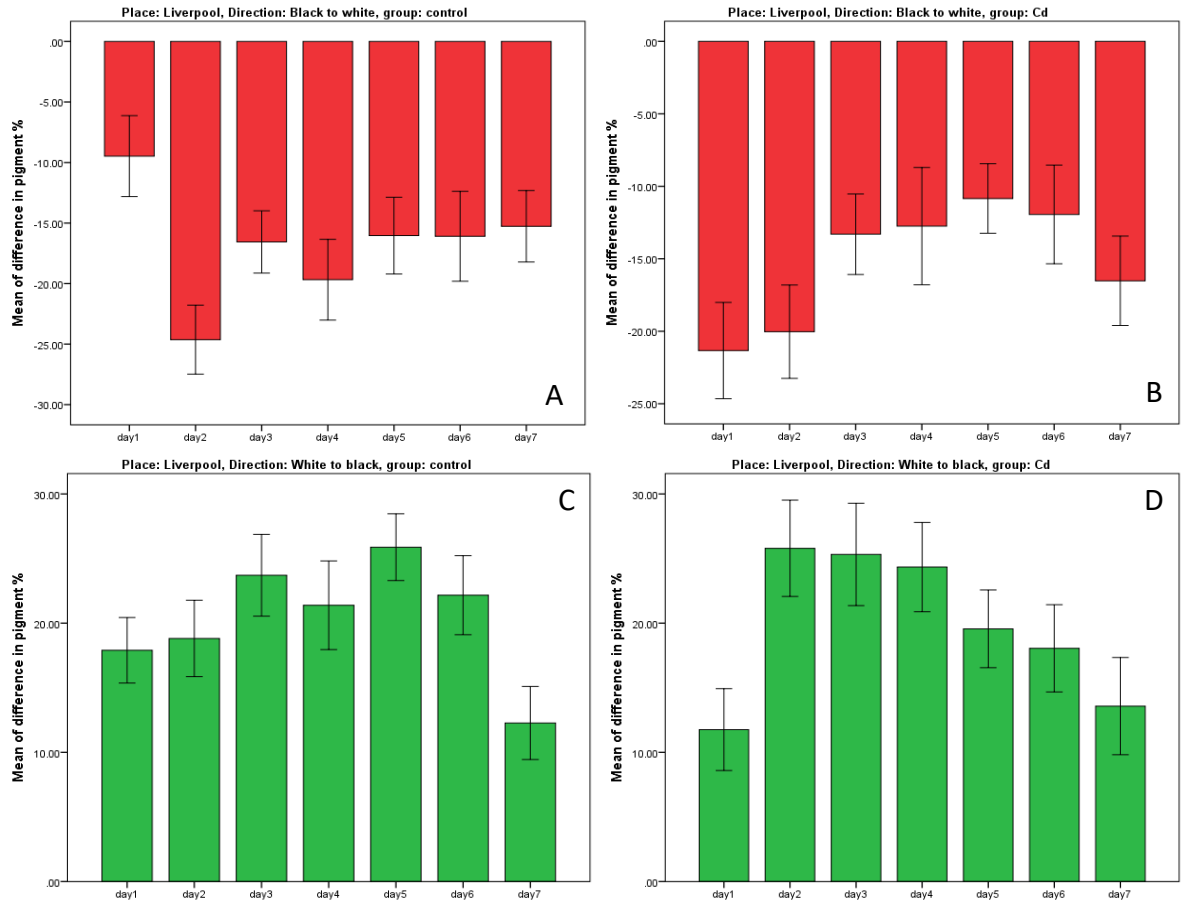

Figure A.3 Difference in pigment cover in black and white sediment between days in Liverpool in B to W direction (A= control, B= Cd) and W to B direction (C= control, D= Cd)

3. Supplementary material for chapter 4

Pairwise comparison showed that the significant changes happened between day 1 and day 6, 11, 15, 17 and 21 in B to W direction (Table A.6). Whereas in W to B direction, the difference in pigment cover in black and white sediment was between day 1 and all other days except for day 15 (Table A.6). It appears that the average of colour change in B to W direction at the last day of the experiment was positive while it was expected to be negative (mean difference: final-initial cover =2.983), suggesting that the shrimp got darker instead of becoming paler when they were moved to white sediment from the black sediment (). In addition to that, in W to B, the mean of difference in pigment was negative (mean=-12.8) in day 21, indicating that the shrimp got paler instead of becoming darker when they were moved from white to the black sediment.

Table A.6 Pairwise comparisons for difference in pigment cover in black or white sediment between days in control group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
B to W	1	-36.956	5.773	.076	**.025**	.045	**.000**	.162	**.005**	**.000**	**.002**
	4	-21.011	4.646		.901	.884	.148	.479	.116	.085	**.024**
	6	-19.972	5.584			.813	.220	.465	.368	**.029**	**.013**
	8	-21.910	4.762				.111	.321	**.037**	**.015**	**.027**
	11	-9.717	5.117					**.010**	.646	.566	.140
	13	-26.737	4.582						**.001**	**.001**	**.004**
	15	-12.082	3.303							.267	.078
	17	-6.470	3.542								.225
	21	2.983	5.809								
W to B	1	41.356	6.795	**.040**	**.004**	**.002**	**.001**	**.018**	.070	**.002**	**.005**
	4	13.788	6.533		.544	.489	.579	.397	.506	.416	.064
	6	9.177	2.779			.770	.970	**.022**	.239	.558	.057
	8	7.361	4.928				.675	**.020**	.108	.964	.050
	11	9.346	2.931					**.009**	.228	.645	.079
	13	22.893	3.918						.563	**.003**	**.009**
	15	17.686	6.091							.169	**.008**
	17	7.079	3.312								.099
	21	-12.800	10.029								

- Bold values were significant (p-value<.05)

In the 5 ppm As(V) group, in day 1 and day 4 the overall performance was better (-30, -28.4) compared to the other days in B to W (Table A.7). The significant difference was determined between day 1 and other days started from day 15 (p-value<.001). Day 17 was noticed to be significantly different from all other days with exception of day 13 and 21 (p-value<.05). Shrimp in day 17 and 21 performed less well in matching their background (mean= -2.5 and 1.4 respectively; Table A.7) compared to day 1. Colour change in day 21 was positive when it was supposed to be negative indicating that the shrimp became darker in white sediment. In W to B a significant difference was found between day 1 and other days started from15 (p-value=.002; Table A.7).

Table A.7 Pairwise comparisons for difference in pigment cover in black or white sediment between days in 5 ppm group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
B to W	1	-30.0	4.2	.796	.160	.154	.160	.097	**.000**	**.000**	**.000**
	4	-28.4	5.9		.266	.282	.127	.179	.128	**.000**	**.002**
	6	-19.4	5.1			.773	.916	.148	.534	**.006**	**.007**
	8	-21.5	3.4				.543	.374	.388	**.000**	**.002**
	11	-18.9	4.3					.445	.692	**.001**	**.010**
	13	-14.0	6.3						.790	.096	**.044**
	15	-16.0	3.6							**.009**	**.006**
	17	-2.5	1.4								.398
	21	1.4	3.9								
W to B	1	21.5	4.7	.185	.884	.521	.806	.969	**.002**	**.004**	**.003**
	4	30.9	4.2		.287	.062	.072	.209	**.002**	**.000**	**.000**
	6	22.7	6.0			.345	.560	.885	.063	**.010**	**.004**
	8	17.8	3.7				.670	.572	.131	**.004**	**.001**
	11	19.6	3.7					.629	.100	**.001**	**.002**
	13	21.9	5.7						.101	**.007**	**.013**
	15	9.4	3.6							.168	.151
	17	3.7	.8								.845
	21	3.3	1.9								

- Bold values were significant (p-value<.05)

In the 10 ppm As(V) group, the shrimp in B to W were matching their background better in day 1, 4, 6 and 8 (mean=-30.89, -24.85, -24.51 and -26.36 respectively) compared to the other days (Table A.8). However, the ability to change colour became significantly low in day 11, 15, 17 and 21 compared to day 1 (Table A.8).

For the shrimp in W to B, only day 21 showed a negative value (mean= -5.24), indicating that overall, in the other days the shrimp were getting paler in black sediment. The pairwise comparisons showed a significant difference between day 1 and day 13, 15, 17 and 21 (Table A.8). A significant difference was found between day 21 and all other days apart from day 15 (Table A.8): shrimp in day 21 were getting even more darker in white sediment than in black sediment (mean= -5.24).

Table A.8 Pairwise comparisons for difference in pigment cover in black or white sediment between days in 10 ppm group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
B to W	1	-30.89	5.28	.434	.453	.495	**.005**	**.021**	**.013**	**.009**	**.011**
	4	-24.85	4.46		.942	.776	.161	.095	**.018**	.070	**.005**
	6	-24.51	4.81			.737	.271	.249	**.006**	.066	**.001**
	8	-26.36	3.41				.077	.119	**.007**	**.002**	**.001**
	11	-16.33	3.17					.912	.158	.556	.094
	13	-16.81	3.35						.093	.548	.067
	15	-7.10	3.56							.086	.449
	17	-14.19	1.14								**.031**
	21	-4.99	3.56								
W to B	1	40.00	5.09	.179	.054	**.002**	.156	**.028**	**.000**	**.000**	**.000**
	4	31.90	5.78		.263	.160	.500	.093	**.004**	**.006**	**.001**
	6	20.58	6.76			.799	.423	.923	.056	.309	**.000**
	8	22.66	5.07				.476	.756	**.019**	**.040**	**.000**
	11	28.56	4.96					.166	**.002**	**.010**	**.001**
	13	19.58	5.85						**.019**	.330	**.009**
	15	3.19	2.90							**.017**	.063
	17	12.56	1.63								**.000**
	21	-5.24	3.11								

- Bold values were significant (p-value<.05)

In the 20 ppm group, colour change ability of shrimp in day 1 was significantly better compared to their performance in day 13 and day 21 in B to W (Table A.9). In day 21, the mean change in colour was very small (mean= -0.167), meaning that the brown shrimp was struggling to change colour from black to white.

With respect to W to B, there was no clear trend colour change between days. However, the difference in pigment cover in black and white sediment in day 21 was statistically less than day 1 (p-value<.002) indicating that the brown shrimp were performing less well (Table A.9). In day 13 for both directions, colour change ability in the shrimp was significantly lower than most other days (Table A.9).

Table A.9 Pairwise comparisons for difference in pigment cover in black or white sediment between days in 20 ppm group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
B to W	1	-28.769	5.618	.159	.234	.136	.201	**.000**	.126	.051	**.004**
	4	-19.587	4.609		.636	.990	.893	**.004**	.866	.131	**.009**
	6	-22.024	5.125			.701	.651	**.001**	.268	.079	**.006**
	8	-19.675	4.912				.924	**.010**	.876	.447	**.021**
	11	-19.008	2.819					**.013**	.954	.220	**.013**
	13	-5.770	2.620						**.016**	.107	.325
	15	-18.573	6.252							.428	**.039**
	17	-13.702	4.125								**.004**
	21	-.167	3.390								
W to B	1	23.069	6.781	.466	.070	.216	.920	**.006**	**.043**	.328	**.032**
	4	27.802	4.775		**.024**	.171	.271	**.001**	**.026**	**.002**	**.000**
	6	12.778	4.466			.771	.195	**.008**	.444	.661	.118
	8	14.799	6.564				.480	.086	.532	.969	.123
	11	22.176	4.071					**.008**	.145	.081	**.001**
	13	3.385	2.450						.081	**.019**	.755
	15	10.638	4.997							.485	.239
	17	15.15	2.976								**.000**
	21	1.902	2.559								

- Bold values were significant (p-value<.05)

In the control group and based on pairwise comparison given in Table A.10, dark pigment cover in black sediment was significantly different between day 1 and day 15, 17 and 21 (p-value= .033, .023 and .022 respectively). This indicted that the shrimp got less dark in day 15, 17 and 21. The difference in dark pigment cover in white sediment was found between day 1 (mean= 34.20) and day 17 and 21 (mean= 52.77 and 53.54 respectively; Table A.10). In white to black direction, the mean of dark pigment cover in white sediment was significantly less (p-value <.05) than any other day (Table A.11).

Table A.10 Pairwise comparisons of dark pigment cover in black and white sediment between days for black to white direction in control group

				p-values using post-hoc test for days							
Sediment colour	day	Mean	Std. Error	4	6	8	11	13	15	17	21
Black sediment	1	71.16	4.73	.101	.056	.125	.047	.479	**.033**	**.023**	**.022**
	4	65.28	2.03		.064	.256	.047	.889	**.044**	**.019**	**.011**
	6	51.60	5.53			.776	**.008**	.229	.198	.859	.776
	8	61.44	3.18				.077	.147	.850	.485	.024
	11	52.20	4.89					.004	.197	.177	.755
	13	65.92	3.73						.251	.114	**.002**
	15	60.73	2.56							.530	.045
	17	59.24	2.56								.097
	21	50.56	3.27								
White sediment	1	34.20	7.10	.326	.795	.593	.360	.586	.075	**.006**	**.030**
	4	44.27	4.01		.025	.289	.664	.283	.335	.205	.108
	6	31.63	5.44			.214	.151	.286	**.030**	**.010**	**.005**
	8	39.53	3.69				.510	.884	.045	.056	.072
	11	42.48	4.95					.499	.216	.161	.143
	13	39.18	2.75						**.003**	**.023**	**.034**
	15	48.64	1.55							.230	.270
	17	52.77	2.68								.844
	21	53.54	3.75								

- Bold values were significant (p-value<.05)

Table A.11 Pairwise comparisons of dark pigment cover in white sediment between days for white to black direction in control group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
White sediment	1	20.31	3.66	**.010**	**.017**	**.030**	**.039**	**.019**	**.029**	**.000**	**.000**
	4	47.48	5.27		.580	.322	.081	**.034**	.295	.767	.743
	6	45.44	6.51			.544	.292	.231	.552	.609	.544
	8	41.64	6.29				.415	.541	.869	.338	.415
	11	38.87	5.48					.913	.654	.199	.164
	13	38.33	3.30						.522	**.020**	**.034**
	15	41.04	5.86							.233	.229
	17	49.57	2.85								.981
	21	49.49	2.60								

- Bold values were significant (p-value<.05)

In terms of 5 ppm As(V) group, the difference between days was found in the dark pigment cover in white sediment between day 1 and day 15 and 17 in white to black direction (Table A.12).

Table A.12 Pairwise comparisons of dark pigment cover in white sediment between days for white to black direction in 5 ppm group

				p-values using post-hoc test for days							
Sediment colour	day	Mean	Std. Error	4	6	8	11	13	15	17	21
White sediment	1	38.63	5.38	.075	.045	.278	.257	.912	**.009**	**.119**	.149
	4	29.41	3.87		.192	.752	.707	.182	**.000**	**.000**	**.013**
	6	22.97	4.98			.065	.496	**.036**	**.001**	**.000**	**.015**
	8	30.73	4.65				.559	.333	**.009**	**.001**	**.035**
	11	27.26	7.17					.065	**.012**	**.003**	**.021**
	13	37.74	6.49						.065	.068	.078
	15	49.18	3.74							.897	.776
	17	48.64	1.70								.740
	21	51.89	10.16								

- Bold values were significant (p-value<.05)

Regarding 10 ppm group and as mentioned in Table 4.4, there was significant difference in dark pigment cover in white sediment in both directions. Pairwise comparison test showed

a significant increase in dark pigment cover in white sediment starting from day 13 of exposing to 10 ppm As(V) till day 21 in black to white direction and starting from day 15 in white to black direction (Table A.13). This indicated that the shrimp was struggling to contract the pigment when they were placed in white sediment.

Table A.13 Pairwise comparisons in dark pigment cover in white sediment between days for both directions in 10 ppm group

				p-values using post-hoc test for days							
Direction	day	Mean	Std. Error	4	6	8	11	13	15	17	21
black to white	1	25.78	3.50	.269	.142	.073	.070	**.002**	**.001**	**.004**	**.000**
	4	30.86	4.08		.482	.228	.360	.244	**.012**	**.037**	**.017**
	6	33.48	4.26			.491	.662	.581	.058	**.024**	**.021**
	8	36.21	4.15				.647	.944	.049	**.039**	**.030**
	11	34.52	3.67					.642	**.021**	**.026**	**.013**
	13	36.58	4.09						**.044**	.181	**.019**
	15	44.98	3.22							.891	.277
	17	44.35	3.19								.112
	21	49.86	2.93								
white to black	1	25.25	5.23	.998	.181	**.033**	.599	.430	**.006**	**.002**	**.003**
	4	25.26	5.17		.122	.062	.468	.365	**.009**	**.004**	**.004**
	6	34.27	4.55			.311	.455	.782	.071	**.002**	**.009**
	8	38.48	5.00				.196	.432	.109	**.007**	**.029**
	11	29.81	5.76					.722	**.009**	**.008**	**.002**
	13	32.22	7.03						.046	**.043**	**.018**
	15	47.86	3.86							.229	.153
	17	56.70	5.12								.470
	21	52.67	2.83								

- Bold values were significant (p-value<.05)

In white to black direction in 20 ppm group, there was a slight decrease in dark pigment cover in black sediment in day 21 compared to day 1 (p-value=.001; Table A.14).

Table A.14 Pairwise comparisons of dark pigment cover in black sediment for white to black direction between days in 20 ppm group

				p-values using post-hoc test for days							
Sediment colour	day	Mean	Std. Error	4	6	8	11	13	15	17	21
Black sediment	1	58.88	3.81	.836	**.029**	.135	.617	.600	.347	.807	**.001**
	4	57.95	4.23		**.031**	.409	.528	.650	.674	.962	**.010**
	6	44.92	4.91			.197	**.010**	.067	**.040**	**.003**	.197
	8	52.72	3.79				.076	.772	.530	.193	**.012**
	11	62.69	5.47					.051	.323	.302	**.002**
	13	54.49	5.88						.913	.452	.070
	15	55.25	4.81							.527	**.021**
	17	57.73	2.88								**.000**
	21	43.68	1.03								

- Bold values were significant (p-value<.05)

University of Salford MANCHESTER

Effects of cadmium and antibiotics on food intake and colour change in the brown shrimp (*Crangon crangon*)

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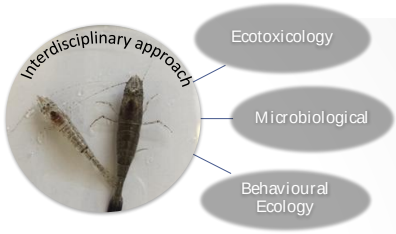
Introduction

Heavy metal contamination due to human activity is affecting terrestrial and aquatic environments. In addition, the increase of incorrect use of antibiotics (clinical and agricultural overuse) may lead to the spread of drug resistant bacteria. The effects of heavy metals on marine organisms have been investigated but little is known about the combined effects of heavy metals and antibiotics.

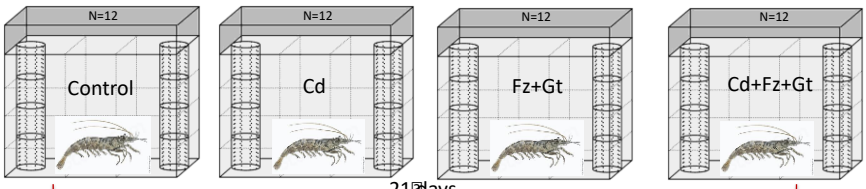
Aim:

- Evaluate the effects of cadmium and the antibiotics (furazolidone (Fz) and gentamicin (Gt)) on physiological and behavioural responses (food intake and colour change) in *C. crangon*.



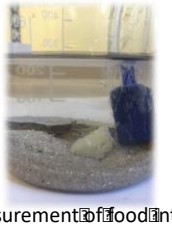
Methodology

The choice of the antibiotics was made using the diffusion assay technique with the gut extract from the shrimp.



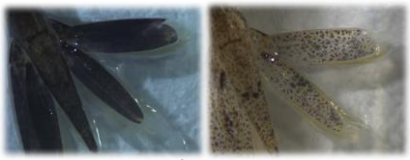
21 days

Every other day



Measurement of food intake

+



Assessment of colour change using Siegenthaler et al. protocol

Acknowledgments:

AA's PhD project is funded by Saudi Arabian Cultural Bureau. Authors are thankful to Paul Oldfield (from Mersey gateway) for his continuous support providing access for sample collection. Special thanks goes to Andrea Lull Tatay and Andjin Siegenthaler for their help.

Figure A.4 SPARC poster

Presented by:
Asma Althomali
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University of Salford MANCHESTER

Supervisors:
Dr Debapriya Mondal
Dr Chiara Benvenuto

Effects of cadmium and antibiotics on food intake and colour change in the brown shrimp (*Crangon crangon*)

Introduction

Crangon crangon is a key component in the aquatic ecosystem and one of the most important commercial species in many countries, especially in the UK[1]. The brown shrimp inhabits estuaries and shallow waters, which are often subject to environmental or anthropogenic pollution[2]. Moreover, the increase of incorrect use of antibiotics (clinical and agricultural overuse) may lead to the spread of drug resistant bacteria. Hence, it is important to know the impact of heavy metals and antibiotics on the brown shrimp and in particular their combined effects, which have not been fully investigated yet.

Aim of study

The aim of this research is to understand the effects of cadmium (Cd) and the antibiotics Furazolidone (FZD) and Gentamicin (GT) on feeding rate and colour change in the brown shrimp.

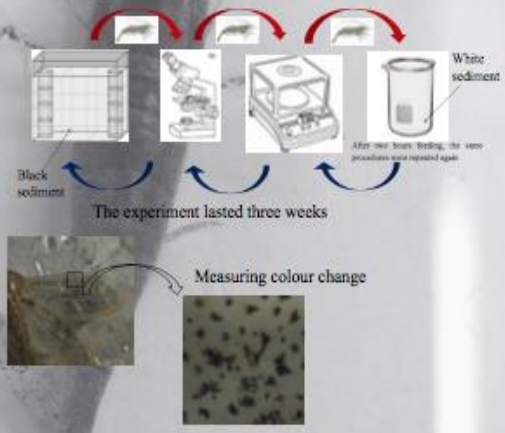
Methodology

Crangon crangon specimens were collected from Dale (South West Pembrokeshire, UK).

The samples were divided into four groups:

- Control: clean artificial water (n = 8)
- Cadmium: 0.04 ppm of Cd^{3} (n = 8)
- Antibiotics: 10 ppm of FZD + 2 ppm of $GT^{4,5}$ (n = 8)
- Antibiotics + cadmium: 10 ppm of FZD + 2 ppm of GT + 0.04 ppm of Cd (n = 8)

Design



Results and discussion

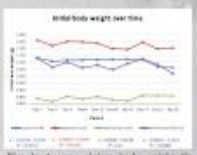


Fig. 1: Average shrimp body weight (in gram) before feeding

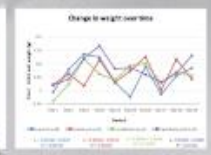


Fig. 2: Average change in shrimp (in gram) before and after feeding

Fig. 3: Average colour change (measured by the percentage of chromatophores in black sediment – percentage of chromatophores in white sediment)

Fig. 4: Average chromatophore coverage in white sediment (all treatments)

With regard to body weight, there was a slight trend of increase in body weight in the antibiotics group and this could be due to the effect of antibiotics on the microbial community in the gut. Whereas, the average weight in the other three groups slightly decreased over time (Fig. 1), but no trend was observed when considering the **change in weight** after each feeding time (Fig. 2).

In terms of colour change, there is an indication that shrimp in the Cd group, when moved from black to white sediment failed to get paler (Fig. 3, 4). Similar results have been reported for fiddler crabs[6].

Conclusion

I have looked at the responses of *C. crangon* to contaminants and antibiotics from two different angles: body weight and colour change. Body weight changes were minor but my results show that Cd might affect the ability of theses shrimp to match the colour of their background. Colour change thus could be a promising biomarker of contamination for this species.

Acknowledgment

AA's PhD project is funded by Saudi Arabian Cultural Bureau. Special thanks goes to Paul Oldfield (Mersey Gateway) for his continuous support on the project, Dr Chloe James, Héctor Abarca Velencoso, Andrea Lull Tatay and Andjin Siegenthaler for their help.

References

[1]Temming, A. & Damm, U. (2002). *Fish. Oceanogr., 11*(1), 45-58.
[2]Buccolieri, A., Buccolieri, G., Cardellicchio, et al., (2006). *Mar. Chem.*, 99(1), 227-235.
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[4]Guo, J. J., Chou, H. N., & Chiu Liao, I. (2003). *Food Addit. Contam.*, 20(3), 229-236.
[5]Manjusha, S., & Sarita, G. B. (2013). *Int. Food Res.*, 20(1), 77-86.
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Figure A.5 SETAC poster

Changing colour in a polluted environment

Asma Althomali, Chiara Benvenuto and Debapriya Mondal

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Soo_maa@hotmail.com

Introduction

The brown shrimp, *Crangon crangon,* utilizes chromatophores (specialized cells containing pigments) to match its background, changing colour either from pale (pigments contracted) to dark (pigments expanded) or *vice versa* to become almost invisible in the environment.

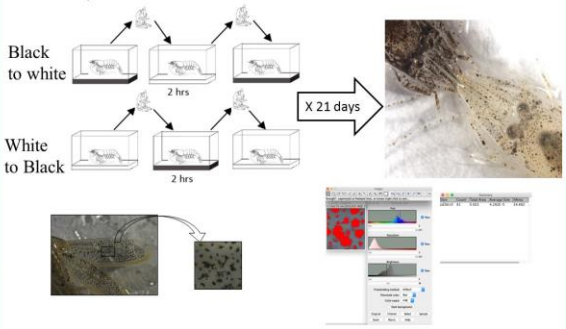
The use of colour change as a behavioural marker of pollution can become an effective tool to assess the initial stages of biological alteration in aquatic organisms. Some marine species have been used to test the impact of external stressors, such as contaminants, on colour change[1,2], but not the brown shrimp. Thus, the aim of this project was to investigate the effect of cadmium (Cd) and arsenic (As) on the colour change ability of this crustacean.

The brown shrimp and its distribution

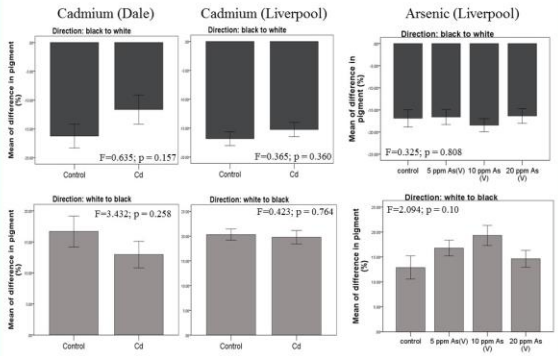
Sampling

Methods

Treatments (0.04 ppm Cd, 5, 10 and 20 ppm As) and control group were kept in the lab, testing animals in two different directions (from black to white and from white to black sediment)



Results



Contrary to expectations, Cd and As did not affect the colour change ability of *C. crangon* (at least not for the doses used). The shrimp continued to match their substrate, showing high inter- and intra-individual variability, thus suggesting that behavioural traits might be challenging to be used as biomarker of toxicity in the environment. Also, lab analyses showed a decrease of inorganic As with time while the less toxic organic form increased, suggesting the possibility of bio-transformation of inorganic to organic As in this shrimp.

Acknowledgments

This project was funded by the Ministry of Higher Education in Saudi Arabia. We are grateful to Hector, Andjin, Michelle, Chinedu, Rachael, and Chayan for their help in the field and lab.

References

[1]Ahmad et al. (2018). Effect of cadmium chloride on general body colouration and chromatophores of stinging cat fish, *Heteropneustes fossilis* (Bloch). *Journal of Applied and Natural Science, 10*(2): 655–660.

[2]Akarte S.R. & Agnihotri U.S. (2013). Chromatophores as an bioindicator for detection of arsenic trioxide in fresh water fish *Channa punctatus*. *International Journal of Pharma and Bio Sciences, 4*(1): 881–888.

Figure A.6 European Society of Evolutionary Biology poster

Behavioural ecotoxicology of colour change

Asma Althomali, Debapriya Mondal and Chiara Benvenuto

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Soo_maa@hotmail.com

University of Salford MANCHESTER

Introduction

The brown shrimp, *Crangon crangon* utilizes chromatophores (specialized cells containing pigments) to match its background, changing its colour either from pale (pigments contracted) to dark (pigments expanded) or *vice versa* to become almost invisible in its environment.

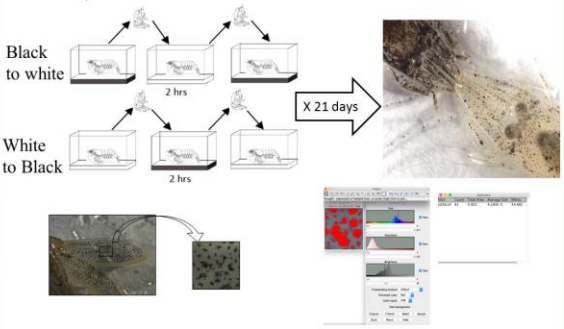
The use of colour change as a behavioural marker of pollution can become an effective tool to assess the initial stages of biological alteration in aquatic organisms. External stressors such as contaminants have been used to investigate their impact on colour change ability of some marine organisms[1,2], however, no attempt has been done with the brown shrimp. Thus, the aim of this project was to investigate the effect of cadmium (Cd) and arsenic (As) on the colour change ability of the brown shrimp.

The brown shrimp and its distribution

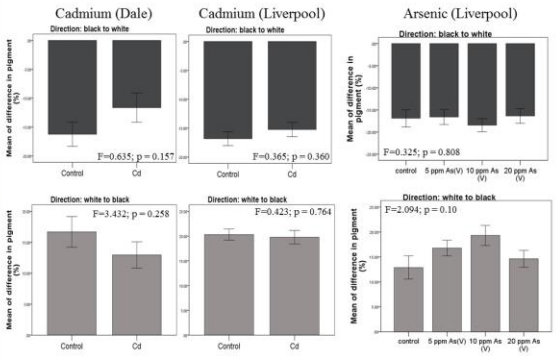
Sampling

Methods

Treatments (0.04 ppm Cd, 5, 10 and 20 ppm As) and control group were kept in the lab, testing animals in two different directions (from black to white and from white to black sediment)



Results



Contrary to expectations, Cd and As did not affect the colour change ability of *C. crangon* (at least not for the doses used). The shrimp continued to match their substrate, showing high inter- and intra-individual variability, thus suggesting that behavioural traits might be challenging to be used as biomarker of toxicity in the environment. Also, lab analyses showed a decrease of inorganic As with time, as the less toxic organic form increased, suggesting the possibility of bio-transformation of inorganic to organic As in this shrimp.

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[2]Akarte S.R. & Agnihotri U.S. (2013). Chromatophores as an bioindicator for detection of arsenic trioxide in fresh water fish *Channa punctatus*. *International Journal of Pharma and Bio Sciences*, *4*(1): 881–888.

Figure A.7 Association for the Study of Animal Behaviour poster

University of Salford MANCHESTER

Research, Innovation and Academic Engagement Ethical Approval Panel

Research Centres Support Team
G0.3 Joule House
University of Salford
M5 4WT

T +44(0)161 295 5278

www.salford.ac.uk/

10 May 2017

Asma Althomali

Dear Asma,

RE: ETHICS APPLICATION ST1617-64 - Effects of contaminants and antibiotics on behavioural and physiological responses in the brown shrimp (*Crangon crangon*)

Based on the information you provided, I am pleased to inform you that your application ST1617-64 has been approved.

If there are any changes to the project and/ or its methodology, please inform the Panel as soon as possible by contacting S&T-ResearchEthics@salford.ac.uk

Yours sincerely,

APHigham.

Dr Anthony Higham
Chair of the Science & Technology Research Ethics Panel

Figure A.8 Ethical approval