Cadmium in Brown Crab Cancer pagurus in Norwegian Waters

Martin Wiech

Avhandling for graden philosophiae doctor (ph.d.)
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Scientific environment

The work for this doctoral thesis was accomplished in the research group Contaminants and Biohazards at the National Institute of Nutrition and Seafood Research (NIFES), which was merged with the Institute of Marine Research, Norway Jan 1st, 2018, and in cooperation with the Department of Biology, University of Bergen. The PhD position was funded by the NIFES/Institute of Marine Research and the work has been supported by monitoring projects funded by the Norwegian Food Safety Authority.

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"Honor to whom honor is due." Romans 13,7

Abstract

The Brown crab *Cancer pagurus* is appreciated as seafood and its fishery is of importance in several European countries. However, findings of high levels of cadmium have increased concern about food safety, and spatial patterns of cadmium levels have been found. Along the Norwegian coast, a sudden spatial increase in cadmium levels in brown crab from Salten region in Northern Norway (ca. 67°N) and northwards has been identified. An earlier study including sediment measurements investigated the reason for the high values in the North, and concluded that it is unlikely that an anthropogenic point source is responsible for the high cadmium levels.

The main aim of this thesis was to investigate further factors that may explain the large variation of cadmium in brown crab, and whether these factors can explain the differences in cadmium levels in brown crab along the Norwegian coast, with focus on physiological factors.

Conflicting values of reported cadmium levels in crab claw meat led us to evaluate the pretreatment of crabs before analyses. A strong effect of cooking and freezing was found, causing a leakage of cadmium from hepatopancreas to claw meat.

The findings in crabs sampled in the North and the South of the Norwegian coast during one year, revealed that the influence of physiological factors on cadmium levels is not very pronounced in comparison to the large differences between crabs from the North and the South. However, there was a correlation between size and cadmium levels in crabs sampled in the North, indicating an accumulation of cadmium over time. As brown crabs are assumed to grow more slowly in the North, this indicates that some of the variation in cadmium between the North and the South can be explained by growth rate. Further evidence for a high potential of brown crab to accumulate cadmium has been found in a lab trial, where cadmium from food and water was traced in brown crab to compare the relative importance of the uptake routes. No depuration of cadmium was observed, indicating a high accumulation potential. Furthermore, the dietary uptake was predicted to contribute at least 98 % to the overall cadmium accumulation in brown crab in Northern Norway. This indicates that foraging and related behavior plays an important role in determining the cadmium levels in crab. As

we found an indication for different foraging patterns in crabs from North and South, this might partly explain the north-south variation.

The field study did not reveal a clear pattern in cadmium levels in brown crab when considering sex, moulting stage, gonad maturation stage, or season, making it difficult to develop mitigation strategies for the crab fishery in the North.

Another aim of this thesis was to assess the risk of exceeding the limit of safe exposure to cadmium by the consumption of brown crab considering different consumption patterns in the Norwegian population. According to our measured cadmium level in cooked crabs from the field study, it is safe to consume crab claw meat regardless of the origin of the crab. The consumption of whole crabs including brown meat in the coastal population, however, was calculated to lead to an intake of cadmium above the tolerable weekly intake. In general, brown meat should be consumed parsimoniously and a legal maximum limit for cadmium in brown meat and mixtures of brown meat and white meat should be considered.

List of publications

Paper 1

Wiech, M., Vik, E., Duinker, A., Frantzen, S., Bakke, S., & Maage, A. (2017). Effects of cooking and freezing practices on the distribution of cadmium in different tissues of the brown crab (*Cancer pagurus*). *Food Control, 75*, 14-20

Paper 2

Wiech, M., Amlund, H., Jensen, K. A., Aldenberg, T., Duinker, A. & Maage, A.. Tracing Simultaneous Cadmium Accumulation from Different Uptake Routes in Brown Crab *Cancer pagurus* by the use of Stable Isotopes. (Submitted to "Aquatic toxicology", Elsevier)

Paper 3

Wiech, M., Frantzen, S., Duinker, A., Rasinger, J. & Maage, A.. Cadmium in Brown Crab *Cancer pagurus* in Norwegian Waters. An Assessment of the Influence of Area, Season, Treatment and Different Physiological Factors and Consequences for Food Safety.

Paper 4

Knutsen, H., Wiech, M., Duinker, & Maage, A.. Cadmium in the shore crab Carcinus maenas along the Norwegian Coast: geographical and seasonal variation and correlation to physiological parameters. (Re-submitted to "Environmental Monitoring and Assessment", Springer)

Contents

Introduction

Brown crab fishery in Norway

The brown crab *Cancer pagurus* is a popular food item in Europe, including Norway and many southern European countries such as France, Spain and Portugal. In general, the importance of crustaceans for fisheries is steadily increasing in accordance with the declining trophic levels in fisheries catches (Molfese, et al., 2014). The brown crab is mainly distributed in the North-East Atlantic and the total European catch was about 42.5 thousand metric tons in 2016. Most of the volume was caught in the United Kingdom (29 500 t), followed by Ireland (7 700 t), Norway (4 900 t) and France (4 200 t), with a total value of 74 million € (EuroStat, 2017). In Norway, which is the northernmost fishery, the brown crab is expanding its distribution northwards and was recently observed at least as far north as 69°44'N (Bakke, et al., 2016). While this fishery still is in an experimental stage and needs further adaptations to be profitable, there is an active commercial fishery at about 68°N in Vesterålen (Bakke, et al., 2016). The main Norwegian brown crab fishery, however, is located in Mid-Norway and Helgeland (63° - 67°N), where 75% of the Norwegian catch is landed (Søvik, et al., 2017; Woll, et al., 2006a). Traditionally, the Norwegian fishery targets mature individuals and peaks from August to October. The highest activity is within 12 nautical miles from land and baited pots are used as gear. The only effort control for the fisheries is an established minimum landing size of 11 cm carapace width for crabs caught from the Swedish border to 59˚30' N and 13 cm carapace width further north (Søvik, et al., 2017).

There is a distinct external sexual dimorphism in brown crab. Female crabs are characterized by broad abdomens with allometric growth from the onset of maturation (Öndes, et al., 2017). The abdomen is provided with four pairs of pleopods. The top of the female carapace is arched compared to males, which have a rather flat carapace. The slim abdomen of the males is only provided with one pair of pleopods morphed to mating organs. In males, claws are generally bigger and grow allometrically with maturation (Tallack, 2007; Öndes, et al., 2017). The brown crab has planktonic larvae that are pelagic for around 60 days at 15-20°C (Nichols, et al., 1982), and field surveys

indicate a larval planktonic phase of 2–3 months (Eaton, et al., 2003). Adults are benthic and mobile. As part of the growth process, crabs periodically shed their worn exoskeleton, replacing it with a new one. Due to this process, the animal periodically undergoes drastic changes in metabolism, and is also able to reproduce lost limbs (Warner, 1977).

Figure 1 Alive male brown crab as offered on the Norwegian market.
Photo: Kai Triebner

It has been shown that crabs have the potential to migrate over long distances with females moving further, most probably for reproduction purposes (Bennett & Brown, 1983; Hunter, et al., 2013; Ungfors, et al., 2007). In fact, females may compensate for larval dispersion in the present current by migration (Ungfors, et al., 2007). However, the lack of genetic variation in brown crab in the Kattegat Skagerrak area, and the absence of a clear population structure in Europe (Pan, M., personal communication, Feb 2018) does not support a hypothesis of compensatory counter-current adult migration (Ungfors, et al., 2009). The lack of genetic structure rather indicates a high degree of genetic mixing over a large area caused by adult or larval movement (McKeown, et al., 2017; Ungfors, et al., 2009). Along the Norwegian coast, a capture-recapture approach indicated less migratory activity in a fjord system in Mid-Norway (Woll, 1995). Recently, brown crabs have been observed down to a depth of 400 m in Norway (Bakke, S., personal communication, Feb 2018) and compared to the rather shallow fishery in Norway, is for example the French fishery regularly targeting brown

crabs in deeper waters (Le Foll, 1982).

The brown crab is assumed to be an opportunistic feeder. However, analysis of stomach contents is difficult as prey items are ground in the gastric mill when entering the stomach. The identification of prey items is therefore difficult and prone to a bias towards animals with parts that are hard to grind and digest (Woll, 1995). Nevertheless, the most frequently detected food items were blue mussels (M*ytilus edulis*) and horse mussels (*Modiolus modiolus*). A difference in ingested prey was found between crabs from different habitats, mirroring the abundance of the prey items (Woll, 1995) and different sized prey items are attacked (Lawton & Hughes, 1985) indicating opportunistic feeding.

Brown crab as seafood

The consumption of seafood has been implemented into the recommendations of a healthy diet in several European countries (NDA, 2014), as seafood is considered to have several beneficial health effects. Associations between increased consumption of seafood and reduced risk of developing coronary heart disease, high blood pressure, stroke, some forms of cancers, rheumatoid arthritis and other inflammatory diseases have been found (Lund, 2013).

Crab white meat from claws and legs is rich of proteins, essential amino acids and elements paired with a low cholesterol and fat content, implicating a well-balanced nutritious food (Barrento, et al., 2009b; Barrento, et al., 2009c; Maulvault, et al., 2012). The most frequent and traditional way to prepare brown crabs in private homes in Portugal is boiling crabs whole (Maulvault, et al., 2013), which also applies to other European countries, including Norway. Muscle meat (mainly from claws and legs) and brown meat (mainly hepatopancreas and gonads) are either consumed separately or as a mixture. In Norway, whole crabs are consumed more frequently than claws only (Bergsten, 2004) and in Portugal, 99.6 % of crab consumers also consume brown meat (Maulvault, et al., 2013). Crabs are mostly sold alive (Figure 1). However, recently, the availability and popularity of ready-to-eat crab products have been increasing in Norway.

During the last years, several studies have risen concern after findings of high values of the toxic element cadmium detected in crabs harvested in Norway, (Frantzen, et al.,

2011; Julshamn, et al., 2013c; Julshamn, et al., 2012; Vik, 2014), Scotland (Barrento, et al., 2009b; Davies, et al., 1981; Falconer, et al., 1986; Maulvault, et al., 2012) and the English channel (Barrento, et al., 2009a).

Cadmium

Cadmium is an element that occurs naturally and is relatively rare, constituting only 0.1 ppm of earth's crust (Wedepohl, 1995). The sources of release into nature are either natural or anthropogenic. Natural release includes mobilization of cadmium during events such as volcanic activity, forest fires or weathering of rocks. The production of metals, fossil fuel combustion and waste incineration are amongst the most important anthropogenic emission sources (UNEP, 2010), also to the aquatic environment. Different estimates of the natural release (Nriagu & Pacyna, 1988; Richardson, et al., 2001; Sigel, et al., 2013) make it difficult to assess the anthropogenic contribution to the overall release. However, emissions of up to 17,000 tons were estimated in 1983 (Nriagu, et al., 1988). Cadmium is found in surface and ground water with concentrations in fresh and saltwater between 0.01 and 0.1 µg/L (Simpson, 1981). In Norway, as in many other European countries, the emission of cadmium has decreased significantly the last 20 years (Miljødirektoratet, 2014). Cadmium is mainly obtained as byproduct in zinc production and its production is therefore dependent on zinc extraction. Nevertheless, the production and use of cadmium almost doubled between 1950 and 1990. Since then, the global production has levelled off. It is used in various products, with batteries representing the major application (UNEP, 2010).

Health effects of cadmium

The potential health hazard of cadmium has long been known. The hazard was originally identified with the occurrence of the Itai-Itai disease in 1955 in Japan, caused by cadmium-polluted rice (Hagino & Kono, 1961). Whilst in this case, the reason was heavy industrial pollution, adverse health effects have also been observed after exposure to rather low doses. The most prominent effects of Cd in the human body is nephrotoxicity and osteotoxicity. Cadmium causes tubular damage, which may lead to complete renal failure (Rani, et al., 2014). After entering the bloodstream, Cd is initially transported to the liver and taken up by hepatocytes, where most of it will be

bound to metallothionein (MT), preventing toxic effects. However, Cd-MT will at least partly be released into the blood stream, when hepatocytes die off and are filtered at the renal glomerulus. From there it is taken up in epithelial cells of the proximal tubule causing damage. Cadmium is efficiently retained in the human kidney with a half-time of 10–30 years (Rani, et al., 2014). Despite that, there is evidence that tubular damage to a certain degree is reversible (EFSA, 2009a).

The osteotoxcity of cadmium is caused by a direct and an indirect mechanism (Rani, et al., 2014). It can stimulate bone resorption and inhibit bone formation by directly acting on osteoclasts or osteoblasts. Further, the renal and gastro-intestinal dysfunction caused by cadmium, can lead to bone damage by potentially hindering the uptake of necessary nutrients. In addition to these effects, cadmium is classified as a human carcinogen and induces some effects typical for endocrine disruptors (Järup & Åkesson, 2009; Satarug, et al., 2010). There is evidence that cadmium exposure leads to genomic instability. The mechanisms are complex and multifactorial. However, an interaction with the DNA-repair mechanism, generation of reactive oxygen species and induction of apoptosis may be most important, indicating a co-genotoxic effect (Rani, et al., 2014).

Human exposure to cadmium

For non-smokers in the general population, diet accounts for approximately 90 % of the total cadmium exposure (EFSA, 2009a; Järup, et al., 2009). Cadmium is abundant in a vast variety of food items and the content varies largely, depending on the environmental contamination and type of food. High levels can be found in offal products, especially from old animals, oil-seeds, cocoa-based products, some wild mushrooms, water mollusks and crustaceans. Generally, animal products like meat, egg, milk and dairy products, contain less cadmium than food from plants (Järup, et al., 2009) and also fish muscle is low in cadmium (Frantzen & Maage, 2016; Julshamn, et al., 2004). Amongst plant based food, basic products like rice, wheat, potatoes, green leafy vegetables and root vegetables show the highest cadmium levels (Järup, et al., 2009). High cadmium levels are particularly caused by the use of phosphate rock for agricultural purposes, containing significant levels of cadmium (Thévenod & Lee, 2013). Food that is consumed in large quantities contribute most to the overall

cadmium exposure. On average, cereals, vegetables and potatoes constitute 80 % of the cadmium intake from food (EFSA, 2012; Järup, et al., 2009). However, some food items, although not being consumed very frequently, may be problematic as they hold high levels of cadmium, such as crustaceans (Järup, et al., 2009). Cadmium is dangerous because of its ubiquity and the chronic long term exposure paired with a long biological half-life exceeding 20-30 years. This means that exposure in childhood actually may affect health in old age (Thévenod, et al., 2013). Already in 1988 the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) for cadmium of 7 µg/kg body weight. After an extensive review a provisional tolerable monthly intake of 25 µg/kg body weight corresponding to a weekly intake of 5.8 µg/kg body weight was established. The European food safety authority (EFSA), however nominated a tolerable weekly intake (TWI) of 2.5 µg/kg body weight (EFSA, 2012).

The European Union applied a maximum level for cadmium in crustaceans excluding brown meat of crab and head and thorax meat of lobster and similar large crustaceans of 0.5 mg/kg ww in the No 1881/2006 (unconsolidated version) (EU, 2006). For crabs and crab-like crustaceans it was specified that the maximum level only applies to muscle meat of the appendages, i.e. claw and leg meat (EU, 2011).

Findings of high cadmium levels in brown crab in Norway

In 2009, Swedish authorities detected high levels of cadmium in muscle meat of brown crab caught in the North of Norway (north of Salten region, Nordland), exceeding the maximum legal limit of 0.5 mg/kg ww (Jensen & Wasmuth, 2010). Accordingly, the National Institute of Nutrition and Seafood Research (NIFES) conducted several investigations on crab in this area, funded by the Norwegian Food Safety Authority. The results revealed a distinct pattern with much higher levels of cadmium in brown crab caught north of 67°19' N (Figure 2) (Frantzen, et al., 2011; Julshamn, et al., 2013c; Julshamn, et al., 2012). Consequently, the Norwegian Food Safety Authority gave advice not to eat crabs caught in Salten and northwards. Fishermen in the area had to

stop commercial crab fishing and also the industry processing crab meat, suffered substantial economic losses.
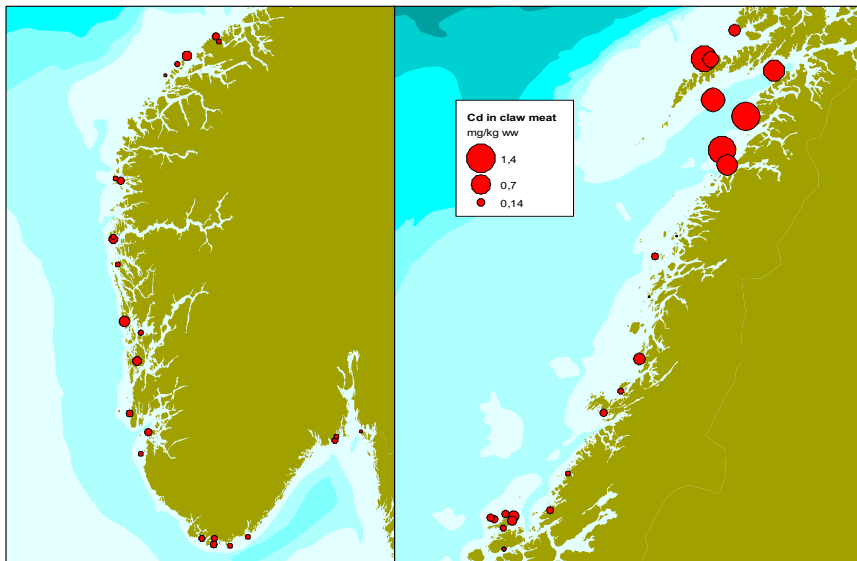

Figure 2 Map of Norway showing concentrations of cadmium (mg/kg wet weight) in claw meat of brown crab captured at different positions along the coast in 2011. Circle sizes indicate the mean concentration for each position as shown in the legend. From Julshamn et al. (2013b).

Food safety considerations

The high values of Cd found in claw meat displayed an issue for fisheries, as exceedance of the legal limit had economic consequences. However, brown meat with much higher cadmium levels, is consumed as well, making it the main food safety issue. This was already identified by others for crabs from UK and Scotland (Barrento, et al., 2009a; Maulvault, et al., 2012). A risk-benefit assessment, addressing the consumption of brown crab in Portugal, considered the risk of the intake of methyl mercury and Cd versus the potential benefits from selenium and EPA + DHA when consuming the different tissues of cooked crab. Combining the consumption frequencies in the Portuguese population and the concentrations found in brown crab, they concluded that muscle meat can be included in a well-balanced diet, while the brown meat should only be consumed parsimoniously (Maulvault, et al., 2013).

No risk assessment considering the Norwegian consumption, was published before June 2015. The Panel on Contaminants of the Norwegian Scientific Committee for

Food safety (VKM) assessed the risk of dietary cadmium exposure in the Norwegian population with special focus on high cadmium food, including brown crab meat (VKM, 2015). To assess the risk, consumption data for the Norwegian population was combined with cadmium levels measured in an earlier study (Julshamn, et al., 2012). They concluded, that the consumption of crab brown meat is of concern, as high consumers are at high risk of exceeding the TWI (VKM, 2015) while the consumption of muscle meat does not pose a risk in the Norwegian population. However, they only considered cadmium levels of crabs being frozen before cooking, which might influence the results. Scenarios were only based on cadmium levels for crabs from the South.

Previous research addressing the high cadmium levels in the North
The North-East Atlantic Ocean has been considered a rather pristine area and the finding of a contaminant gradient with increasing values in the North got much attention, both in public and research. Several studies were conducted to investigate mainly the sources of the Cd found in crabs in the North. Both natural and anthropogenic sources were suggested.

The run-off from bedrock is considered a natural source of cadmium and it was investigated by analyzing naturally occurring cadmium in bedrock, ground and surface water. No differences in cadmium run-off were found between areas with high or low levels of cadmium in crab (Finne, 2013). In an attempt to identify anthropogenic contamination, sediment samples from the region with high concentrations in crab were analyzed. Only low levels of cadmium were found, and it was concluded that local sources of cadmium pollution are very unlikely to cause the high values in crabs (Falk, 2012). To investigate, whether the high values of cadmium in crab were caused by high concentrations of cadmium in seawater, a study was conducted using blue mussels *Mytilus edulis* as indicator for cadmium levels in seawater (Foldøy Tverdal, 2012). Cadmium levels in blue mussels from the Salten area were low, and no correlation was found between levels in blues mussels and brown crab in the affected region. It was concluded that the high values of cadmium in crabs in the North are probably not directly caused by high values of cadmium in the seawater. Also, a study of fish from the Salten area showed no elevated Cd levels in fillet or liver of tusk *Brosme brosme*,

Atlantic halibut *Hippoglossus hippoglossus* or redfish *Sebastes marinus* (Julshamn, et al., 2013a).

Falk (2014) excluded fish feed from fish farms as the main source for cadmium after having analyzed cadmium in crabs, sediment, blue mussels, polychaetes and seawater around three fish farms, without finding any significantly increased Cd levels.

Another study focused the cadmium concentration of macrofauna amongst different taxonomical groups and trophic levels in the area with high levels of cadmium in brown crab. No clear relationship between the level of cadmium in prey organisms and brown crab itself was found. However, several potential prey organisms were identified with high levels of cadmium (Ness, 2014).

A literature study suggested an emphasized upwelling of deep-sea water rich in nutrients and cadmium, being the reason for the high levels of cadmium in brown crab (Falk & Nøst, 2013). Because of its special topography, it can be expected that the affected coastal region is exposed to a pronounced upwelling and since it is assumed that deep-sea water is rich in cadmium, it could be the starting point for high values in crab. However, this theory needs further experimental backup and it delivers no explanation, why other organisms exposed to the cadmium rich deep-sea water in the same area do not exhibit increased levels of cadmium to the same extent as brown crabs do. In another survey, cadmium was measured in brown crabs sampled in 20 localities from Salten to Vesterålen (Frantzen, et al., 2015). As expected, high levels of cadmium were found with a large inter-individual variation. Interestingly, no clear difference in cadmium levels was seen between brown crabs caught in inner fjord and outer coast localities, potentially more exposed to upwelling deep-sea water.

2. Objectives and Methodology

As no conclusive explanation for the gradient of cadmium concentration in the brown crab in the North-East Atlantic Ocean along the Norwegian coast has been found, the main objectives of this work have been:

1. To identify the main parameters influencing cadmium levels in brown crab and to determine which of these parameters can explain the difference between crabs from the North and the South of Norway.

2. To identify possible mitigation strategies to avoid the catch of crabs high in cadmium content in the North.

3. To assess the risk from cadmium exposure due to brown crab consumption in the Norwegian population, and evaluate possible mitigation strategies.

3. Methodological Approach

Paper 1) Effects of Cooking and Freezing Practices on the Distribution of Cadmium in Different Tissues of the Brown Crab (*Cancer pagurus*)

To address our objectives, a robust sample preparation procedure had to be established to obtain reliable and comparable results. The importance of sampling and sample preparation as basis for reliable measurements is underestimated and often poorly addressed in scientific literature. This can be illustrated by comparing the sample treatment in various studies on crab measuring cadmium in different tissues. Crabs were treated in multiple ways before sample dissection. While some crabs were sampled after thawing (Davies, et al., 1981), cooking (Bolam, et al., 2016; Foldøy Tverdal, 2012; Frantzen, et al., 2011) and some fresh (Barrento, et al., 2009a; Barrento, et al., 2009b; Bjerregaard & Depledge, 2002; Bolam, et al., 2016; Ervik, et al., 2017), some crabs underwent combinations of different treatments (Julshamn, et al., 2013c; Julshamn, et al., 2012). In other studies, pretreatment was not described at all (Bjerregaard, et al., 2005; Rainbow, et al., 2000) and in several instances it is not clearly stated if individuals have been frozen before sample preparation (Barrento, et al., 2009c; Frantzen, et al., 2011; Maulvault, et al., 2012; Noël, et al., 2011). Without knowing the effect of the different procedures on cadmium levels in the crab, it was difficult to compare findings in the different studies. This also became obvious, in the case of the measurements of high cadmium levels in Northern Norway. While Julshamn, et al. (2012) and Frantzen, et al. (2011) consistently found high concentrations of cadmium in claw meat, it was claimed by some stakeholders of the fisheries, that measurements of crab claw meat from the North, conducted by an independent service provider, showed very low concentrations. Doubt was risen on the reliability of the existing data.

Furthermore, the existing risk assessment for the Norwegian population (VKM, 2015), was based on samples of frozen and cooked crabs, which does not conform to the commercial or traditional way of cooking brown crab. Traditionally, fresh and alive crabs are boiled in salted water. In the processing industry fresh crabs are either cooked whole or claws are removed and steamed separately from the rest. The EU limit of

0.5 mg Cd/kg applies to unprocessed white meat from crab appendages.

To develop a standardized and appropriate sampling procedure as solid basis for further studies, a laboratory study on the effect of different cooking and freezing methods on the concentration of cadmium in brown crab was conducted. First, the effect of cooking and freezing whole crabs was investigated, before the effects of treating claws and cephalothorax separately were addressed. This gave us the opportunity to address objective 3, as first attempt addressing the risk from consuming brown crab caught in Norway, using cadmium levels from crab treated similarly to the traditional and commercial cooking method.

Paper 2) Tracing Simultaneous Cadmium Accumulation in Brown Crab *Cancer pagurus* from Different Uptake Routes using Stable Isotopes
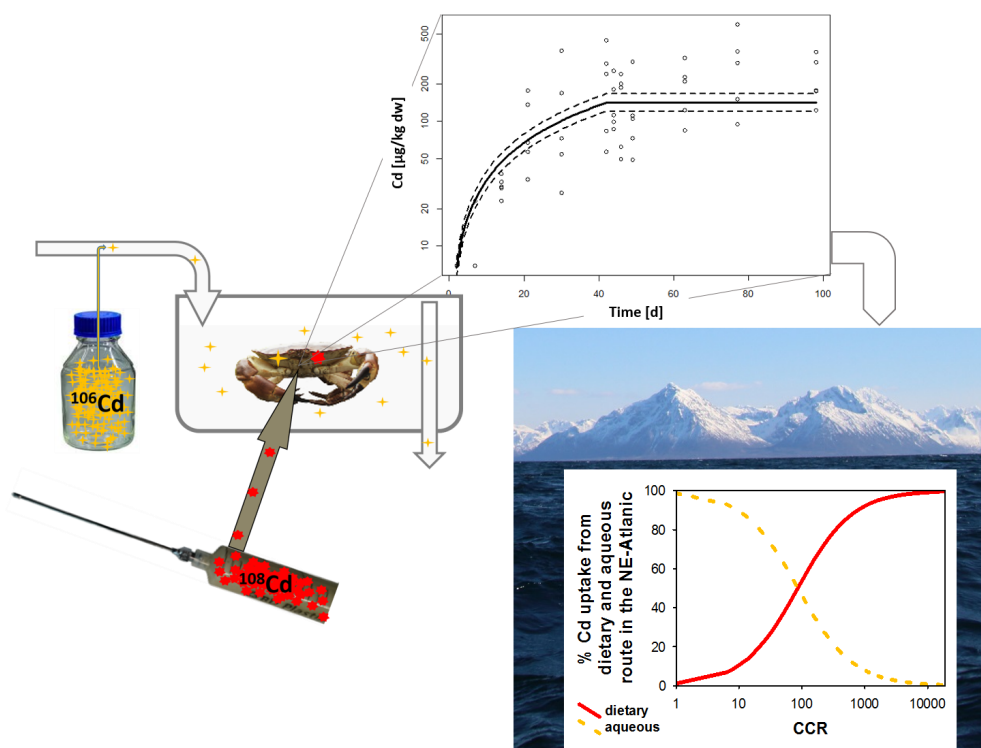

Figure 3 Schematic illustration of Paper 2. Accumulation parameters for dietary and aqueous accumulation of Cd in brown crab were established tracing different stable isotopes and used in a modelling approach to predict the importance of the uptake routes in the case of Northern Norway. CCR denotes the cadmium concentration ratio between feed and seawater.

Aquatic invertebrates take up trace metals via two different routes: From solution or from diet, and the rate of uptake and relative importance depends on the metal and/or species considered (Rainbow & Luoma, 2011a). In addition, the uptake may vary with physiological stage of the animal, physicochemical conditions of the surrounding medium and by bioavailability of trace metals in diet (Rainbow, et al., 2011a). Most studies investigating the uptake in crab looked at the uptake from solution, and only a limited number of studies addressed the uptake from diet (Bjerregaard, et al., 2005). For brown crab, the uptake of cadmium via both routes was poorly investigated (Davies, et al., 1981). To get a better understanding on the importance of the different uptake routes for the total cadmium accumulation, giving an indication what the main source of cadmium in brown crab actually is, a laboratory study was conducted. One widely accepted way to compare the relative importance of the uptake routes and predict concentrations in different invertebrates at different conditions is the use of biodynamic modeling (Luoma & Rainbow, 2005; Wang, et al., 1996). This approach simplifies the accumulation of trace metals to a very limited number of parameters which can be measured in laboratory trials. The steady state accumulation is assumed to be the uptake from water plus the uptake from diet minus depuration and growth (Luoma, et al., 2005). Uptake from diet and water is further the product of the uptake rate constant and concentration in diet or water, respectively. Both, uptake rate constants and the depuration rate constant can be established using lab trials. The uptake rate constant from water is mostly derived directly in lab trials under certain conditions. The uptake rate constant from food, however, is expressed as diet ingestion rate in the experiment multiplied by assimilation efficiency, more practical to measure. The use of long-term exposure has been criticized, as the measured uptake in the exposure actually is the sum of uptake and depuration, as the animal after a certain time will simultaneously eliminate trace metal (Reinfelder, et al., 1998). This has recently been confirmed in HP in a freshwater shrimp (Cresswell, et al., 2017). The depuration rate of cadmium from HP was much lower after long-term exposure compared to short-term exposure (Cresswell, et al., 2017). However, the suggested under-estimation of the uptake because of simultaneous elimination should in a sufficient long-term

exposure be balanced out by a decreased elimination in the elimination phase, as less trace metal will be left.

In the present literature radiotracers were often used to determine the accumulation parameters. The experimental work with radiotracers, however, requires elaborate permissions and handling licenses for disposal procedures and work is cumbersome because of the potential hazard for personnel. It is therefore difficult to find a lab fulfilling the necessary requirements to conduct a trial with radiotracers in large animals like crab, with high demand for water that has to be disposed of. Also the availability of pure radiotracers can be limited and they are expensive (Croteau, et al., 2004). Furthermore, logistics for sample shipment is difficult and requires permission and expensive technical measures. It was therefore decided to use stable isotopes in our experiment, being much easier to handle. However, availability of standards with a high enrichment of stable cadmium isotopes turned out to also be limited. Other difficulties directly connected to the use of stable isotopes were mainly analytical. An analytical issue when using ICP-MS are the polyatomic interferences on all cadmium masses in different analyzed tissues. This was addressed with an analytical setup using an ICP-MS instrument with collision/reaction cell, where a reaction gas can be used to get rid of ions potentially leading to interferences. As a part of the natural isotope distribution, the stable isotopes of an element are abundant wherever natural cadmium is present in the experiment. This means that high background concentrations have to be expected when using them in a laboratory trial. To get control of this issue, a mathematical correction was used addressing all input of natural background and also the contributions of the impurities in the standards used to spike water and food. The final solution equations were calculated with a computer program and although the approach appears to be complex, it is practically handy. Applying these equations made it possible to establish accumulation curves for both uptake routes in all tissues except for the dietary uptake in hemolymph.

When studying the ratio of amount of tracer in feed to the amount taken up in the animal, it is crucial to know how much feed actually was ingested. This is especially challenging in crab, as they tend to crush all prey when eating it. As a consequence, it

is difficult to estimate the actual digested amount of food and in addition, tracer could leak into the water. In our case, gavage feeding was chosen as the most accurate and practical, though time-consuming method. In rodents, where gavage-feeding is frequently applied (Atcha, et al., 2010), holding the animals in a certain way, makes it easy to insert the needle into their mouth. For crab however, it is crucial that they open their mouth parts voluntarily to be able to insert the gavage needle without harm. The application of a few drops of water flavored with shrimp powder on the mouth parts was found to be an effective stimuli and animated the crabs to move their mouth parts and the syringe could gently be inserted. Also the consistency of the food is important. If the applied food is too liquid, it can easily run out of the crab's stomach after feeding. Additionally, feed should be homogeneous and sieved, as particles will block the needle, making an accurate feeding impossible. As crabs in a pre-trial were occasionally spitting out the feed if directly put back into the water, they were kept out of the water for at least 30 seconds after feeding and washed with seawater to avoid tracer from eventually spilled feed to enter the experimental tank.

To estimate the accumulation parameters, the obtained experimental data was fitted to the standard bioaccumulation equation (OECD, 2012). As hepatopancreas (HP) is the organ containing about 90 % of the total cadmium body burden in crab (Bjerregaard, et al., 2002), for simplicity, a one-compartmental model was used, assuming that the gross amount of ingested cadmium from both routes will be accumulated in HP. The freely available R-package bcmfR (Aldenberg, 2017) developed to evaluate bioconcentration studies in fish according to OECD 305 was used for modelling the accumulation parameters. However, due to a modified setup, the package had to be modified. In contrast to OECD 305, aqueous and dietary uptake was traced simultaneously and we therefore applied the model for the aqueous uptake also on the dietary approach. In other studies establishing accumulation parameters in invertebrates, mostly separate experiments for the estimation of dietary and aqueous uptake and elimination rate constants are conducted (Lee & Fisher, 2016; Wang, et al., 1996) (Bjerregaard, et al., 2005).

Further, the uptake of cadmium in HP observed from both routes, was not followed by a marked elimination and the best model fit was found, assuming the elimination rate

constant being zero.

The estimated accumulation parameters were used to predict the relative importance of the two uptake routes. To make a prediction for crabs at the Norwegian coast, the importance of the uptake routes was calculated considering a wide range of combinations of cadmium concentrations observed earlier in natural potential prey organisms (Ness, 2014) and seawater (Falk, 2015) in Northern Norway.

Paper 3) Cadmium in Brown Crab *Cancer pagurus* in Norwegian Waters. Effects of Area, Season, Cooking and Different Physiological Factors and Consequences for Food Safety

While most investigations targeting the high cadmium values in brown crabs in Northern Norway mainly have focused on the potential source of cadmium, it was ignored that accumulation of cadmium in invertebrates also can be influenced by factors other than this. This has clearly been shown for the shore crab *Carcinus maenas*. Other than pure concentration in water and feed (Bjerregaard, 1990; Jennings & Rainbow, 1979; Pedersen, et al., 2014; Wright, 1977a, 1977b), many physiological factors like crab size (Bjerregaard & Depledge, 1994) stage in the moulting cycle (Bondgaard, et al., 2000; Nørum, et al., 2005) ovarian stage (Bondgaard, et al., 2000) and the feeding status of the crab (Bjerregaard, 1991; Styrishave, et al., 2000) have been shown to affect the accumulation of cadmium. Furthermore, environmental factors like temperature and salinity have been shown to have an influence (Hutcheson, 1974; O'Hara, 1973). Therefore, crabs were sampled in the field from one locality in the North of Norway (Vesterålen), known for high levels of cadmium in crab and one locality in the South of Norway (Sotra). The physiological factors size, sex, moulting stage, gonad maturation stage, condition and tissue hydration were recorded for each crab to study the effect of different physiological factors on the cadmium concentration and whether these effects vary between different areas. The intention was to sample crabs every second month throughout one year at both stations for comparison. By sampling crabs during one whole year, we addressed objective two by investigating if there were times of the year with lower cadmium levels. This could potentially provide a time window in which crab fishing could take place. To assess the risk from exposure

to cadmium when consuming brown crab (objective 3), we combined cadmium levels measured in freshly cooked crabs with available consumption data for the Norwegian population to identify consumer groups in risk of exceeding the tolerable weekly intake set by the European food safety authority to 2.5 µg/kg bw (EFSA, 2009b). The risk of exceeding the tolerable weekly intake was assessed for two different cases. First assuming brown crab as the only source of cadmium exposure in the diet of the consumers and second, by considering the exposure to cadmium from other food stuff estimated for the European population (EFSA, 2012).

Paper 4) Cadmium in the shore crab *Carcinus maenas along* the Norwegian Coast: Geographical and Seasonal Variation and Correlation to Physiological Parameters

The pattern with high values of cadmium in animals North of Salten region along the Norwegian coast has not been observed in blue mussel *Mytilus edulis* (Foldøy Tverdal, 2012; Frantzen, et al., 2011) known to be a good indicator species for cadmium pollution (Phillips, 1977). Also the finfish species Atlantic cod *Gadus morhua*, Atlantic halibut *Hippoglossus hippoglossus*, redfish *Sebastes marinus*, and tusk *Brosme brosme*, did not show elevated concentrations in the North (Julshamn, et al., 2013d), although known for high trophic levels and relatively high mercury concentrations, generally indicating potential for biomagnification. However, it has been shown that cadmium, although not biomagnified considering all trophic levels in an ecosystem, actually was biomagnified within a benthic submodel (Signa, et al., 2017b). This together with high levels of cadmium in a wide range of crab species (Bolam, et al., 2016; Hutcheson, 1974; Noël, et al., 2011; Rouleau, et al., 2001), indicate that there might be common characteristics in benthic food webs and especially crab, enhancing the accumulation of cadmium. Brown crab and shore crab *Carcinus maenas*, have partly overlapping ecological niches, many characteristics in common and both are known to be efficient cadmium accumulators (Bjerregaard, et al., 2005). To investigate if this is sufficient to cause the same pattern in cadmium levels along the Norwegian coast, we conducted a comparative study sampling shore crab *Carcinus maenas* at foyr different locations along the Norwegian coast. Two in the North, where high values

have been found in brown crab and two in the South. Also here the effect of physiological parameters and seasonal variation on cadmium was investigated.

4. Results and Discussion

4.1 Factors explaining variation in cadmium in brown crab

High variation in cadmium levels in brown crab is a common finding in all conducted studies within this thesis and the present literature (Barrento, et al., 2009a; Barrento, et al., 2009b; Croteau, et al., 2005; Davies, et al., 1981; Falconer, et al., 1986; FSA, 2013; Maulvault, et al., 2012; Maulvault, et al., 2013; Maulvault, et al., 2011). While some factors causing variation could be identified or confirmed, the effect of other investigated factors was not as clear. It could be underscored that the crab's local origin and the tissue analyzed does explain much of the total variation in cadmium levels (Paper 1 and 3). Furthermore, sampling practice was found to be a crucial factor explaining much variation between differently treated samples (Paper 1 and 3).

However, also when considering these factors, much inter-individual-variation in cadmium levels was observed. Addressing different physiological factors showed that the variation and potential covariation of different factors make it difficult to disentangle the effects on cadmium concentrations. Other factors, which could not be covered in this thesis, such as opportunistic feeding, might have contributed to the large variation in cadmium levels.

4.1.1 Sampling practices and nomenclature

The comparison of the effects of different cooking and sampling procedures on the distribution of cadmium between different tissues in brown crab revealed strong effects. In Paper 1 cooking crabs induced a leakage of cadmium from hepatopancreas (HP) to claw meat. The transfer was pronounced by freezing the crabs prior to cooking, probably due to a bursting of HP cells. Also freezing and thawing in itself led to a significant leakage of cadmium from HP to claw meat to a comparable extent as cooking. When claws were boiled or thawed separately from carapace, cadmium levels were low. Consequently the amount of cadmium in brown meat, consisting of HP and gonad, was reduced after cooking whole crabs. While the cadmium concentrations in claw meat were significantly increased after cooking whole crabs, freezing and cooking

claws separately did not change the concentrations. This was confirmed by the findings in Paper 3.
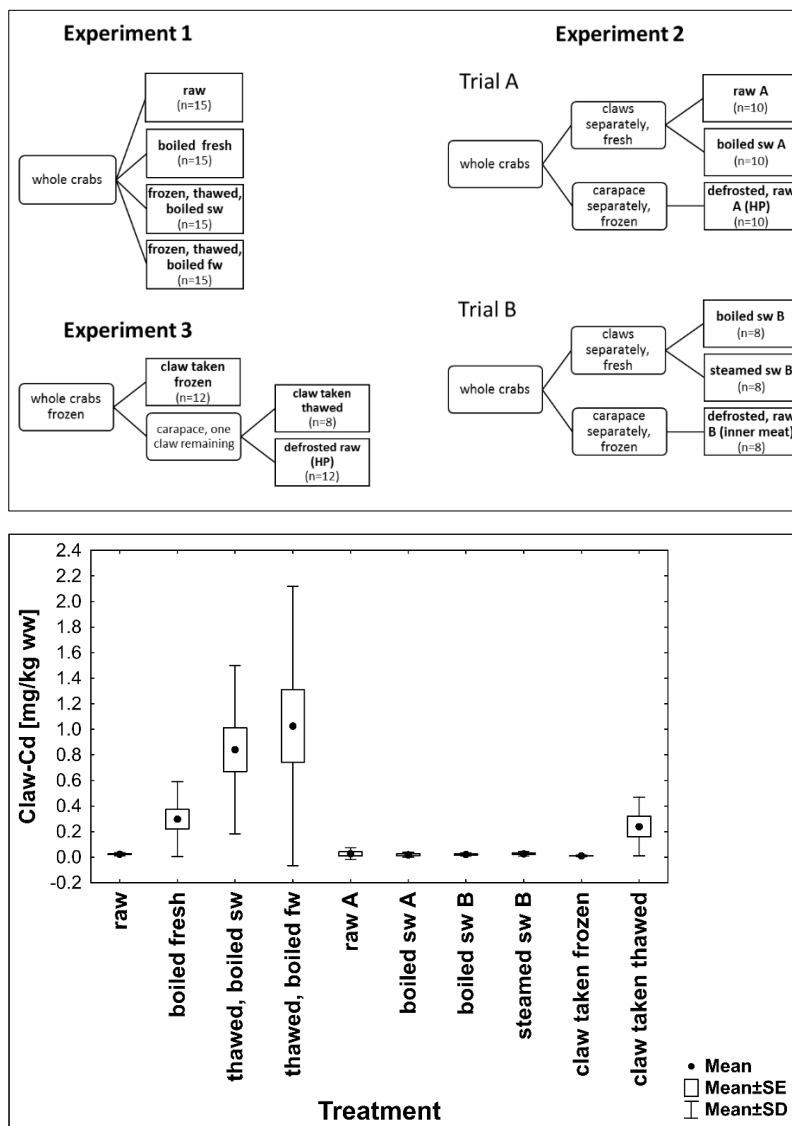

Figure 4 Schematic illustration of the cooking and freezing practices applied in Paper 1 (upper panel) and the corresponding cadmium measurements in claw meat of crabs sampled in the North (lower panel).

These findings emphasized the importance of sampling procedure. When levels of

cadmium in crabs in the field are studied, it is crucial to choose an appropriate sampling and processing method mirroring the real concentrations. As freezing and thawing already have been shown to influence cadmium levels significantly, the dissection of fresh animals should be favored. This however, can be challenging depending on sampling area and available logistics. When analyzing crabs for assessing food quality or risk assessment, crabs should be boiled whole and fresh in salted water to mimic the most common way of cooking crabs.

For the study presented in Paper 3 in brown crab, we therefore decided to be on site when brown crabs were landed, to be able to dissect crabs freshly and freshly cooked, respectively. This was also based on personal experience with shipping of alive brown crabs over long distances, which can be challenging with the available logistics in Norway. The large size of the animals and long distances make fast transport challenging and costly. Also in commercial trade, the handling of alive crab is challenging and can lead to high mortality rates (Barrento, et al., 2008) and induces stress (Barrento, et al., 2011; Woll, et al., 2010), which should be avoided due to welfare reasons. Further, stress responses might influence the metabolism of trace metals and lead to mortality, which could lead to a leakage of cadmium similar to what was observed while thawing crabs. Dead cells, especially of HP in the presence of digestive enzymes, will be broken down and potentially release cadmium, which again can redistribute within the crab. Furthermore, tissue hydration may be altered during transport.

The observed differences in sample handling make it difficult to compare earlier reported cadmium levels and draw conclusions, as much of the variation found in earlier studies might be due to different sampling procedures. Noël et al. (2011), for example analyzed crabs from France, United Kingdom, Netherlands and Ireland and found a huge variation of cadmium levels in white meat (<0.020 mg/kg - 0.587 mg/kg ww). Sample preparation in terms of cooking, freezing or analyzing fresh samples, was not explicitly described in this study and sampling was performed by inspectors at the final consumer level, at different types of facilities. Considering the strong effect of cooking and freezing and the high chance that differently treated products are offered

at consumer level, a large part of the variation found, is likely to be caused by different processing and sampling procedures.

Another issue making it difficult to compare levels of contaminants or nutrients in different studies of crab, is the inconsistent use of nomenclature for organs and tissues. Especially the terms 'brown meat' and 'white meat', describing the main edible tissues, are often not exactly defined, or defined in different ways. In literature on brown crab, the following terms can be found for the muscle meat: white meat, either not further specified (Maulvault, et al., 2013; Noël, et al., 2011) or defined as taken from legs and claws (Bolam, et al., 2016), claw muscle (Davies, et al., 1981), muscle meat, defined as muscle from the claw (Barrento, et al., 2009b) (Barrento, et al., 2009c), muscle, not further specified (Maulvault, et al., 2012; Maulvault, et al., 2011) and claw meat (Paper 1) The differences between the different "white meats" might not be crucial, as there is not much reason to assume different cadmium values in the different muscle tissues. However, the proximity of HP to muscle meat within the cephalothorax and similarly the walking legs, may cause higher values, especially for processed crabs.

When considering 'brown meat', the definition is of obvious importance, as the different tissues falling under the definition vary greatly in cadmium level. The main part of brown meat is gonad and HP, and in Paper 1 a mean ratio of over 1 000 in cadmium concentration was found between these tissues. Furthermore, especially in females, the amount of gonad tissue varies widely according to the gonad maturation stage.

The following definitions of brown meat can be found in the literature on brown crab: 'brown meat', not further specified (Maulvault, et al., 2012; Maulvault, et al., 2013), 'brown meat with thorax' (Noël, et al., 2011), 'including the reproductive organ, as well as the digestive organ' (HP) (Bolam, et al., 2016), 'gonads and HP' (Maulvault, et al., 2011) and 'hepatopancreas or hard roe' (Ervik, et al., 2017). Ervik et al (2017) sampled brown crab in an inshore region in Mid-Norway and at one location a mean cadmium concentration of 23.19 µg/kg dw was reported with an extraordinary large standard deviation of 64.18 µg/kg dw, which is very likely caused by the sampling of only 1g of tissue consisting of hepatopancreas or roe, known to vary significantly in cadmium levels. Further, the term 'hard roe' indicates that crabs might have been

cooked before sampling.

In Norway, according to experience, brown meat is often referred to as all edible tissue in the cephalothorax except muscle. This also includes other tissues than gonad and brown meat. Especially in early postmolt crabs and crabs with low meat yield, the sub-epidermal connective tissue actually is a significant part of the brown meat and may lead to a dilution of cadmium. Therefore, we referred to the analyzed tissue as 'inner meat'. As none of the earlier studies referred to the sub-epidermal tissue, it is unclear if it was regarded a part of brown meat or not. Further, some studies only consider hepatopancreas (Barrento, et al., 2009c) or HP and gonads separately (Barrento, et al., 2009a; Barrento, et al., 2009b). With reference to the unclear terminology, there is a need to harmonize protocols and being precise in the description of the sampled tissue and applied sampling procedure, to allow precise comparisons between sampling procedures and study findings.

4.1.2 Physiological factors

Size and Age

The results of paper 3 indicate a correlation between size and cadmium levels in brown crab. In crabs sampled in the North, a weak correlation was found for size and cadmium concentration in HP. Based on the total amount of cadmium, however, the correlation was clear. For crabs from the South, no such correlation was found. A similar pattern as in brown crab in the North was also observed in shore crab (Paper 4). In our study with brown crab, only crabs above the legal size limit of 13 cm carapace width (CW) were sampled. The correlation between size and cadmium was therefore probably masked by limited variation in size (Paper 3). In a master thesis of Lindborg (2017), conducted in connection to the present work, crabs between 90 and 180 mm CW were sampled at two sampling locations from the Norwegian coast, one in the North (Senja, 69 N), and one in the South (Sotra, see Paper 3) and analyzed for cadmium. Similar to the findings in Paper 3, there was a weak correlation between size and cadmium concentration in HP in the North and a clear correlation between size and the total amount of cadmium in HP. The findings from both studies suggest an accumulation of cadmium in HP of brown crab over time, not clearly visible as an increase in

concentration, probably due to growth dilution. An accumulation over time is reasonable considering the high assimilation efficiency and slow excretion of cadmium in brown crab (Paper 2) and shore crab (Bjerregaard, et al., 2005). In shore crab, cadmium concentration in HP based on dry weight, but not wet weight, was positively correlated to the total weight of the crab (Bjerregaard, et al., 2002).

The fact that no correlation between size and cadmium level was found in brown crabs from the South both in Paper 3 and by Lindborg (2017) can be due to different growth rates in the two areas, expressed as moulting frequency (Bakke, S., personal communication, Feb 2018). If moulting occurs less frequently in the North, the same variation in size may represent a wider age range in the North than in the South, explaining why Cd in brown crab showed correlation with size in the North and not in the South.

As growth rate in crabs can vary between individuals (Eriksen & Moen, 1993) two crabs at the same size might be of very different age. Further, higher temperatures lead to shorter intermoult periods in crustaceans (Passano, 1960), potentially triggering different growth rates in latitudinally separated populations. Therefore, age rather than size can be considered being the more relevant parameter to assess bioaccumulation. Hence, the intention in the study of Lindborg (2017) was to have the age determined for a certain number of crabs similar in size, and to correlate age with cadmium levels. Recently, a considerable effort has been spent to develop methods for the direct age determination of crustaceans. Kilada et al. (2012) proposed a promising direct ageing method similar to the common method of otolith readings in fish. However, the challenge with crustaceans was to identify a structure that is retained unchanged during the moults. The calcified structures on eyestalk and gastric mill ossicles have been proposed to be suitable, as they in addition show discernable growth bands. The count of growth bands in four crustacean species obtained comparable age estimates, for the respective individuals, as length-frequency analysis. However, a direct validation of the periodicity of the growth bands in organisms of known age was still missing (Kilada, et al., 2015). For brown crab, this method also obtained promising result, however until now without validation (Sheridan, et al., 2015). The attempt to determine the age of the crabs sampled in the study of Lindborg (2017) however, turned out to be

challenging. Different persons counting the growth bands on the same ossicles got different results and the correlation between age and size was very weak, also within crabs from the same location. It was therefore decided not to use the results further, because of the high uncertainty connected to the method. Sheridan et al. (2016) followed the fate of the ossicles in Norway lobster *Nephrops norvegicus* throughout the moulting cycle, in order to investigate their utility for age determination. By staining the ossicles and dissecting animals after moulting, they found ossicles being detached and loose within the stomach of the individuals and stained material was later found incorporated in the newly calcifies ossicles. They concluded that the growth bands are unlikely to be of annual periodicity as previously interpreted and that gastric mill ossicles probably cannot be used to directly determine the age of Norway lobster and the same may be the case for a number of decapod crustacean species. Further scientific work on this field is highly warranted.

Moulting and gonad maturation

Neither the factors moulting stage nor gonad maturation stage had a clear influence on the cadmium levels in brown crab. This is in contrast to investigations on the uptake of cadmium in shore crab, where clear differences in uptake were seen for crabs at different moulting and gonad maturation stages (Bondgaard & Bjerregaard, 2005; Bondgaard, et al., 2000; Nissen, et al., 2005; Nørum, et al., 2005). However, most of these studies mainly focused on the cadmium uptake from water. The results from Paper 2 and an earlier study on shore crab (Bjerregaard, et al., 2005), suggest that the accumulation of cadmium from diet is more important for the overall accumulation. Hence, significant effects of moulting and gonad maturation might not be found in the overall accumulation of crabs sampled in the field. Further, it cannot necessarily be expected that differences in uptake between crabs at certain moulting or gonad maturation stages in the laboratory are mirrored in the levels of cadmium found in the field. The cadmium level measured in a crab at a certain time is the result of the accumulation of cadmium during the whole lifespan of the crab and thereby the sum of the cadmium accumulation throughout multiple moulting and gonad maturation cycles.

Condition

The condition of the crab, measured as hepatosomatic index (HSI), was moderately negatively correlated to the cadmium concentration based on dry weight in North ($r=-0.36$) and South ($r=-0.42$), while no correlation was found for the amount of cadmium and cadmium concentration based on wet weight. This indicates a dilution of cadmium with increasing condition, as the constant amount of cadmium will be distributed in a growing HP. However, as the result of study 2 revealed a high importance of the dietary route combined with a high assimilation efficiency of cadmium from feed, an increase of the total amount of cadmium could be expected with increasing condition, resulting from continuous feed intake. This effect might be masked by the fact that crabs are opportunistic feeders and prey on organisms having varying levels of cadmium (Ness, 2014). This leads to large inter-individual variation in cadmium intake, reflected in variation in cadmium levels between the crabs. Further, due to the moulting cycle, crabs are building up their condition rather stepwise due to the total depletion of reserves after building up the new exoskeleton after ecdysis (Warner, 1977). Moreover, the condition can vary according to season and feed availability. Therefore, the large inter-individual variation might also be caused by the fact that the condition of the crab during the recent moutling cycle or feeding season, might not correspond to the condition during passed moulting cycles or feeding seasons. This means that crabs sampled in recent good condition, may have eaten and accumulated little cadmium in the previous moulting cycles, as moulting is not only triggered by condition, but also external factors such as light regime and temperature (Warner, 1977). The cadmium accumulated during one season or moulting cycle is probably only a relativelty small part of the total cadmium burden.

Sex

There was no differences in cadmium levels in HP between male and female crabs, although differences in migratory habits of crabs have been shown (Bennett, et al., 1983; Hunter, et al., 2013; Karlsson & Christiansen, 1996; Ungfors, et al., 2007). For brown crab, as an opportunistic feeder (Woll, 1995), different migration patterns will result in the consumption of different prey items at different locations. Males and females may also be exposed to other concentrations of metals dissolved in water

including different physicochemical conditions of sediment and water, potentially influencing accumulation (Signa, et al., 2017a). It is known from other regions that female crabs tend to migrate further (Hunter, et al., 2013; Ungfors, et al., 2007) and a study using microsatellite genotyping on brown crab also suggests differences in migration between males and females in a fjord at the Swedish west coast (McKeown, et al., 2017). While females showed no distinct genetic differences to crabs sampled in other regions of the North-East Atlantic Ocean, the males were distinctly different, suggesting limited gene flow and thereby migration.

Further, the sexual dimorphism with larger claws in males connected to allometric growth (Öndes, et al., 2017), could lead to the consumption of different prey. However, also earlier studies on brown crab have not reported different cadmium levels in HP between males and females in HP in brown crab (Barrento, et al., 2009a) and burrowing crab *Neohelice granulate* (Beltrame, et al., 2010). Differences were, however, found in muscle and gills with higher concentrations in females (Barrento, et al., 2009a). Higher cadmium concentrations in claw meat of females was also observed in the results of Paper 3, while the total cadmium content was the same. This is probably due to a larger growth dilution of cadmium in claws in male brown crabs due to allometric growth. The higher concentration cadmium in gills of the female crabs may be caused by a higher exposure to Cd when migrating to deeper waters.

Season

The cadmium levels in crab from North and South did not follow a clear seasonal pattern. A tendency to higher levels in February and July in the North and in October in the South was however seen. Examining the corresponding changes in physiological parameters revealed one interesting clue. While no clear seasonal pattern was visible in the physiological parameters themselves for crabs sampled in the South, in the North, the condition was lower in July, however not significantly, corresponding to the trend in high cadmium levels. This underlines the negative correlation between condition and cadmium levels discussed earlier. No clear patterns were seen for the other assessed physiological parameters underlining the huge inter-individual variation in crab, making it difficult to obtain representative results on a population level. The fact that crabs migrate, makes it difficult to ensure that a representative part of the population is

sampled throughout field studies. Further, as we only used one type of gear (baited pots), gear selectivity can lead to sampling bias. For baited pots, gear avoidance behavior is known for ovigerous crabs (Howard, 1982) and actively foraging crabs will be most vulnerable. Further, soak time and type of bait have an influence on the catchability (Bennett, 1974).

The sampling procedure itself revealed another interesting difference between crabs in the North and South. In the South, brown crabs were caught all year round in rather shallow water (5 - 40 m). In the North however, considerable effort was spent to catch brown crab in April, without success. This could be due to a lack of experience in fishing at this time of the year, as the fishing of brown crab in this region is restricted to autumn and early winter. Only one attempt in deeper water (80 – 140 m) delivered two crabs. Sufficient catches at fishing depths and places common for the crab fisheries in autumn, could not be obtained before July. This finding in combination with the observed lower condition in July indicates that crabs from the North might have different migratory patterns, probably linked to lower water temperatures. The water temperature along the Norwegian coast generally decreases with increasing latitude and the mean temperature for 2015 to 2017 at Sognesjøen (61 °N), a station close to our sampling site in southern Norway, was 10.0 °C, ranging from 5.8° to 15.6 °C at a depth of 5 m. At a station in the proximity of our site in the North, Eggum (68 °N), a mean temperature of 8.3 °C, ranging from 4.7 ° to 12.3 °C during the same time and at the same depth, was measured (IMR, 2018). Crabs in the South seem to be foraging and moving at shallower waters to a certain degree all year round, making them available for trap fisheries. The low catchability of crabs in the North when the water temperature is at its lowest, may have two explanations: Either crabs stop foraging, as indicated by observations of recreational divers, spotting crabs dug into sediment during spring and earlier found evidence that crabs do not feed at all at temperatures below 5 °C (Karlsson, et al., 1996). Or, they are migrating to deeper waters for feeding, comparable to what is observed in for example French waters (Le Foll, 1982). A combination of the two is also possible. The lower HSI supports that crabs are not foraging. However, it is also possible that crabs are not foraging as actively in deeper water or that prey is less abundant there.

4.1.3 Dietary and aqueous cadmium uptake

The results from Paper 2 revealed that the dietary route dominates the accumulation of cadmium in HP in brown crab for representative concentrations in water and diet found in the North and most likely the whole distribution range of brown crab. The relative importance of the dietary route was predicted to be at least 98 % in our model, considering a wide range of naturally occurring combinations of concentrations in potential feed organisms and seawater in Northern Norway. The assimilation efficiency α from cadmium in food in HP was also determined to be 98 %.

This means that even though our modelled parameters might carry some uncertainty and the uptake at only one set of physicochemical condition was measured, the dietary uptake can be considered as more important. This is in line with the findings in brown crab of Davies et al. (Davies, et al., 1981) who exposed crabs to cadmium in water (10 µg/L) and a high concentration in feed (58 mg/kg) for approximately 300 days. Also for shore crab, Bjerregaard et al. (2005) concluded that diet is the main uptake route for cadmium, as the increase of cadmium in crabs sampled at different months could not be explained by an accumulation of cadmium from water measured in a laboratory study.

The model predictions of the importance of the uptake route might bear uncertainty because of variability in the model parameters. The applied ingestion rate will vary with food accessibility and quality as well as environmental conditions such as temperature (Woll, et al., 2006b). Assimilation efficiency has been shown to depend on food type and chemical form of cadmium (Rainbow, et al., 2011b) and also variation in geochemical parameters such as salinity can influence the uptake of trace metals (Bjerregaard, et al., 1994). Furthermore, physiological parameters might influence the uptake. However, considering the clear result of the model prediction, the dietary route can be regarded to be more important than the aqueous uptake.

Using a biodynamic modelling approach assuming steady-state conditions, the determined accumulation parameters like those determined in Paper 2, can further be used to predict concentrations of cadmium in brown crab in the field (Lee, et al., 2016; Luoma, et al., 2005; Wang, et al., 1996). The predicted value can then be compared to values found in the field to validate the model output.

The total steady state concentration of cadmium in HP of crab (C_{SS}) can then be described as:

$$C_{SS} = C_{SSwater}+C_{SSfood}= \frac{(k_w \cdot C_w)}{k_e+ g} + \frac{(\alpha \cdot I \cdot C_f)}{k_e+ g},$$

Where k_w is the uptake rate constant from water determined to be 0.0067 L · g crab^{-1} · d^{-1}, C_w and C_f being the cadmium concentration in seawater [µg/L] and feed [µg/g dw], I being the feeding rate of 0.08 g_{feed} · g_{HP}^{-1} · day^{-1} dw from Woll et al (2006b), adjusted for HP and dry weight (Paper 2). The elimination rate constant k_e was set to zero, according to the best model fit and as no clear depuration of cadmium from HP was observed after the exposure phase. Therefore, the growth rate g is crucial for the output. However, estimates of the growth rate of brown crabs are scarce adding uncertainty to the prediction. One rough estimate found in the literature is that crabs at around 200 g in Mid-Norway are about 8 years old (Eriksen, et al., 1993), corresponding to a growth rate of 0.07 g · d^{-1}.

The mean cadmium concentration in seawater recently measured in the Salten region was 0.05 µg/L (Falk, 2015) and concentrations in blue mussel and horse mussel (*Modiolus modiolus*) identified as the most frequently consumed food items of brown crab were 0.75 and 11.4 µg/g dw (Duinker, et al., 2016). Applying these concentrations in the model, steady state concentrations of 0.84 and 12.8 µg/g dw were predicted, respectively. These values are relatively low compared to the highly variable concentrations found in the North and South with means ± SD of 68 ± 67 µg/g dw and 23 ± 25 µg/g dw, respectively (Paper 3). For small filter feeders and plankton, validations of the biodynamic model resulted in more accurate predictions. (Luoma, et al., 2005). However, variations in the trace metal concentrations in the diet are probably not as pronounced and the environmental conditions more stable for plankton and filter feeders in comparison to a mobile opportunistic feeder like brown crab. Considering the uncertainty in the input parameters in the present model prediction, some deviation in the predicted concentration can be expected. Better estimates for the growth rate, ingestion rate and foraging preferences are needed to make a validation of the model, using measurements of cadmium levels in brown crab sampled in the field, more reasonable. The accumulation parameters itself are also somewhat uncertain, as the

uptake and elimination of trace metals in invertebrates is complex and can be influenced by a variety of factors such as physicochemical properties of the environment and physiological parameters of the crab difficult to cover completely in laboratory studies. Further, even though considerable effort was spent to use physiologically similar crabs in the experiment and environmental conditions, with cadmium exposure similar for all crabs, rather large variation was seen in the uptake. This indicates inter-individual differences in uptake of cadmium in brown crab.

In conclusion, Paper 2 revealed that cadmium in brown crab is mainly accumulated via the dietary route, which clearly indicates, that foraging behavior is important for the accumulation of cadmium in this species.

4.1.4 Cadmium accumulation in shore crab vs brown crab

The pattern in the brown crab with high cadmium levels in the North was not reflected in the shore crab. Only males from one station in the North showed significantly higher cadmium levels than at the other locations (Paper 4). This suggests, that the difference in cadmium levels in brown crab from North and South are rather due to certain characteristics of the species itself, than being connected to characteristics of the area, at least in shallow waters. Different cadmium accumulation patterns among taxa and species can be explained by factors such as: species-specific differences in bioaccumulation dynamics, differences in metal exposures by different habitat characteristics or dietary preferences, different foraging behaviors and different food web structure and trophic position (Croteau, et al., 2005). Shore crab and brown crab have similar feeding habitats. However, clear differences can be seen in the depth distribution of the two species; i.e. from the tidal zone down to a few meters in shore crab (Klassen & Locke, 2007) and from the tidal zone down to several hundred meters in brown crab (Le Foll, 1982). Furthermore, a comparative study in juvenile brown and shore crab showed that brown crab tended to feed on larger prey, most probably because of the more powerful chelae (Mascaro & Seed, 2001) and it can be assumed that this difference is even pronounced in adult animals. If differences in foraging habitat are causing a variation between the spatial cadmium patterns between different species, this supports the suggestion that differences in foraging behavior also can

explain the variation between different areas within one species, the brown crab. Another reason for the absence of the spatial cadmium pattern in shore crab, could be that shore crab might be better adapted to the climatic conditions. At our northern sampling location, brown crab lives near its northern limit of distribution, where pronounced biological responses to seasonal temperature fluctuations may be expected. Brown crab, arrived according to local fishermen in the North about 20 years ago and the earlier mentioned lower moulting frequency suggests suboptimal conditions. In contrast, at the southern location, brown crab inhabits an area where temperatures may be closer to ideal all year round. Shore crab, however, is known for its wide distribution range all along the Norwgeian coast and tolerance of temperature and salinity (Klassen, et al., 2007). Shore crab might therefore not grow slower in the North and crabs at the same size may have had the same time to accumulate cadmium. Further the shore crab may not need to migrate, because of difficult foraging at cold temperatures, as indicated for brown crab (Karlsson, et al., 1996).

4.2 Difference between North and South

Our results indicate that the sudden spatial increase in cadmium levels in brown crab in the North of the Norwegian coast is a complex, multifactorial phenomenon. Nevertheless, important factors could be identified potentially playing an important role in cadmium accumulation. Combined with earlier observations, our findings suggest some explanations for the differences in cadmium levels in brown crab between the areas north and south of about 67°N.

An earlier study measuring the cadmium level in sediments in the North came to the conclusion that the high levels of cadmium in crab are probably not caused by an anthropogenic point source (Falk, 2012). This finding is strengthened by the fact that neither blue mussels (Foldøy Tverdal, 2012), finfish (Julshamn, et al., 2013a; Julshamn, et al., 2013e), (Julshamn, et al., 2013a), nor the closely related shore crab showed the same pattern with high cadmium levels in the North. Regarding riverine input of cadmium in Norway, the release of cadmium in 2015 was actually higher in the South with 1.1 t to Skagerrak, 0.61 t to the North Sea, 0.2 t to the Norwegian Sea

and 0.3 to the Barents Sea in 2015 (Skarbøvik, et al., 2016). Neither was there a trend towards higher concentrations in the North visible in Cd levels in seawater at different depths across the North-East Atlantic Ocean (Danielsson, et al., 1985).

The finding in Paper 2 that most cadmium is accumulated from diet indicates that foraging and linked factors are important in understanding the variation in cadmium levels.

Combining these results with the finding in Paper 3, indicating different patterns of migration and foraging for crabs from North and South, this could be the key to understanding the different cadmium concentrations in brown crab found in North and South. As different prey organisms will be abundant at different habitats and depths, the possible downward migration of crab might bring them into contact with organisms potentially holding high amounts of cadmium, like for example porifera (Ness, 2014) and as deep-sea water is known to be rich in cadmium (Janssen, et al., 2014; Xu & Morel, 2013), also species abundant in shallower waters might be higher in cadmium in deeper waters.

Low accumulation of cadmium from seawater in brown crab means that differences in cadmium in crab from different regions cannot be explained by variations in direct uptake of cadmium from seawater. However, as trace metals have the potential to biomagnify over several trophic levels, the earlier suggested hypothesis that upwelling of cadmium rich deep-sea water is causing the high values in the North (Falk, et al., 2013) cannot be ruled out. Although biomagnification of trace metals except methyl-mercury is not expected (Fisher & Reinfelder, 1995), cadmium biomagnification over several trophic levels in seawater has been described earlier. In a subtropical lagoon (SE Gulf of California), 20 of 31 trophic interactions resulted in biomagnification factors > 1.0 for cadmium (Ruelas-Inzunza & Páez-Osuna, 2008) and also within the Greenland part of the Arctic a general pattern of cadmium biomagnification was found (Dietz, et al., 2000). On the contrary, cadmium concentrations were lower in higher trophic levels in a southern Baltic ecosystem (Szefer, 1991) and also in a food web in the Mediterranean Sea, cadmium concentrations in species at higher trophic levels were lower. In a freshwater food web, a higher cadmium accumulation was shown in fish than in invertebrates (Croteau, et al., 2005). Cheung and Wang (2008) found cadmium

to biomagnify distinctly in food webs with gastropods as top predators in different marine environments. This is in accordance with the recent study of Signa et al. (2017b) in a highly contaminated area of the Mediterranean Sea in a benthic food web. Interestingly, when considering all the components of the food web, not only the benthic part, no biomagnification could be found. This indicates that the detection of biomagnification in a food web clearly depends on the considered species and parts of the food web. Findings in gastropods suggest that biomagnification of cadmium is more common in benthic food-webs than in pelagic food-webs and that certain predators, like gastropods, are prone to biomagnify cadmium (Signa, et al., 2017b).

Comparing cadmium concentrations found in Paper 1 and 3 with concentrations found in the potential feed organisms for crab in Northern Norway (Ness, 2014), showed that brown crab itself is amongst the organisms with the highest concentrations. In combination with the high assimilation efficiency found in Paper 2, this make it very likely that brown crab is a top predator for a benthic cadmium biomagnifying food web, similar to gastropods.

Whether an organism biomagnifies a certain trace element depends on the underlying physiological handling mechanisms (Rainbow, et al., 2011a) and can be independent of the trophic position. While some invertebrates directly regulate the excess of trace metal concentrations by balancing uptake with excretion, some store it in detoxified form. Some organisms excrete the detoxified metals, like amphipod crustaceans with copper. Others show no significant excretion, such as barnacles for cadmium, similar to crabs.

An indication for the accumulation of cadmium in HP of brown crab over time was found in paper 3. Further, a recent study on the moulting frequency of brown crab along the Norwegian coast suggests that crabs in the North moult less often and therefore most probably have a slower total growth (Bakke, S., personal communication, Feb 2018). This supports the hypothesis that a lower growth rate in the North could lead to higher cadmium levels in crabs compared to crabs at the same size in the South.

Moulting may also play a role as a mechanism of trace metal depuration. Bergey and Weis (2007) measured the content of copper, lead and zinc in fiddler crab *Uca pugnax*

and in the respective exuvia immediately after ecdysis. They found a significant decrease in all metals, however the decrease was less in crabs coming from a clean compared to a contaminated site. While the total body burden elimination for copper was 12 and 3 % at a contaminated versus clean site, was it 76 and 56 % for copper and 22 and 8% for zinc, respectively. The large difference between the different metals could partly be explained by the fact that most of the total body burden of lead is located in the exoskeleton, while zinc and copper are mainly abundant in soft tissue. For cadmium, mainly accumulated in HP, the elimination during moulting will most likely not be as pronounced as for lead. However, if there is some elimination taking place at ecdysis, the higher moulting frequency, and thereby elimination of cadmium in the crabs in the South, might contribute to the lower values of cadmium.

A comparison of the cadmium levels measured in Paper 3 and literature values on brown crab from Scotland and the English channel (Barrento, et al., 2009a; Barrento, et al., 2009b; Maulvault, et al., 2012) indicates that the cadmium levels in brown crab from the North actually are not high compared to other areas, while brown crab from the South are low in comparison. In other studies crabs were purchased at fish mongers and a high meat yield and condition of the crabs can be assumed. According to our finding of a negative correlation between condition and cadmium levels, this should result in lower concentrations, even underlining that actually brown crabs from the South of Norway are unusual with their lower values.

4.3 Implications for the brown crab fishery

Considering commercial fishing and that crabs are processed and cooked as whole, the results of Paper 3 indicate that capture area (North or South) is the most important factor influencing the concentration of cadmium in HP. Neither season nor other physiological factors had a strong influence. This makes it difficult to develop mitigation strategies for fisheries other than avoiding fishing in the North. Only fishing south of Salten provides a catch of crabs with low enough cadmium concentrations in HP to guarantee cadmium values in claw meat below the legal limit of 0.5 mg/L (Paper 3). Neither fishing at a certain period of the year nor sorting by a certain physiological

stage in the North of Norway would be sufficient to guarantee cadmium levels below the legal limit in claw meat.

However, as HP was identified as source of cadmium redistributing in the animal, and also being most crucial for the risk when consuming crab (Paper 3), a suitable mitigation strategy in the North could be the avoidance of HP in processing. According to the results of Paper 1, with low findings of cadmium in crab claw treated separately and in raw gonad, there is potential to use crab claw meat as a food resource on commercial basis also in the North. It can be ethically questioned whether or not it is acceptable to harvest a crab, if only a minor part will be processed. However, according to own experience, brown crab is caught as by-catch in net fisheries targeting finfish. For example, in the early period of the large yearly fishery on Atlantic cod at the spawning ground in the Lofoten area, brown crab is a very common by-catch which is discarded and often killed or mutilated prior to release, as they are entangled in the nets, making it cumbersome and time consuming to release them alive. High catches of crab in the North have also been reported in net-fishery aiming for halibut and monkfish in autumn (Bakke, et al., 2016).

Thus, claw meat of crabs caught as bycatch in other fisheries could safely be utilized as food if claws are removed before processing. As it is illegal and unethical to remove claws from live crabs, brown crabs should first be killed humanely. With some degree of training, this can be done quickly by the method described in Paper 1 to 4.

4.4 Risk assessment and implications for human consumption

Our results from Paper 1 regarding the change in concentrations after cooking and thawing are crucial when the risk of exceeding dietary reference values in a certain population wants to be assessed. The findings in Paper 1 clearly show that a relevant risk assessment has to take into account how the food item in question actually is processed before consumption, as concentrations can be heavily affected. So far, this is not implemented in legislation and the awareness is low.

The risk assessment presented in Paper 3 is the first using cadmium concentrations measured in freshly boiled crabs to assess the risk of critical cadmium exposure linked

to the consumption of brown crab.

Consumer data from the Norwegian population was combined with the measured cadmium values in brown crabs from North and South. Even when considering the exposure of cadmium from consumption of brown crab in addition to exposure from other foodstuffs, crab claw meat of crabs cooked whole can be safely consumed regardless of where the crabs were caught. Eating whole brown crab from the South as frequent as an average Norwegian consumer, would not lead to an exposure higher than the tolerable weekly intake. However, consumers at the coast, having a higher consumption frequency, would slightly exceed the TWI, while heavy coastal consumers are exposed to three times the TWI. Consumption of the same number of crabs from the North leads to an exceedance of the TWI in all considered consumption patterns and the heavy consumers ingest 13 times the TWI. As most of the crabs commercially landed in Norway are caught south of the critical are (Søvik, et al., 2017), the consumption of crabs with low levels comparable to our findings in the South, are more likely. However, it has been shown, that the contribution of self-caught seafood is especially high for people living along the Norwegian coast (Meltzer, et al., 2002). Consequently, consumers living along the coast of Northern Norway, eating self-caught crabs, are at risk.

Table 1 Calculated exposure to cadmium in the Norwegian population given as percentage of the TWI of 0.73 mg Cd/kg body weight, accounting for the contribution of Cd from other foodstuff in the European population. Different consumption patterns and concentrations in brown crab along the Norwegian coast and consumed tissue were considered. Red indicates an exceedance of the TWI.

% of TWI	Whole crabs		Claw meat	
	North	South	North	South
Avgerage consumption	295	85	19	2
Coastal consumption	353	102	19	2
High coastal consumption	1237	357	82	8

The current advice of the Norwegian food safety authority to avoid eating crabs from the North is reasonable, although the consumption of claw meat can be regarded as safe

regarding cadmium. However, it does not protect the coastal consumers of brown crab in Norway. Additionally, it seems like the popularity of ready-to-eat products is increasing often containing a mixture of crab brown and white meat. This probably changes the consumption patterns. Further, it can be discussed if the current legal maximum limit protects consumers against high cadmium exposure from consumption of brown crab. It only applies for muscle meat, which has been identified not to be of concern. For the actual hazard, cadmium in brown meat, no legal limit is established. Such a limit would be more protective for consumers and could be set considering current consumer patterns and processing methods and should also apply for mixtures of brown and white meat.

5.   Conclusions

The results of this thesis showed that the main factors explaining the large inter-individual variation of cadmium levels in brown crab are sampling area and sample treatment. Crabs sampled in the north of the Norwegian coast showed significantly higher values than crabs from the South, confirming findings from earlier studies. Cooking and freezing crabs caused a pronounced leakage of cadmium from the hepatopancreas to the claw meat and thereby altered the cadmium distribution.

No clear pattern was seen in the cadmium levels throughout the year in the North and the South. However, in the North, a trend towards higher concentrations was accompanied by a somewhat reduced condition of the crabs, supported by a negative correlation between condition and cadmium concentrations, indicating growth dilution with increasing condition.

The variation in other physiological parameters did not have a strong influence on the cadmium levels. An indication for an accumulation of cadmium over time was observed in the North as a correlation between crab size and total amount of cadmium. Taking into consideration that brown crabs in the North have been shown to have a lower moulting frequency than brown crabs in the South, this could explain the differences between crabs in the North and the South. If crabs are accumulating cadmium over time, a lower growth rate in the North will result in more time for the crab to accumulate cadmium in comparison to crabs of the same size in the South.

In the conducted laboratory study, crabs were found to be highly efficient in assimilating cadmium from ingested feed and no elimination was observed. This underlines the assumption of an accumulation over time. The predicted importance of dietary uptake to the overall accumulation was 98 %.

Sampling of crabs during the year indicated a difference in migration or foraging between crabs in the North and the South. Crabs in the North were not catchable during the coldest period of the year. Considering the importance of the dietary uptake and opportunistic feeding behavior in crabs, a difference in foraging will potentially lead to differences in cadmium levels.

In the comparative study with shore crab, no congruent pattern was found, indicating

that the high accumulation in the North is specific for brown crab, and that an anthropogenic point source is rather unlikely to cause the high levels of cadmium.

As no clear differences in season and physiological parameters were found, no mitigation strategy could be developed. However, the risk assessment revealed that the consumption of claw meat is safe in terms of cadmium regardless of where the brown crabs are caught. Whole crabs and brown meat, however, should be consumed parsimoniously and consumers living along the coast in particular are in danger of exceeding the tolerable weekly intake of cadmium, as they generally consume more brown crab. The establishment of a legal limit for cadmium in the brown meat of crabs would be an important step to ensure consumer protection.

6.   Future perspectives

The evidence we found of the importance of foraging behavior for cadmium levels in crab can potentially explain some of the differences in cadmium found along the Norwegian coast. Further stomach analyses should be conducted, coupled with DNA analysis to get a better idea of the different prey items in North and South. Another approach to find out more about foraging would be using stable nitrogen and carbon isotopes to estimate the trophic level of the crabs in different regions to see if there is a correlation with cadmium levels. This could be expanded on by sampling organisms from several trophic levels to investigate the biomagnification of cadmium, as has been done elsewhere (Signa, et al., 2017b). It has also been shown that some links between dietary regimes and metal accumulation are better detected using fatty acid profiles and a complementary use of stable isotopes and fatty acids is preferable (Le Croizier, et al., 2016).

Since brown crabs are opportunistic feeders, their dietary composition probably highly depends on migratory patterns. However, especially along the Norwegian coast, information about the migration of crabs is scarce. This issue could be addressed with capture-recapture studies using electronic data storage tags, which have shown to be useful for tracking brown crab in the English channel (Hunter, et al., 2013). An alternative approach to investigate the migration patterns is acoustic tracking with ultrasonic transmitters such as those used in a brown crab study on the west coast of Sweden (Ungfors, et al., 2007).

Modern isotopic analysis using multi-collector inductively coupled plasma-mass spectrometry (MC-ICP-MS) has been proven to be a promising tool to trace mercury pollution in brown crabs, by comparing isotopic signatures of the mercury present in crab to the possible sources of mercury (Rua-Ibarz, et al., 2016). There are first attempts to apply the same technique to cadmium, and MC-ICP-MS would be a promising tool to trace back the high cadmium values in Northern Norway to sources like cadmium rich deep-sea water, or anthropogenic sources like mining activity or dumped ammunition.

In our investigations we focused mainly on biota, however physicochemical characteristics of the environment can also be important for the uptake of trace metals and cadmium, and has been shown to be more important for bioaccumulation than pure sediment contamination in a highly contaminated area of the Mediterranean sea (Signa, et al., 2017a). The importance of physicochemical characteristics could be addressed by measuring potentially important parameters like pH; total organic carbon, redox potential and grain size in sediment and water samples from the different areas.

As some crabs showed exceptionally high levels of cadmium, the question of the toxicity of cadmium to brown crab itself could be raised. While the recent literature on the toxicity of cadmium in sub-lethal doses suggests no behavioral changes in other crab species (Blewett, et al., 2017), there is evidence for toxicity on the cellular level, and altered gene expression (Zhou, et al., 2016; Zhou, et al., 2017). As cadmium exposure can also cause cell damage and apoptosis in the hepatopancreas of crab (Lin, et al., 2017), it could lead to feedback mechanisms by releasing detoxified cadmium, causing even more damage. These toxic effects might in turn also influence cadmium accumulation. The high variation in cadmium in crabs gives a good opportunity to investigate dose-response mechanisms using genetic tools in crabs from the field, while laboratory exposure experiments can be difficult because of the varying background concentrations.

So far only organisms abundant in shallow water have been comparatively analyzed for cadmium in the north and south of the Norwegian coast. An investigation of the level of cadmium in Norway lobster *Nephrops norvegicus*, which lives at water depths similar to where brown crab is assumed to migrate to in spring in the North, could deliver valuable information. However, because of a low abundance of Norway lobster in the North, a first fishing attempt was not successful and more effort would be needed to obtain samples from North.

With regard to risk assessment, the bioavailability of cadmium in crab to human beings is an issue important to address. In rats it has been shown that the uptake of cadmium from crab meat is only half of that from a diet fortified with cadmium chloride (Maage

& Julshamn, 1987). In another experiment, cadmium absorption was increased in diets with low calcium and protein content, which is not the case for crab meat (Thévenod, et al., 2013). In humans, up to 8% of ingested cadmium is absorbed, and uptake depends on individual differences such as age, body stores of iron, calcium, zinc, pregnancy history and lactation. However, absorption is also dependent on the levels of ions (zinc, calcium) and other dietary components ingested with cadmium (Thévenod, et al., 2013), and due to the strong binding to MT, the absorption of cadmium from crab brown meat may be difficult. In an *in vitro* study, a trend was visible towards higher bioaccessibility of cadmium from crab meat, when it was cooked. To assess the real risk from cadmium when consuming brown crabs, further studies are needed to investigate exposure, depending on the food type cadmium is ingested with.

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Paper 1

CrossMark

Effects of cooking and freezing practices on the distribution of cadmium in different tissues of the brown crab (*Cancer pagurus*)

Martin Wiech [a], [*], Eirin Vik [a], Arne Duinker [a], Sylvia Frantzen [a], Snorre Bakke [b], Amund Maage [a]

[a] National Institute of Nutrition and Seafood Research, PO Box 2029, Nordnes, N-5817, Bergen, Norway
[b] Møreforskning Ålesund, PO Box 5075, Larsgården, N-6021, Ålesund, Norway

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ABSTRACT

Increased cadmium concentrations in claw meat were demonstrated after cooking and freezing practices of whole brown crabs. This was investigated in crabs from two different locations along the Norwegian coast, one with normal and one with high cadmium concentration. For both locations, in whole crabs, samples of fresh raw claw meat showed lowest values followed by raw-boiled and frozen-thawed-boiled. Cadmium levels in separately cooked claws were comparable to the low values in claws from raw whole crab. Claws taken from frozen crabs before thawing had low values compared to claws taken off the carapace after thawing. This clearly indicates a transfer from hepatopancreas to claw meat, which potentially induces biases when measuring and monitoring Cd levels in crabs. Further, different cooking and storing practices might have profound effects on cadmium intake from eating crabs since concentrations above regulatory limits were found following common household and commercial practices.

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1. Introduction

The edible crab *Cancer pagurus* is an appreciated food item in different countries and its fishery is of significant economic value in different European countries. In the English channel, the catch of crustaceans steadily increases, partly replacing the catch of large finfish (Molfese, Beare, & Hall-Spencer, 2014) which shows the future importance of the crab fishery. In Norway, the annual catch of brown crab tripled within a decade (1993–2003) and has been stable around 5–6000 tons ever since (Norwegian Directorate of Fisheries, 2016). White crab meat from claws and legs is a well-balanced nutritious food, rich in proteins, amino acids and essential elements with a low cholesterol and fat content (Barrento et al., 2009b; Barrento et al., 2009c). However, findings of high values of

cadmium above the legal limit of 0.5 mg/kg ww set by the European Commission (EU, 2006) in claw meat (Julshamn, Nilsen, Valdersnes, & Frantzen, 2012), and even higher values in the commonly consumed hepatopancreas (HP) (Maulvault, Cardoso, Nunes, & Marques, 2013), have raised concerns about food safety (Maulvault et al., 2012a; Noël et al., 2011). High values of cadmium have been found in claws and HP in crabs harvested in Norway (Julshamn et al., 2012), Scotland (Davies et al., 1981; Maulvault et al., 2012a) and the English channel (Barrento et al., 2009a). Measurements along the Norwegian coast have shown a clear pattern with higher values of cadmium in brown crab meat in the north of Norway (Julshamn et al., 2012) which eventually led to a breakdown of the crab fishery in the Salten region. However, crabs are also caught north of this area and commercially processed to different products. In food processing, crabs are either steamed as whole, or claws are taken off and the different body parts processed separately. The traditional and most frequently applied method for preparing crabs in private homes in Portugal is boiling crabs as a whole (Maulvault et al., 2013), which also applies to other European countries.

The toxicity of cadmium towards humans is well known with

* Corresponding author. National Institute of Nutrition and Seafood Research, PO box 129, Nordnes, N-5817, Bergen, Norway.
E-mail addresses: Martin.Wiech@nifes.no (M. Wiech), eirin.vik1@gmail.com (E. Vik), arne.duinker@nifes.no (A. Duinker), sylvia.frantzen@nifes.no (S. Frantzen), snorre.bakke@moreforsk.no (S. Bakke), amund.maage@nifes.no (A. Maage).

renal and bone diseases as most common symptoms (Järup & Åkesson, 2009). Further evidence also implicates cadmium as a risk factor for diseases in other tissues and organ systems, and also at low concentrations (Satarug, Garrett, Sens, & Sens, 2010). The European food safety authority EFSA has determined a tolerable weekly intake (TWI) for Cd of 2.5 μg/kg body weight as a long term intake limit to avoid harmful effects (EFSA, 2009). At present, the intake of Cd by the average European is already close to the TWI without considering consumption of crabs (EFSA, 2012).

Most studies focusing on toxic elements in crabs as well as the current legal limits consider raw tissues, even though they are almost exclusively consumed cooked. The cadmium content varies substantially between different organs in many consumed crab species, with much higher concentrations in HP than in muscle (Davies et al., 1981; Noël et al., 2011; Nørum, Bondgaard, Pedersen, & Bjerregaard, 2005; Rouleau, Gobeil, & Tjälve, 2001). Mobility of Cd under different cooking processes has been shown in fish (Atta, El-Sebaie, Noaman, & Kassab, 1997; Ersoy, Yanar, Küçükgülmez, & Çelik, 2006), mussels (Houlbrèque et al., 2011; Metian et al., 2009) and crustaceans (Abd-Allah & Abdallah, 2006; Jorhem, Engman, Sundström, & Thim, 1994). Although located in different body parts of the crab, there is limited physical barrier between the HP, located in the carapace and the claw meat. Thus, there is a risk in the edible crab that cadmium from HP may contaminate claw meat and other tissues while cooking. Contamination of claw meat by Cd during cooking may impose an increased risk for reaching the legal limit and thereby a critical intake level of Cd. So far, only one study looked at the effect of cooking on the Cd concentration in crab claw meat (Maulvault et al., 2012a) and no difference between raw and cooked crabs was found. However, the brown meat cadmium level was relatively low (mean 5.6–8.4 mg/kg wet weight (ww)) compared to crabs from Northern Norway (mean 16–18 mg/kg ww) (Julshamn et al., 2012). Further, the process of freezing also needs to be considered as it has been shown to influence the cadmium level in saucer scallop (Francesconi, Moore, & Joll, 1993) and sunfish samples (Ney & Martin, 1985). Studies focusing on the natural level of Cd in crabs and processes underlying the accumulation of cadmium, have not always contemplated the effect of sample treatments like freezing. If a transfer occurs during sample treatment, it could have a significant effect on analytical results and thereby study outcomes.

This study was conducted to assess the influence of different cooking and sample preparation methods on the level of cadmium measured in the claw meat of crabs. The aim was to assess 1) the difference in Cd concentrations in HP and claw meat between raw, whole boiled and thawed and whole boiled crabs from geographical areas with high and normal Cd levels, 2) the effect on Cd concentrations in separately boiled or steamed claws and 3) the effect of freezing and thawing on Cd concentrations.

2. Material and methods

2.1. Biological material

Three experiments were performed to elucidate the effects of different cooking and sampling methods. In experiment 1, 60 female crabs were sampled in Vesterålen, Northern Norway (68.7 N, 15.1 E) 05 Nov 2013, where earlier investigations have shown that crabs contain elevated levels of cadmium, and around Hitra, Southern Norway (63.5 N, 9.2 E) 01 Dec 2013. For experiments 2 and 3, 18 crabs were sampled 18 Nov 2014 and 12 crabs were sampled 19–22 Sep 2015, respectively, in Vesterålen, Northern Norway (68.7 N, 15.1 E). Crabs were captured using baited crab pots.

2.2. Experiment 1: effect of freezing and cooking crabs

In each treatment of experiment 1, we used fifteen crabs. Samples of claw meat and brown meat were taken from each crab. Brown meat consisted solely of HP and gonad at different maturation states. In treatment 1 ('raw'), samples were taken from raw, fresh crabs. Before samples were taken, crabs were euthanized by sticking according to best practice regulations Codex Alimentarius (WHO/FAO, 2012). Gill samples were also taken. In treatment 2 ('boiled fresh'), samples were taken after boiling the whole crabs for 15–25 min in 8 L of salted water (50 g NaCl/L). In treatment 3 ('thawed, boiled sw') and treatment 4 ('thawed, boiled fw'), whole crabs were frozen and thawed before being boiled in salted water (50 g NaCl/L) or fresh water respectively. In treatments 2 to 4, several crabs were cooked together in one pot and the water was renewed after each cooking.

2.3. Experiment 2: effect of cooking claws separately

In experiment 2, we investigated, whether the exoskeleton could serve as a source of cadmium, and used frozen crab carapaces and claws cooled on ice. The experiment consisted of two trials. In trial A (n = 10), claw meat of one of the two claws from each crab was analyzed raw ('raw A') and the second claw was boiled ('boiled sw A') separately in salted water (50 g NaCl/L) before sampling. The boiling water was renewed after each cooking to avoid contamination. We took HP samples from the thawed carapace ('defrosted, raw A'). In trial B (n = 8), one claw was boiled ('boiled sw B') for 20 min in salted water (50 g NaCl/L) and the remaining claw was steamed ('steamed sw B') for 15 min over boiling salted water (50 g NaCl/L). The complete inner meat, consisting of HP, gonad and connective tissue (excluding stomach), of the thawed carapace was taken as raw sample ('defrosted, raw B'), as this was done in earlier studies on Cd levels in crabs in Norway (Julshamn et al., 2012). In addition, we took samples of the exoskeleton from all claws after the respective treatments.

2.4. Experiment 3: effect of freezing and thawing

In experiment 3, the aim was to determine if leakage of cadmium occurs during freezing and thawing. Whole crabs (n = 12) were received frozen. One claw was removed from the carapace while still frozen and thawed separately ('claw taken frozen') (n = 12), while the second claw was removed after the crab had thawed ('claw taken thawed') (n = 8). HP was taken after thawing ('defrosted raw'). Samples from claw meat and HP were analyzed.

A schematic illustration of the different treatments in the experiments is given in Fig. 1. In all three experiments, all liquid was collected when dissecting the raw claw meat, and the carapace was emptied of liquid before samples of HP or brown meat were taken. To prevent contamination, all used stainless steel dissection instruments were cleaned between each sample and samples were directly transferred to sampling tubes, avoiding contact with other external surfaces.

2.5. Chemical analyses

After freeze-drying and homogenization of the samples, the analysis of cadmium was performed using ICP-MS as described by Julshamn et al. (2007). In brief, we used ICP-MS (Agilent 7500c) after digestion of samples in a microwave oven (Milstone-MLS-1200). The method is accredited by the Norwegian Accreditation Authority according to NS-EN 17025 and was controlled by use of standard reference material (CRM, Tort 2, National Research
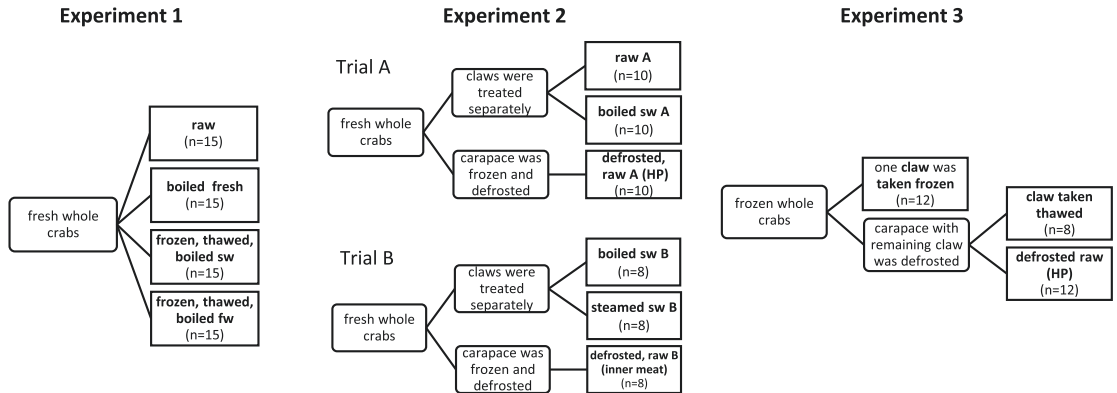
Fig. 1. Schematic illustration of the cooking and freezing practices applied in the different experiments.

Council, Canada). The LOQ_{dw} was set to 0.005 mg/kg dw with standard sample size (0.2 g). The wet weight based LOQ for each individual samples (LOQ_{ww}) was determined as: $LOQ_{ww} = LOQ_{dw}$ x % dry matter$_{sample}$/100.

2.6. Data treatment

In a pilot study it was found that the Cd concentration in gonads represented less than 1% of the Cd concentration in brown meat of raw crabs (0.031 ± 0.032 mg/kg ww (mean ± SD)). Thus, for experiment 1, Cd concentration in HP was calculated using the ratio of dry weight of brown meat neglecting the Cd concentration in gonad:

$$Cd_{HP} = Cd_{brown\ meat} \times dry\ weight_{brown\ meat}/dry\ weight_{HP}$$

With Cd_{HP} being the calculated concentration of Cd in HP and $Cd_{brown\ meat}$ being the actual measured concentration of Cd in brown meat consisting of HP and gonad. The same was assumed for boiled and thawed and boiled crabs.

Differences between the Cd concentration in the different treatments were tested with ANOVA and whenever necessary followed by multiple comparison testing (Tukey HSD). The significance level was 0.05. If homoscedasticity requirements were not fulfilled, data was log-transformed before use. For experiment 1, Pearson's linear correlation coefficient was calculated for Cd in HP and Cd in claws. All statistical analyses were performed with STATISTICA 12 (©Statsoft, Tulsa, USA).

3. Results

The results from all three experiments are presented in Table 1.

A clear difference in Cd concentrations between crabs from Northern and Southern Norway was found for both, claw and brown meat, with higher concentrations in crabs from Northern Norway. This is in line with the results from the national monitoring program (Julshamn et al., 2012).

3.1. Experiment 1: effect of freezing and cooking crabs

The different treatments in experiment 1 led to significantly different Cd values in claw meat. In crabs from Northern Norway, claw meat from raw crabs was lowest with 0.024 mg/kg ww followed by boiled crabs with 0.30 mg/kg ww and highest in crabs thawed and boiled in either freshwater (0.84 mg/kg ww) or salted

water (1.0 mg/kg ww). The difference between boiling crabs in fresh or salted water was not significant. The same pattern was found for crabs from Southern Norway with 0.007 mg/kg ww for raw, 0.065 mg/kg ww for boiled and 0.16 mg/kg ww and 0.10 mg/kg ww for crabs thawed and boiled in freshwater and salted water respectively.

In crabs from Northern Norway, the different treatments caused no statistically significant difference in the Cd concentration in the HP. However, there was a trend with lower concentrations in HP of crabs that were thawed and boiled than in HP of raw crabs. In crabs from Southern Norway, the concentration in HP in freshly boiled crabs was significantly higher than in thawed and boiled crabs in freshwater. Otherwise, no clear trend was seen.

For both locations, the total Cd content in brown meat (consisting of both gonads and HP) showed higher values in raw crabs compared to thawed and boiled crabs. In crabs from Northern Norway, boiling of fresh crabs also resulted in lower values than in raw crabs. Gills showed a significantly higher Cd concentration in crabs form Northern Norway with 0.54 ± 0.14 mg/kg ww (mean ± SD) compared to Southern Norway with 0.36 ± 0.09 mg/kg ww (mean ± SD).

In the crabs from Northern Norway, with generally higher Cd concentrations in HP, strong correlations between the concentration of Cd in HP and claw meat were found in all treatments (coefficients of correlation between 0.65 and 0.95, Table 1). In crabs from Southern Norway, only the correlation between the Cd concentrations in HP and claw meat from raw crabs was statistically significant (r = 0.70).

3.2. Experiment 2: effect of cooking claws separately

Meat from claws, which were cooked separately from the carapace in trial A and B, showed no elevated Cd concentrations compared to raw claw meat with values between 0.020 and 0.027 mg/kg ww.

The Cd concentration in HP in thawed crabs was significantly higher than the concentration in the whole inner meat consisting of HP, gonads and other edible tissues, excluding the stomach.

We found very low Cd concentrations in the exoskeleton of the separately cooked claws with only three values above LOQ (0.04 mg/kg ww) and a maximum of 0.013 mg/kg ww.

3.3. Experiment 3: effect of freezing and thawing

In frozen claws thawed separately from the carapace, the

Table 1
Weight, carapace width (CW) and measured cadmium concentrations in the different tissues of the brown crabs and the correlation between HP and claw meat concentrations. Different letters indicate statistically significant differences within each experiment and column.

Treatment	Weight [g]	CW [cm]	Claw Cd [mg/kg ww]	HP Cd [mg/kg ww]	Total brown meat Cd [mg]	Correlation between HP Cd and Claw Cd	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	r	p
Experiment 1							
Northern Norway							
Raw	589 ± 76 ac	15.6 ± 0.7 ac	0.024 ± 0.012 a	38 ± 28 a	1.15 ± 0.76 a	0.72	<0.01
Boiled fresh	473 ± 51 b	14.4 ± 0.5 b	0.30 ± 0.29 b	44 ± 42 a	0.45 ± 0.26 b	0.95	<0.01
Thawed, boiled sw	553 ± 68 c	15.1 ± 0.7 ac	0.84 ± 0.66 c	22 ± 16 a	0.41 ± 0.28 b	0.95	<0.01
Thawed, boiled fw	592 ± 104 c	15.8 ± 1.0 c	1.0 ± 1.1 c	26 ± 16 a	0.43 ± 0.25 b	0.65	<0.01
Southern Norway							
Raw	492 ± 63 abc	15.1 ± 0.7 abc	0.007 ± 0.005 a	8.4 ± 4.9 ac	0.21 ± 0.14 a	0.70	<0.01
Boiled fresh	555 ± 86 b	15.6 ± 0.8 b	0.065 ± 0.075 b	12 ± 11 ab	0.16 ± 0.12 a	0.33	0.23
Thawed, boiled sw	462 ± 39 c	14.7 ± 0.4 c	0.16 ± 0.09 c	6.8 ± 5.1 ac	0.08 ± 0.07 b	0.39	0.15
Thawed, boiled fw	450 ± 51 c	14.5 ± 0.7 c	0.10 ± 0.08 c	5.7 ± 2.3 c	0.08 ± 0.10 b	0.16	0.58
Experiment 2							
Raw A			0.027 ± 0.047 a				
Boiled sw A			0.020 ± 0.019 a				
Boiled sw B			0.022 ± 0.012 a				
Steamed sw B			0.027 ± 0.019 a				
Defrosted, raw A	459 ± 99 a	14.9 ± 1.1 a		15 ± 9.5 a			
Defrosted, raw B	390 ± 114 a	14.3 ± 1.2 a		5.7 ± 2.3 b			
Experiment 3							
Claw taken frozen			0.011 ± 0.007 a				
Claw taken thawed			0.24 ± 0.23 b				
Defrosted raw	379 ± 93	14.0 ± 0.9		11 ± 11			

concentration of cadmium in the meat was low with a mean of 0.011 mg/kg ww and thereby significantly lower than the concentration in the meat from the claw that was thawing while attached to the carapace with 0.24 mg/kg ww.

4. Discussion

4.1. Effect of cooking method and sample preparation

4.1.1. Claw meat

Our Cd levels found in raw claw meat (Table 1) are in line with other findings of 0.01 (Maulvault et al., 2012a) and levels of about 0.03 mg/kg ww (Barrento et al., 2009c).

Both, cooking and thawing significantly increased Cd values in the claw meat. In Experiment 1, for both locations, boiling whole fresh crabs resulted in about ten—fold higher claw meat Cd concentrations compared to raw claw meat. Freezing and thawing before boiling enhanced this effect, causing at least a further doubling of the claw meat Cd concentrations. In Experiment 3, the freezing and thawing process also increased the Cd concentration in claws that were attached to the carapace during thawing. This clearly indicates a transfer and redistribution of Cd from HP, with its high concentrations, into the claw meat during these processes. Cd in raw crabs is mainly found in the soluble cytosolic fraction bound to metallothionein (MT) (Pedersen et al., 1994). During freezing, water expands and ice crystals form, leading to cell bursting and leakage of the cadmium during thawing. In cooking processes, additionally free Cd ions are partly released, as, although MTs are described as heat stable, the metal link (metal-SH) is vulnerable to heat (Bragigand & Berthet, 2003). As no rise in Cd levels was found once claws were thawed and boiled separately, it can be concluded that the Cd is not transferred from the exoskeleton. In accordance with our findings, Jorhem et al. (1994) noticed a reduction of cadmium concentrations in crayfish HP and a rise of Cd in abdominal muscle comparing raw and boiled crayfish. As high brown meat to white meat Cd ratios are known for other widely consumed crustaceans like Lobster *H. gammarus*, with 101, spider crab *M. squinado*

with 28.5 (Noël et al., 2011), and snow crab *Chiononectes opilio* with 10 (Rouleau et al., 2001), a transfer of Cd into the white meat during cooking is probable for all these important crustacean species.

In similar experiments with bivalves, the Cd concentration in the cooking water increased and cooking processes concentrated Cd in the soft tissue by loss of water (Houlbrèque et al., 2011; Metian et al., 2009). When boiling crayfish, Jorhem et al. (1994) found no changes in heavy metal concentration in the water. The differences in anatomy of bivalves and decapod crustaceans might explain this difference. Bivalves, once the shell opens during boiling, expose a big surface of the inner organs to the cooking water. The almost closed exoskeleton of crabs instead, only allows a limited movement of boiling water in and out of the exoskeleton. Cadmium from HP may therefore to a higher degree redistribute inside the crabs.

4.1.2. Brown meat

Experiment 1 showed that for both locations, the total cadmium content in brown meat (taking into account the wet weight of the total brown meat consisting of both gonad and HP), is highest in the raw crabs with lower values in boiled and thawed and boiled crabs, although not significantly for freshly boiled crabs from Southern Norway. In HP, the levels of Cd were lower in the thawed, boiled treatments, however, not significantly and the values in freshly boiled crabs were even slightly higher than in raw crabs. In an earlier study by Maulvault et al. (2011), an increase in brown meat Cd levels was found after cooking, while in another study conducted by the same group no difference in the brown meat Cd content after cooking was found (Maulvault et al., 2012a). The degree of change in Cd levels in HP due to cooking seems to be less clear. Probably, this is partly related to the large inter-individual variation in Cd levels (see 4.2). The assumption made in calculating the concentration of Cd in HP, that the gonad of the crabs does not contain any Cd might bias the results in the freshly boiled and thawed and boiled crabs. As seen with the claw meat, it is possible that there was some transfer of Cd from HP to the gonad while cooking and thawing. Thereby, the concentrations in HP

might be overestimated and the effect of the treatments under-estimated. The degree of leakage from HP to gonad should be investigated further in future studies, as the female gonad is commonly consumed as a part of the brown meat or could be used separately if taken from raw crabs.

In crabs from Northern Norway, concentrations of Cd in HP and claw meat were strongly correlated in all treatments. In crabs from Southern Norway, correlations between Cd in claw meat and HP were not that pronounced, probably because of lower concentrations in the HP. High variations between the individual crabs might also mask the statistical significance of the effects. Furthermore, several crabs were cooked together in one pot, and although the leakage out of the body is not expected to be pronounced while boiling crabs, it might be enough for some interaction of the concentrations between different crabs through the boiling water. Other factors during crab handling could also influence the final cadmium levels in the different organs. The position and orientation of the crabs while freezing, thawing and cooling off after cooking, as well as lost legs and claws while and before thawing and cooking, could influence the distribution of the liquids and hereby Cd concentrations. Hence, the need to have a detailed standardized protocol for how to process crabs for analysis is evident. There was no difference between the salted water and freshwater treatment, showing that the variation in salt used for preparation in households is of no importance for the Cd content.

4.2. Natural variation of Cd in the edible crab

Cd measurements exhibit a high variability with a mean SD of 80% in raw HP and 87% in raw claw meat. This is confirmed by findings in other studies measuring Cd in the edible crab with a mean SD of 61% in raw HP and 63% in raw claw meat (Barrento et al., 2009b) and 137% in raw HP and 93% in raw claw meat (Maulvault et al., 2012a). Noël et al. (2011) measured Cd levels in crabs originating from France, United Kingdom, Netherlands and Ireland. The values for white meat ranged from <0.020 mg/kg ww and up to 0.587 mg/kg ww underlining the high variability. However, the sample preparation is not explicitly described in this study and the leakage processes demonstrated in our study might have contributed to the high variation. In an investigation by the Food Standard Agency UK (2013) of crabs caught and retailed in UK, cadmium concentrations in HP from whole crabs ranged from 0.61 to 16 mg/kg ww. Here, cooked and fresh samples of different crab species were evaluated together. The high variation might be explained by different biological and natural factors. In the green crab Carcinus maenas the accumulation of cadmium depends on different physiological parameters like ion concentrations, hydration level and volume of tissues which can be interpreted as condition of the crab (Bjerregaard, 1991; Bjerregaard & Depledge, 2002) and is thereby linked to feeding conditions. Also the stage in the moulting cycle and ovarian maturation influences the Cd accumulation in green crabs. In post-moult stages, they accumulated Cd at much higher rates and accumulation decreased during ovarian maturation when exposed to Cd in water (Bondgaard, Nørum, & Bjerregaard, 2000; Nørum et al., 2005). Furthermore age and growth rate could influence the values like seen in fish (Giguère, Campbell, Hare, McDonald, & Rasmussen, 2004).

Our mean value in HP in raw crabs from Southern Norway of 8.4 mg/kg ww is in line with earlier findings of 5.6 mg/kg ww (Maulvault et al., 2011), about 8 mg/kg ww (Maulvault et al., 2012a) and 6—28 mg/kg ww (Barrento et al., 2009b) in crabs from Scotland, and the findings of Noël et al. (2011) in crabs from different countries (UK, FR, IE) with a mean value of 12.8 mg/kg ww. However, our values for Northern Norway with an average of 38 mg/kg ww and a maximum of 87.8 mg/kg ww were exceptionally high. As similar

differences were found earlier along the Norwegian coast (Julshamn et al., 2012), it seems to be a consistent pattern. Further investigations are needed to clarify why this is the case. Dry weight contents in the different tissues were quite stable and showed no significant differences within the different trials and experiments. Accordingly, dry weight and wet weight based results show the same pattern. In contrast, Maulvault et al. (2012a) found higher dry matter contents after steaming compared to boiling (summer 19% and 15%, spring 9 and 5%) and argues for a leaching of water during the cooking process. Measurement in gills of raw crabs showed significantly higher Cd values in Northern Norway compared to Southern Norway. This might be caused by higher concentrations of Cd in the North, as it has been shown in fish that higher Cd concentrations in water results in higher concentration in gills (Giguère et al., 2004). The higher values in the gastro-intestinal tract, here HP, than in gills however, suggest a more important uptake of Cd from food than water. This has been demonstrated in lab experiments in brown crabs (Davies et al., 1981) and green crabs (Bjerregaard, Bjørn, Nørum, & Pedersen, 2005).

4.3. Implications for human consumption and study design

The Cd concentrations in all our analyzed HP and brown meat samples were higher than the EU limit of 0.5 mg/kg set for unprocessed white meat from crab appendages (EU, 2006, 2011), with a total mean value exceeding the EU maximum level by a factor of 35. Considering the high values of Cd found in brown meat in crabs from Norway, a person of 70 kg only needs to consume as little as 4 g of HP from a freshly boiled crab from Northern Norway or 15 g from a freshly boiled crab from Southern Norway, to reach the TWI of 2.5 μg/kg body weight set by EFSA (EFSA, 2009). Also the mean levels in claw meat from the thawed and boiled crabs from Northern Norway and some single values in the cooked crabs of experiment 1 and thawed crabs of experiment 3 exceeded the legal limit of 0.5 mg/kg ww. In the Portuguese and Norwegian population, crabs are mostly consumed in the coastal area with portions of up to 200 g of muscle and inner meat consumed per meal (Bergsten, 2004; Maulvault et al., 2013). Taking into account these consumer habits together with the potential of high catch rates in some regions, people fishing crabs for recreation and consuming their own catch, are in high danger of heavy Cd exposure. In industrial processing, meat of crabs from different origin is often mixed. This, however, is not the case in small-scale recreational fishing, and a repeated exposure to values as high as our maximum individual value of 174 mg/kg ww in HP of a freshly cooked crab can take place. A questionnaire amongst recreational fishermen in the inner Oslofjord showed that over 45% did not know about any contamination in different kinds of fish (Holt, 2015). Thus, low risk perception or lack of knowledge might fortify the risk of high Cd exposure.

The risk of exceeding the TWI is highest when brown meat is consumed (99.6% of the consumers in Portugal (Maulvault et al., 2013)), but should not be neglected either, if white meat in crabs from Northern Norway is consumed, given that crabs are prepared in the traditional way of boiling the whole crabs. An assessment based on the existing consumer data in the Norwegian population, considering the Cd intake from other sources then crab meat, concluded that consumers of high amounts of crab brown meat and especially adolescences are at high risk of exceeding the TWI (VKM, 2015). In contrast, treatments based on cooking the claws separately, result in a safe product in regards to Cd. We agree with the conclusion of Maulvault et al. (2013) that white crab meat is a healthy food item if adequate processing methods are used. Considering our findings, even crabs with high values in the HP can be processed if claws are treated separately from HP. Furthermore,

gonads of the females could be safely consumed if taken from raw crabs. Our findings emphasize the importance of choosing the right sample preparation method coinciding with the aim of the study. If conclusions about food safety regarding to Cd are drawn, samples must be prepared according to common household or commercially used practices. Otherwise, values are prone to overestimation (HP) or underestimation (white meat) and cannot serve as the basis of risk assessment. Similar findings are present for other heavy metals in seafood (Atta et al., 1997; Ersoy et al., 2006; Jorhem et al., 1994) and also other characteristics like chemical composition, fatty acids profile, macro and trace elements (Maulvault et al., 2012b).

5. Conclusions

This study evidenced the influence of cooking and freezing on the Cd content in claw and brown meat of the edible crab. The results strongly suggest that the process of cooking crabs whole leads to a leakage of Cd from HP to claw meat. In crabs with high levels of Cd in HP this resulted in values in claw meat above the maximum legal limit. Freezing and thawing enforced the effect with even higher values after cooking the crabs whole. Claw meat from separately cooked claws had Cd values comparable to raw claw meat, which were low and unproblematic for human consumption. Furthermore, the freezing and thawing process of whole crabs from Northern Norway with high levels of Cd in HP led to values of Cd in raw claw meat above the maximum legal limit. Thus, if natural levels of Cd are investigated, care should be taken if freezing of the samples is necessary. Our results strongly suggest the consideration of the cooking process when assessing food safety of the edible crab regarding Cd.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Paper 2

Tracing Simultaneous Cadmium Accumulation from Different Uptake Routes in Brown Crab *Cancer pagurus* by the use of Stable Isotopes

Martin Wiech[a,d], Heidi Amlund[a], Karl Andreas Jensen[b], Tom Aldenberg[c], Arne Duinker[a] and Amund Maage[a,d]

[a]Institute of Marine Research, P.O. Box 1870, Nordnes, NO-5817 Bergen, Norway

[b]Institute of Environmental Sciences, Norwegian University of Life Sciences, P.O. Box 5003, NO-1432 Ås, Norway

[c]National Institute for Public Health and the Environment, P.O. Box 1, NL-3720 BA Bilthoven, The Netherlands

[d]University of Bergen, P.O. Box 7800, NO-5020 Bergen, Norway

Abstract

High concentrations of cadmium in brown crab are an issue of food safety, and large variations between different areas have been found. To investigate the relative importance of dietary and aqueous uptake regarding the overall accumulation in brown crab, we used stable isotopes to trace the uptake from both routes simultaneously in the same animals. We demonstrated that the analytical challenges regarding background concentrations of natural isotope distribution and polyatomic interferences in the different matrices can be overcome with an appropriate analytical setup and modern mathematical corrections using a computer software. Cadmium was accumulated from both routes and was found in all measured organs at the end of the exposure phase. The obtained data was used to establish accumulation curves for both uptake routes and estimate accumulation parameters for hepatopancreas, as the most important organ in crab regarding total cadmium body burden. Using the estimated parameters in combination with naturally relevant cadmium concentrations in seawater and diet in a model, allowed us to predict the relative importance of the aqueous and dietary uptake route to the total hepatopancreas burden. According to the prediction, the dietary route is the main route of uptake in brown crab with a minimum of 98% of the accumulated cadmium in hepatopancreas originating from diet. Future studies addressing the source and accumulation of cadmium in crab should therefore focus on the uptake from feed and factors connected to foraging.

Key words**:** *Cancer pagurus*; cadmium; accumulation; stable isotope; gavage feeding; kinetic modelling

Corresponding author: Martin Wiech, Institute of Marine Research, P.O. Box 1870, Nordnes, NO-5817 Bergen, Norway, tel: +47 45159792; email: Martin.Wiech@hi.no

1. Introduction

The brown crab (*Cancer pagurus*) is an appreciated seafood species with an increasing value and a global catch of about 50 000t (FAO, 2017) with about 5 000 tons harvested in Norway in 2016 (Søvik, et al., 2017). However, elevated concentrations of cadmium (Cd) in the hepatopancreas (HP) and claw meat of cooked brown crab in several European countries (Barrento, et al., 2009; Julshamn, et al., 2012; Maulvault, et al., 2012) has become a food safety concern (Maulvault, et al., 2012; Noël, et al., 2011). In the North-East Atlantic Ocean, an interesting pattern was seen in crabs caught at the Norwegian Coast. The highest Cd values were found in crab in the North and claw meat concentrations have regularly been found to exceed the current legal limit of 0.5 mg/kg ww set by European Union (Julshamn, et al., 2012; Wiech, et al., 2017). The coast of Northern Norway is regarded a rather pristine area and the occurrence of high concentrations of Cd in crab therefore arouse public concern and scientific interest in finding the reason for the high levels. In general terms, trace elements, except methyl-mercury are not expected to biomagnify along the food chain (Fisher & Reinfelder, 1995). To elucidate the cause of the high Cd levels, it is important to understand how Cd is taken up and retained in brown crab.

The uptake of metals in crab can occur via two different routes: from water over the gills, or via the dietary route from ingested diet. The importance of these routes regarding the overall metal concentration at equilibrium can be determined using a kinetic model when assimilation efficiency, ingestion rate, and unidirectional uptake and elimination rate constants are known for the species in question (Luoma & Rainbow, 2005; Wang, et al., 1996).

To produce data sufficient for a reliable parameter estimation, radioisotopes have often been used to trace the accumulation of metals. However, the use of radioisotopes has some drawbacks (see Croteau et al (2004)) and since the recent developments in inductively coupled plasma mass spectrometry instrumentation (ICP-MS), the use of stable isotopes has become a good alternative. The use of stable isotopes has proven to be adequate to investigate the uptake of metals from water and feed in bivalves (Croteau, et al., 2004; Strady, et al., 2011). In *Daphnia magna* also interaction effects of metals were successfully studied using stable isotopes (Komjarova & Blust, 2008, 2009). Strady et al. (2011) have further shown the potential of using stable isotopes to simultaneously trace aqueous and dietary uptake in the same animals in the case of oysters. A prerequisite for simultaneous tracing is that the uptake from the different routes is non-competitive. In crab, Cd is mainly present in HP and almost entirely bound to metallothionein (MT) (Pedersen, et al., 1994; Pedersen, et al., 1998). As the binding capacity for Cd ions in MT is limited, expression is induced at a certain level (Pedersen, et al., 2014) and

exceedance could lead to a competitive accumulation of Cd.

One challenge when using stable isotope tracing lies within the chemical analysis. Stable isotopes, are part of the natural isotope distribution of an element and are therefore abundant wherever natural Cd is present in the experiment. Therefore, high background concentration is expected. Another analytical issue when using ICP-MS are polyatomic interferences on all Cd masses in the different tissues. These challenges need to be addressed to enable the detection of Cd in tissues of animals exposed to low naturally relevant concentrations.

Aqueous uptake of Cd in branchuryan crabs has been studied closely in the green crab *Carcinus meanas*, a species partly sharing the habitat with brown crab. Various factors such as temperature, salinity, exposure concentration, calcium concentration, moulting stage, ovarian stage and feeding status influencing the uptake of Cd from water, have been identified (see Bjerregaard, et al. (2005)). The dietary uptake route has not been studied equally well (Pedersen, et al., 2014), although a comparative study indicated that the uptake from feed contributes most to the overall Cd accumulation in green crab (Bjerregaard, et al., 2005).

A recent study has quantified the Cd concentrations in green crabs along the Norwegian coast and found a different pattern between green and brown crab. For green crab, there was no clear difference in Cd concentrations between crabs from North and South (Knutsen, et al., under review), as seen in brown crab (Julshamn, et al., 2012). This indicates that there might be differences in uptake and elimination processes in the two species, as already known for other crab species (Rainbow & Black, 2005a, 2005b). The accumulation of Cd in brown crab, although commercially important, has not gotten much attention. To our knowledge, only Davies et al. (1981) investigated the uptake of Cd from feed and water in brown crab and concluded that dietary uptake exceeds aqueous uptake. However, deep-freezing of crabs before dissection make the results uncertain, as this can have a significant influence on the Cd concentrations in the different organs and can mask the actual distribution of Cd (Wiech, et al., 2017). In general, the importance of the different uptake routes in brown and green crab have been estimated based on assimilation efficiencies for dietary uptake and concentration factors for aqueous uptake, often only considering data from the end of the exposure phase (Bjerregaard, et al., 2005). Further, concentrations of Cd in prey and seawater, and ingestion rates under natural conditions were not taken into account (Davies, et al., 1981) adding uncertainty to the results and making a direct comparison of uptake routes difficult.

In the present study, we wanted (1) to determine accumulation parameters of Cd in brown crab from aqueous and dietary route at the same time in the same animal, by (2) applying the method of stable isotope tracing. To address the observation in Northern Norway, (3) the importance of the different uptake routes was estimated using a modelling approach.
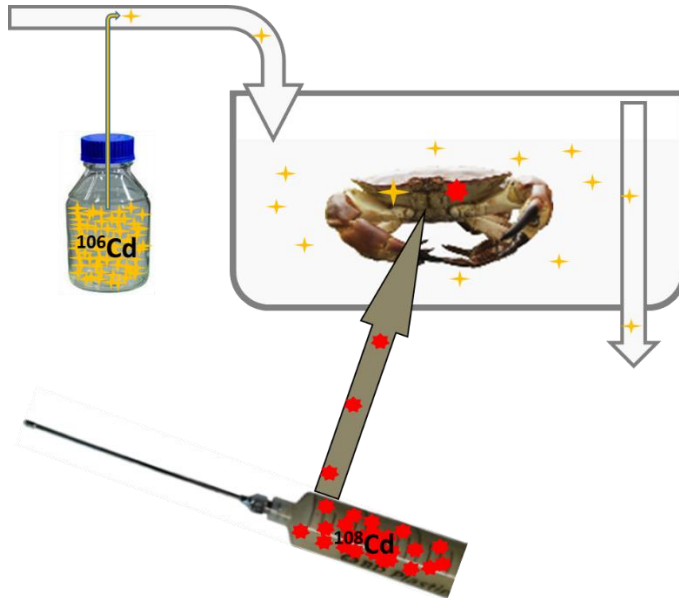
2. Material and Methods

Figure 1. Schematic illustration of the methodological approach, where Cd accumulation from water and feed was traced simultaneously in the same animal using two different stable Cd isotopes.

2.1 Experimental Animals

Female, intermoult brown crabs (*Cancer pagurus*) (n=156) with a carapace width of 131 ± 5 mm (mean $\pm$ SD), caught with baited traps in September 2016 around the southern tip of Sotra, Norway, were used in the experiment. Prior to the experimental period, crabs were acclimated to the laboratory conditions at Austevoll Research Station, Institute of Marine Research, Norway, for minimum five days, before the controlled feeding regime was established. The claws of the crabs were tied with a rubber band to avoid cannibalism and provide safety for the personnel handling the animals. Each of the rubber bands was carrying a number for identification of individual crabs. The animal handling and experimental protocols were approved by the Norwegian Food Safety Authority (FOTS ID 8845) and performed in accordance with the Norwegian and European law for the use of animals in experiments.

2.2 Experimental Setup

During the experimental period of 96 days starting 04 Oct 2016, the crabs were maintained in two 900 L tanks (control and exposure) in two levels of plastic baskets (34x25x16 cm) at a maximum density of 32 crabs/m^2. Crabs were mainly kept in darkness with only slight exposure

to the natural light regime. Seawater was taken from 160 m water depth, sand-filtered and continuously exchanged at least ten times daily, and the pressure regulated using valves with flow-meters. Water temperature was measured daily and ranged from 7.2 to 9.0 ºC during the experimental period. Salinity was measured to 35 ppt and pH to 8.0 at start and end of the experiment. Aeration with air stones was used to obtain a sufficient oxygen saturation (> 88 %) and a homogenous mixture of the water. To minimize potential desorption of Cd from feces to the water, the tank was flushed and cleaned two to three times a week.

2.3 Feeding

Gavage feeding was applied in order to know the exact amount of feed ingested. Crabs from control and exposure tank (see 2.4 for exposure) were taken out of the water and fed individually with 6 mL feed per week, by feeding them two or three times with 2 or 3 mL, respectively (Ingestion rate I: 2.36 $mg_{feed} \cdot g_{crab}^{-1} \cdot day^{-1}$ ww or 9.39 $mg_{feed} \cdot g_{HP}^{-1} \cdot day^{-1}$ dw) using a disposable plastic syringe with gavage needle (15G, 1.8 x 80 mm, Jørgen Kruuse A/S, Denmark). The feed was a slurry prepared from codfish powder (cooked, dried and micro milled cod fillet, Seagarden AS, Norway) sieved through 200 µm and mixed with deionized water in a blender to a dry weight content of 22.5 %. Gavage feeding is only possible when crabs are moving their mouth parts voluntarily, which can take minutes. A few drops of deionized water flavored with shrimp powder (Seagarden AS) was an effective stimuli for the crabs to open their mouth parts and the feeding time could be shorten to approximately under one minute per crab. To impede crabs from spitting out the feed, they were kept out of the water for minimum 30 seconds after feeding.

2.4 Exposure

Crabs in the exposure tank (n=78) were exposed to Cd in seawater (0.5 µg [106]Cd/L) and in feed (1 mg [108]Cd/kg wet weight) (Figure 1) for 42 days, followed by a depuration phase of 56 days. To obtain an accurate concentration in feed, a stock solution enriched with [108]Cd (Neonest AB/BuyIsotope.com, Stockholm, Sweden) was added and the mixture homogenized by stirring. For the spiked sea water, a stock solution enriched with [106]Cd (Neonest AB/BuyIsotope.com, Stockholm, Sweden) was dosed using a peristaltic pump (Watson-Marlow). Flow was checked daily. In addition, during the exposure phase, weekly water samples from the exposure tank were measured in the exposure phase and a concentration of 0.518 ± 0.010 µg [106]Cd/L (mean ± SD, n=7) and a maximum of 0.002 µg [108]Cd/L (n=7) was found. During the depuration phase the highest measured [106/108]Cd concentration in water was 0.002 µg/L (n=6). In the control tank,

the highest measured concentration of $^{106/108}$Cd concentration was 0.001 µg/L (n=4). The enriched feed contained 1.01 ± 0.03 mg ^{108}Cd /kg ww (mean ± SD, n=5).

2.5 Sampling

Samples of HP, gills, hemolymph, claw meat and gonad were collected from five individuals per treatment on day 0, 2, 7, 14, 21, 30 and 42 in the exposure phase and at day 2, 4, 7, 21, 35 and 56 in the depuration phase. Hemolymph was drawn through the arthodial membrane of the posterior pereiopod using a disposible syringe. Then, crabs were humanely sacrificed (WHO/FAO, 2012) piercing the two main nerve ganglia according to Baker (1955). Crabs were dissected fresh. Gills were squeezed to remove the contained liquid and blotted dry using tissue paper. All samples were kept on ice during sampling and frozen as soon as possible after and kept at -20°C until ICP-MS analysis. For HP a subsample was kept at -80°C for the measurement of MT.

Total weight and carapace width (CW) before and after the experiment, gonad maturation stage according to Haig et al. (2016), and gonad and HP weight was determined for each crab. Further, gonadosomatic index (weight of gonad/CW^2 ·100) and hepatosomatoc index (weight of HP/ CW^2 ·100) were calculated. To assess if there were statistically significant physiological differences between crabs sampled at the different sampling days, data was analyzed using ANOVA. Data was checked for homoscedasticity (Levene`s test) and log-transformed, if necessary. For categorical parameters (gonad maturation stage), non-parametric statistics was applied. The significance level was p=0.05. The analysis was done using STATISTICA 12 (©Statsoft, Tulsa, USA).

2.6 Chemical Analysis

2.6.1 ICP-MS Analysis

All isotopes of Cd were measured with a tandem quadrupole Agilent 8800 ICP-MS with collision/reaction cell (CRC). The use of NH$_3$ as reaction gas was found to be the most efficient for removing polyatomic interferences on all Cd isotopes. ^{103}Rh was used as online internal standard. A control standard was analyzed every ten samples as drift check, both for concentration and mass bias. Masses 106, 108, 110, 112, 113, 114 and 116 were corrected from isobar overlapping from Pd, In and Sn. All isotope ratios were measured in pulse detector mode. Samples were diluted if concentrations exceeded the pulse mode limit (< 1.2 Mcps/s) due to nonlinear calibration between puls and analog mode. The instrumental setup is shown in Appendix A1.

2.6.2 Sample Preparation

Tissue samples were freeze-dried (Freezone, Labconco, US) before being homogenized. Approximately 0.2 g (dry weight) of the homogenized sample and certified reference material (CRM) was microwave digested (Ethos, Milestone, Italy) with 2 mL HNO_3 and 0.5 mL 30% H_2O_2. After digestion, samples were diluted to 50 mL with deionized water. The CRM were Lobster HP (TORT 3, National Research Council Canada) and Oyster Tissue (1566b, National Institute of Standards and Technology, USA). All HP, gill and hemolymph samples were analyzed while for claw meat and gonad only samples taken at day 42 were analyzed.

2.6.3 Calibration

A Cd standard with a naturally abundant isotopic composition (Inorganic Ventures, Christiansburg, USA) was used for calibration and the Cd concentration (w/V) for each isotope was calculated from the natural mass percent abundance. Using this calibration, mass bias corrected isotope ratio (IR), cps_1/cps_2 equals C_1/C_2, where cps denotes counts per second on the detector and C denotes the isotope concentration (w/V) of isotope 1 and 2. Cadmium concentrations, for all isotopes measured in TORT-3 (n=7) and NIST 1566b (n=6) were within the certified ranges.

2.6.4 Correction for background Cd

The ^{114}Cd is the naturally most abundant isotope and was selected for correcting the natural contributions of ^{106}Cd and ^{108}Cd and for estimating the natural total Cd concentration. The enriched ^{106}Cd and ^{108}Cd isotope standards used for spiking of water and feed (enrichment levels of 73.1 and 69.9 mass %), also contained all other Cd isotopes. Thus, contributions from the isotope standards on ^{114}Cd, the contribution from ^{106}Cd isotope standard on ^{108}Cd and the contribution from ^{108}Cd isotope standard on ^{106}Cd had to be corrected for. This was accomplished using equation 1 to 9 as input to solve equation 10 to 12 to calculate Cd in the crab accumulated from water ($^{106}Cd_w$), feed ($^{108}Cd_f$) and the natural background concentration ($^{114}Cd_n$). The equations were solved using the software wxMaxima 16.12.0 (http://andrejv.github.io/wxmaxima). The input equations were:

$$C_{114} = C_{w,114} + C_{n,114} + C_{f,114}$$
(1)

$$C_{106} = C_{w,106} + C_{n,106} + C_{f,106}$$
(2)

$$C_{108} = C_{w,108} + C_{n,108} + C_{f,108}$$
(3)

$$IR_{f,108} = C_{f,108} / C_{f,114}$$
(4)

$$IR_{w,106} = C_{w,106} / C_{w,114}$$

(5)

$$IR_{n,106} = C_{n,106} / C_{n,114}$$

(6)

$$IR_{n,108} = C_{n,108} / C_{n,114}$$

(7)

$$IR_{f,106} = C_{f,106} / C_{f,114}$$

(8)

$$IR_{w,108} = C_{w,108} / C_{w,114}$$

(9)

where C denotes total measured concentration (w/V) of the respective isotopes and w, n and f are contributions from isotope standard in water, natural contributions and contributions from isotope standard in feed, respectively. IR denotes the measured mass bias corrected isotopic ratio from original water, feed and natural isotope ratio before exposure. The solution equations were:

$$^{106}Cd_w = -\frac{C_{114}(IR_{f,108}IR_{n,106}IR_{w,106} - IR_{f,106}IR_{n,108}IR_{w,106}) + C_{106}(IR_{n,108}R_{w,106} - IR_{f,108}IR_{w,106}) + C_{108}(IR_{f,106} - IR_{n,106})IR_{w,106}}{IR_{n,106}IR_{w,108} - IR_{f,106}IR_{w,108} + IR_{f,108}(IR_{w,106} - IR_{n,106}) - IR_{n,108}IR_{w,106} + IR_{f,106}IR_{n,108}}$$

(10)

$$^{108}Cd_f = -\frac{C_{114}IR_{f,108}(IR_{n,108}IR_{w,106} - IR_{n,106}IR_{w,108}) + C_{106}IR_{f,108}(IR_{w,108} - IR_{n,108}) + C_{108}IR_{f,108}(IR_{n,106} - IR_{w,106})}{IR_{n,106}IR_{w,108} - IR_{f,106}IR_{w,108} + IR_{f,108}(IR_{w,106} - IR_{n,106}) - IR_{n,108}IR_{w,106} + IR_{f,106}IR_{n,108}}$$

(11)

$$^{114}Cd_n = \frac{C_{114}(IR_{f,108}IR_{w,106} - IR_{f,106}IR_{w,108}) + C_{106}(IR_{w,108} - IR_{f,108}) + C_{108}(IR_{f,106} - IR_{w,106})}{IR_{n,106}IR_{w,108} - IR_{f,106}IR_{w,108} + IR_{f,108}(IR_{w,106} - IR_{n,106}) - IR_{n,108}IR_{w,106} + IR_{f,106}IR_{n,108}}$$

(12)

The natural total concentration of Cd in samples are calculated from $^{114}Cd_n$ multiplied by 1/natural abundance of ^{114}Cd. Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on control group concentrations using equations 10 and 11. LOQs increased with increasing concentration and range of natural Cd in the control group (Appendix A2). Concentrations of naturally abundant Cd from the samples had no significant effect on the results as they were subtracted by using the equations for the added enriched isotopes on the treatment group.

2.7 Determination of Metallothionein

The concentration of MT in HP was examined in five crabs of the exposure group at start (t=0d) and end (t=42d) of the exposure phase. Metallothionein was measured spectrophotometrically at wavelength 412 nm after extraction and derivatization of thiols with Ellman`s reagent DTNB as described by Viarengo et al. (1997). The method was shown to be suitable for green crab (Pedersen, et al., 1997). A standard curve was made using glutathione. Assuming a fixed ratio of thiol groups between glutathione and MT of 1:19 and a molecular weight for MT of 5800 (Overnell, 1986), the concentration of MT could be calculated.

2.8 Modelling the Accumulation Parameters

Crabs accumulate about 90% of the total Cd body burden in HP (Bjerregaard & Depledge, 2002). Therfore, the accumulation in HP was used as a proxy for the accumulation of Cd in the whole crab. All modelling was based on data of Cd concentrations in HP.

To estimate the parameters describing the accumulation of Cd in HP for both uptake routes (aqueous and dietary), data was fitted to standard bioaccumulation equations derived from OECD TG 305 (2012) and related guidance document (OECD, 2017) :

$$Cd_{\text{crab}}(t) = \begin{cases} C_{\text{input}} \cdot \dfrac{k_1}{k_2} \cdot \left(1 - \exp(-k_2 \cdot t)\right) & \text{for } 0 \leq t < t_{\text{dep}} \\ Cd_{\text{crab}}\left(t_{\text{dep}}\right) \cdot \exp\left(-k_2 \cdot \left(t - t_{\text{dep}}\right)\right) & \text{for } t \geq t_{\text{dep}} \end{cases} \qquad (13)$$

We neglected growth of the crab, since it was zero during the course of the experiment. To apply the generic model for either exposure route, we adapted the exposure concentration and uptake rate constant to the respective experimental condition as follows:

$Cd_{\text{crab}}(t)$ (aqueous): $Cd_{\text{crab}}^{\text{w}}$, Cd concentration in HP of the crab over time [µg ^{106}Cd · kg crab^{-1}],

         (dietary): $Cd_{\text{crab}}^{\text{f}}$, Cd concentration in HP of the crab over time [µg ^{108}Cd · kg crab^{-1}],

C_{input} (aqueous): C_{w}, water exposure concentration [µg ^{106}Cd · L^{-1}],

         (dietary): C_{f} feed exposure concentration [µg ^{108}Cd · kg feed^{-1}],

k_1 (aqueous): k_{w}, uptake rate constant from water [L · kg crab^{-1} · d^{-1}],

         (dietary): k_{f}, uptake rate constant from feed [kg feed · kg crab^{-1} · d^{-1}],

k_2 (both aqueous and dietary): k_{e}, elimination rate constant [d^{-1}],

t independent variable time [d],

t_{dep} onset of the depuration phase [d].

At $t = 0$, the initial concentration in the crab equals zero:

$Cd_{\text{crab}}(0) = 0$,

while at the onset of the depuration phase, i.e. $t = t_{\text{dep}}$, one has, in the generic form:

$$Cd_{\text{crab}}\left(t_{\text{dep}}\right) = C_{\text{input}} \cdot \frac{k_1}{k_2} \cdot \left(1 - \exp(-k_2 \cdot t_{\text{dep}})\right) \qquad (14)$$

which follows from equation 13, first part, when t approaches t_{dep}.

Generally, when $k_2 > 0$, the accumulation curve of Cd in the crab during uptake phase will

be concave and increasing, while the depuration curve of Cd in the crab over time will be convex and decreasing.

However, because of the shape of our data in the depuration phase (Figure 3) and the statistical analysis of the applied models (Appendix A3), two versions of the model were applied: one with k_2 being unconstrained, i.e. allowed to have any value, and one with k_2 constrained to be zero. For the case of a constrained elimination rate, i.e. $k_2 \rightarrow 0$, elimination is assumed to be negligible.

Since, with k_2 very small, we have $\left(1 - \exp(-k_2 \cdot t)\right) \approx \left(1 - (1 - k_2 \cdot t)\right) = k_2 \cdot t$, the approximate model equations (generic form) become:

$$Cd_{\text{crab}}(t) = \begin{cases} C_{\text{input}} \cdot k_1 \cdot t & \text{for } 0 \leq t < t_{\text{dep}} \\ C_{\text{input}} \cdot k_1 \cdot t_{\text{dep}} & \text{for } t \geq t_{\text{dep}} \end{cases}$$

(15)

In this case, the uptake curve will essentially be linear over time, with the level of accumulation nearly constant from the onset of the depuration phase onwards.

For the dietary uptake route, we additionally considered the sub-model

$$k_{\text{f}} = \alpha \cdot I$$

(16)

with I the ingestion rate of feed in the experiment [kg feed $\cdot$ kg crab$^{-1} \cdot$ d^{-1}], and α the assimilation efficiency, as a dimensionless constant.

2.8.1 *Model fitting and statistical analysis*

The model equations (13) define Cd concentration in crab as a function of time, with separate branches relating to the respective uptake and elimination phases. The model is nonlinear in the unknown parameters, k_1 (k_w or k_f, respectively) and k_2 (k_e in both cases), hence, fitting the models to the measured time series basically is a problem of nonlinear regression. Initially, we allowed k_e to be fitted separately for each of the routes, before constraining it to zero.

We used the R-package bcmfR_0.3-2.zip, as distributed by OECD (Aldenberg, 2017) with additional enhancements for the negligible elimination rate case, and supplementary routines for summarizing regression output, as well as estimating parameter and prediction uncertainty. The predictive limits of the model fits were calculated with the Bayesian bootstrap (Rubin, 1981, 1987).

The fitting procedure used was the nonlinear least squares regression function nls from the base R-package stats. Both untransformed Cd accumulation/depuration data were fitted, as well as

log10-transformed accumulation/depuration data with the method of 'transform-both-sides' (Ritz & Streibig, 2008).

The regression quality was assessed through the Shapiro-Wilk test for normality of the regression residuals and Q-Q plots that compares the distribution of the standardized residuals to a standard Normal distribution. We employed implementations for both assessments from the R-package nlstools (Baty, et al., 2015).

2.9 Modelling the Relative Importance of the Uptake Routes

To compare the relative importance of the aqueous route RI_{water} and dietary route RI_{feed} to the overall accumulation of Cd in crab at different feed and water concentrations, we consider $k_e = 0$ and defined them as:

$$RI_{water}(t)$$
$$= \frac{Cd_{crab}^{w}(t)}{Cd_{crab}^{w}(t) \; + \; Cd_{crab}^{f}(t)} \tag{17}$$

and

$$RI_{feed}(t)$$
$$= \frac{Cd_{crab}^{f}(t)}{Cd_{crab}^{w}(t) \; + \; Cd_{crab}^{f}(t)} \text{, respectively.} \tag{18}$$

Adapting the generic equation 15 to the respective uptake route and substituting into equation 17 and 18 for the respective route, (t) cancels and the relative importance of the aqueous route RI_{water} becomes:

$$RI_{water} =$$
$$\frac{k_w \cdot C_w}{k_w \cdot C_w + k_f \cdot C_f} \tag{19}$$

and the relative importance of the dietary route RI_{feed} becomes

$$RI_{feed} =$$
$$\frac{k_f \cdot C_f}{k_w \cdot C_w + k_f \cdot C_f} \text{, respectively} \tag{20}$$

Considering equation 16, we got the final equations used for the calculations of the relative importance of the uptake routes in percent (Figure 4):

$$RI_{\text{water}} = \frac{k_{\text{w}} \cdot C_{\text{w}}}{k_{\text{w}} \cdot C_{\text{w}} + \alpha \cdot I \cdot C_{\text{f}}} \cdot 100$$

and

$$RI_{\text{feed}} = \frac{\alpha \cdot I \cdot C_{\text{f}}}{k_{\text{w}} \cdot C_{\text{w}} + \alpha \cdot I \cdot C_{\text{f}}} \cdot 100$$

with I being adapted to a more natural feeding rate of 79.4 $\text{mg}_{\text{feed}} \cdot \text{g}_{\text{HP}}^{-1} \cdot \text{day}^{-1}$ dw according to Woll et al (2006) and adjusted to HP and dry weight according to the ratio of total crab weight and HP weight and average dry weight content from crabs used in the present study. To illustrate the relative importance of the uptake routes of Cd for brown crab at different concentrations found in feed [µg/kg dw] and seawater [µg/L], the concentrations were illustrated as cadmium concentration ratio (CCR) being $C_{\text{f}}/C_{\text{w}}$.

3. Results and Discussion

Although low and environmentally relevant concentrations were used in water and feed and the background Cd concentrations in the wild-caught crabs were high and strongly varying, it was possible to reliably detect and quantify even low contributions of Cd from both uptake routes in all measured tissues except claw meat (Figure 3 and Appendix A4). This was made possible by using mathematical corrections preventing the natural concentrations of Cd to outweigh the signal from spiked and accumulated Cd isotopes. The issue of plasma formed polyatomic mass interferences on all Cd isotopes was overcome using NH_3 as reaction gas in the CRC.
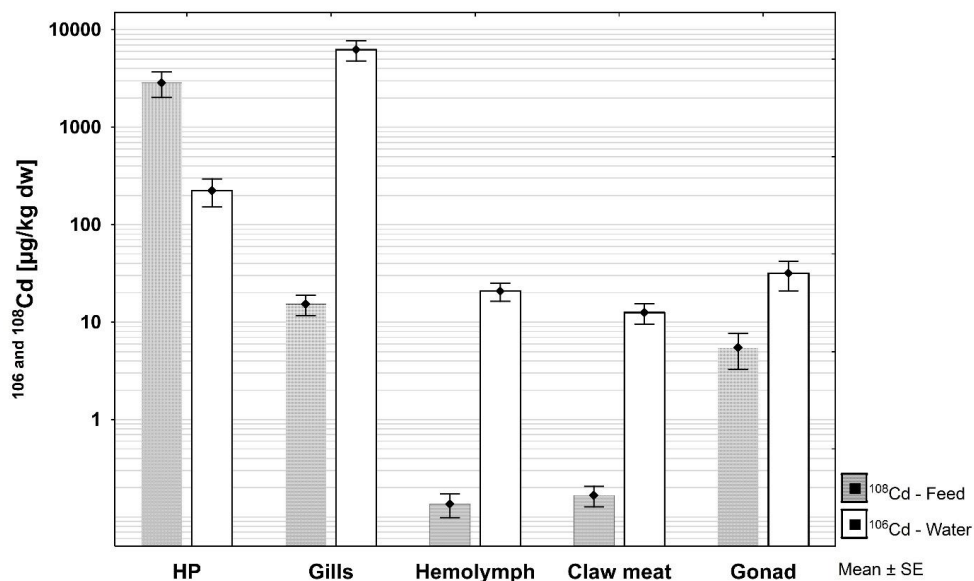
Figure 2. Distribution of Cd taken up from feed and water in the different tissues after 42 days of exposure. Filled columns show mean concentrations of ^{108}Cd taken up from feed and the clear columns concentrations of ^{106}Cd taken up from water. Whiskers represent standard errors (n=5).

At the end of the exposure phase, most Cd from feed was accumulated in HP followed by gills, gonad, hemolymph and claw meat (all concentrations < LOQ) with concentrations of 2850 ± 1870 µg/kg dw, 15.3 ± 8.0 µg/kg dw, 5.47 ± 4.96 µg/kg dw, and 0.14 ± 0.08 µg/kg dw (2 values < LOQ) (mean ± SD, n=5), respectively. A similar tissue distribution was found in green crab fed six meals of ^{109}Cd labelled blue mussels soft parts over 11 days (Bjerregaard, et al., 2005). Considering the total body burden, HP was by far the organ accumulating most Cd with 91 ± 4 % of the total body burden of the traced Cd. Much less Cd was found in the other organs with on average 0.03 to 4.6 % (Bjerregaard, et al., 2005).

Cadmium accumulated from water, was mainly found in gills at the end of the exposure phase,

followed by HP, gonad, hemolymph and claw meat with concentrations of 6235 ± 3240 µg/kg dw, 224 ± 159 µg/kg dw, 31.6 ± 23.8 µg/kg dw, 20.8 ± 9.8 µg/kg dw and 12.5 ± 6.7 µg/kg dw (n=5) (mean ± SD), respectively.

Gills were also found to have higher Cd concentrations than HP in green crabs after exposure to 100 pm ^{109}Cd/mL for 27 d (Bjerregaard & Depledge, 1994). In that study, the concentration in muscle was much higher than in hemolymph (Bjerregaard, et al., 1994). The difference to our studies, might be due to the fact that we only analyzed muscle meat from claw, which might contain lower Cd concentrations than muscle meat from other parts of the crab like the thoracic sternum, as the proximity to the HP might lead to higher Cd concentrations. Norway lobster, *Nephrops norvegicus,* also accumulated Cd from feed mainly in HP, however when exposed to Cd in seawater, the concentration in gills were not higher than in HP (Canli & Furness, 1995).

The concentration factor (C_w/C_{HP}) for the aqueous uptake of Cd in HP was 10.6 ± 7.6 (mean ± SD, n=5) at the end of the exposure phase, which is comparable to findings in green crab using similar exposure conditions with factors of 6.9 ± 7.5 and 6.5 ± 5.1 at concentrations of 173 and 800 ng/L respectively (Bjerregaard, et al., 2005).
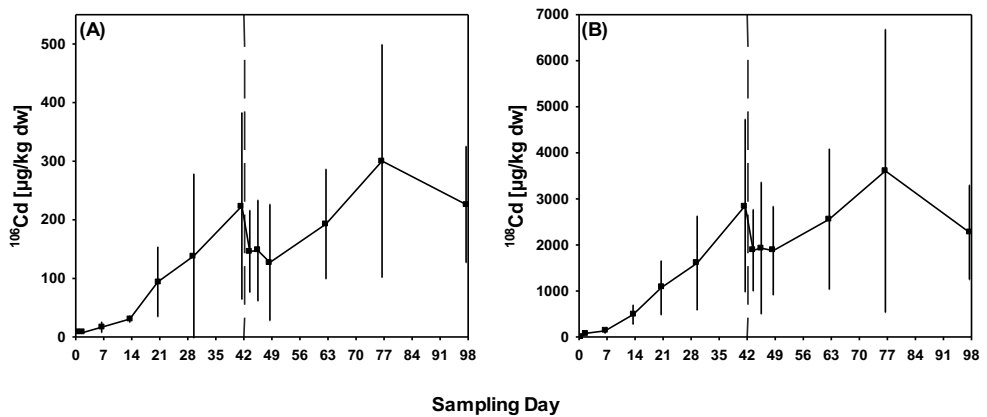

Figure 3. Accumulation curve of mean concentrations of Cd from water (A) and feed (B) in HP of brown crab. The vertical dashed line marks the end of the exposure phase. Error bars indicate the standard deviation of the five samples taken per sampling day.

The Cd concentration in HP increased continuously during the exposure phase of 42 days and steady state was not reached. During the depuration phase, no clear pattern of decrease in Cd concentration was seen. Rather a temporary increase was observed. However, none of the concentrations from the sampling days of the depuration phase were significantly different (P > 0.32), which might be due to high inter-individual variation. High inter-individual variation

in Cd concentrations is common in brown crab sampled in the field (Wiech, et al., 2017). Similar patterns with increasing concentrations of Cd during the depuration phase have also been observed earlier. After an exposure of seven days to Cd in water, the concentration of Cd in HP of the freshwater prawn *Macrobrachium australiense*, was first rapidly decreasing within two days and subsequently was increasing again until day 7, where it was stable until day 21 (Cresswell, et al., 2017). In fish, Cd concentrations in liver and kidney increased and concentrations in the gut and white muscle decreased simultaneously, representing a redistribution of Cd within the organism (Harrison & Klaverkamp, 1989; Wicklund Glynn, et al., 1992). In the present study, an internal redistribution of Cd from other organs into HP is not very likely. The only considerable amount of Cd at the end of the exposure phase was seen in gills and these concentrations also increased during the depuration phase (Appendix A4). The hemolymph concentrations were rather low and rapidly decreased to a negligible level after exposure. If a redistribution would have taken place, it should have been visible as an increase in the hemolymph concentrations, acting as intermediate organ for Cd transported between for example gills and HP (Bjerregaard, 1990; Cresswell, et al., 2017). It cannot be ruled out that Cd was accumulated in tissues not analyzed and a redistribution from these tissues occurred. However, it is not likely, as it has been shown for green crab that Cd does not accumulate in considerable amounts in other tissues than those measured here (Bjerregaard, et al., 2005). This is confirmed by the high assimilation efficiency of 98 % in HP, which means that most of the Cd from feed was accumulated there.

Reinfelder et al (1998) discussed that in accumulation experiments with long-term exposures, as the present study, especially for metals, a substantial elimination can occur already during the exposure phase. This can result in a lower elimination rate after exposure ended and might partly explain the low depuration observed in the present experiment. In freshwater prawn *Macrobrachium australiense* it was recently shown, that the depuration rate of Cd from HP was much lower after long-term compared to short-term exposure (Cresswell, et al., 2017).

The transport of Cd from hemolymph to HP is strongly dependent on the physiological condition in green crab (Bjerregaard, 1990). We found no physiological differences in crabs at the different sampling days. However, inter-individual differences were present in crabs from the same sampling day, which might be connected to the feeding stage of the animals. As all crabs had the same feeding regime while being in the lab, and no weight change during the experiment was seen, it is possible that the former feeding conditions are reflected in the physiological differences and thereby contributed to the high inter-individual variation in Cd.

The concentrations and induction of MT in HP was used as an indicator of the handling capacity of Cd in the crab, to examine if there was a competitive uptake between the two uptake routes. Cadmium in HP is mainly present in the soluble cytosolic fraction and almost entirely bound to MT. In green crab, a dietary exposure to 5.1 mg Cd/kg ww for 18 days led to an induction of MT, while 1.1 and 3.1 mg Cd/kg ww did not (Pedersen, et al., 2014). This indicates that the binding capacity of present MT for Cd ions was reached at the highest concentration. Exceeding this capacity and inducing MT, could lead to a change in the handling and accumulation of Cd and thereby a competitive uptake of Cd. Consequently, the importance of the two routes could be prone to under- or overestimation. In our study, there were no statistically significant difference between the MT concentrations in HP at start and end of the exposure phase with 20.2 ± 2.1 nmnol/g ww (mean $\pm$ SD, n=5)) at day 0 and 16.1 ± 3.7 nmol/g ww (mean $\pm$ SD, n=5) at day 42, respectively (P > 0.05). Levels are comparable to findings in green crab (Pedersen, et al., 2014). The stable MT concentration, found in the present study indicated that there was no competition for binding sites on MT of the Cd accumulated in HP, and that the measured Cd concentrations reflect the real uptake for both uptake routes, although traced simultaneously in the same animals.

The present study demonstrates that the use of stable isotopes when studying trace metal uptake has several advantages compared to radiotracers, as already discussed by Croteau et al (2004). Advantages, such as low costs for the tracer and low handling hazard and less restrictions, become especially important when using large laboratory animals such as fish or crab, with high water and space demand. Further advantages of the method relate to the correction for background Cd. This makes it possible to use wild-caught animals in laboratory experiments, which might be necessary when larger animals or species difficult to raise in captivity are studied. Wild-caught organisms or parts of organisms enriched with a stable isotope can in this way be used as feed, almost regardless natural background concentrations. This is especially useful when using wild-caught filter feeders, such as blue mussels enriched with stable isotopes as feed to study trophic transfer. Further, is it no longer necessary to use pure stable isotope standards for enrichment, which often are expensive and difficult to obtain, as we can correct for the content of the other isotopes. The correction also enables the use of natural water with its possible background contamination and further, laboratory equipment does not have to undergo laborious cleaning to avoid background contamination. Restrictions however, could arise when total element exposures are too high, as the accumulation from different uptake routes might be competitive and toxic effects might arise influencing the accumulation. Further

the LOQ increases with ascending background concentrations.

Since there is no need for a control group to correct the background concentrations against, experimental animals in the control group can be reduced to the number necessary to control for other effects, as for example to study if possible toxic effects observed in exposed animals are due to the exposure. Both uptake routes can also be studied in the same animals simultaneously, making two different treatment groups redundant.

In conclusion, the introduced methodology makes accumulation studies using stable isotopes a robust alternative to radiotracers.

The modelling based on the standard bioaccumulation equations with additional adaptations to estimate the accumulation parameters, delivered reasonable results for both routes. The case with constrained k_e to zero and $\log_{10}$ transformed data resulted in a better model fit for both uptake routes (Appendix A3) and k_w and k_f were determined to be 6.721 ± 0.567 L · kg crab^{-1} · d^{-1} (mean $\pm$ SE) and 0.0092 ± 0.0008 kg feed · kg crab^{-1} · d^{-1} (mean $\pm$ SE). Using equation 16, we calculated the mean assimilation efficiency α in HP to 98 % for the dietary route. This corresponds to similarly high values reported for green crab with 91 ± 4 % (Bjerregaard, et al., 2005) and $81 - 96$ % (Pedersen, et al., 2014). This means that almost all Cd administered in the feed was accumulated in HP. One factor facilitating the uptake from feed in the present study might be the chemical form of Cd. It has been shown earlier that trophical availability of Cd in crustaceans depends on the chemical form (Rainbow, et al., 2011) and as the Cd in our study was spiked to the processed feed as watery solution, it was probably easier accessible than Cd in natural prey. However, as the assimilation efficiency in green crab fed with blue mussels exposed to Cd for spiking, was equally high (Bjerregaard, et al., 2005), crab seems to have a high digestive power making it efficient in taking up Cd from diet.

To predict the relative importance of the uptake routes to the total accumulation of Cd in brown crab at different concentrations in diet and water in the field, we used a modelling approach based on estimated accumulation parameters for the two uptake routes. To be able to make a prediction for brown crab along the Norwegian coast, the respective CCR was estimated. Knowledge on the feeding habits of brown crab is limited and stomach analysis difficult due to the fact that prey items are masticated and ground in the gastric mill when entering the stomach. Therefore, analysis is prone to overestimation of animals holding parts difficult to grind and digest (Woll, 1995). Nevertheless, the two most frequently found feed items in the stomachs, were blue mussel (M*ytilus edulis*) and horse mussel (*Modiolus modiolus*) (Woll, 1995), with mean Cd values along the Norwegian coast of 0.12 mg Cd/kg ww and 2.3 mg Cd/kg ww

(Duinker, et al., 2016) corresponding to about 0.75 and 11.4 mg/kg dw, respectively. In a recent investigation on Cd in seawater in the North of the Norwegian coastline, concentrations were measured to 0.05 ± 0.07 µg/L (mean $\pm$ SD, n=18))(Falk, 2015). Considering these data, an average CCR of 15 000 for blue mussels 228 000 for horse mussel can be expected, corresponding to an importance of diet to more than 99% for both cases according to the modelling (Figure 4). An attempt to map the total range of Cd concentrations in the potential feed organisms for crabs in Northern Norway, found concentrations between 0.4 and 11 mg/kg dw (Ness, 2014) resulting in CCRs between 8 000 and 220 000. This corresponds to a relative importance of the dietary route of at least 98% for all the considered CCRs. Therefore, we suggest, based on the output of our model, that the large difference in Cd between crabs from the South and North of the Norwegian coast, can rather be explained by differences in foraging than differences in water concentrations. The CCR for Cd between a crabs diet and seawater can be considered to be equally high in other regions of the brown crab's distribution. It is therefore reasonable to assume that the dietary route contributes most to the overall Cd uptake in brown crab in general. When investigating differences in Cd between different locations in decapods, it should therefore be focused on factors connected to foraging and feed preference, potentially connected to migratory patterns. Our findings suggest that dietary uptake of Cd in decapods should be investigated further and that knowledge on dietary exposure is crucial to understand Cd accumulation in crab.
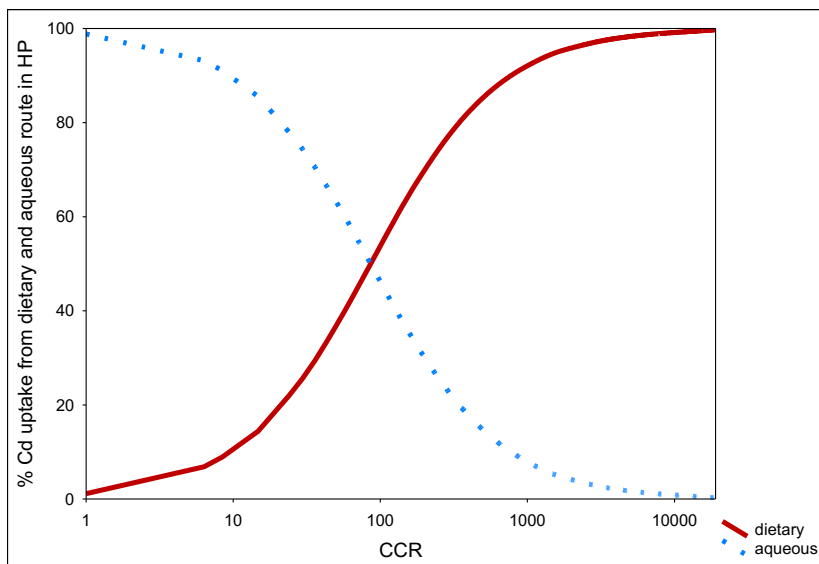
Figure 4. Prediction of the relative importance of dietary and aqueous uptake route to the total Cd hepatopancreas burden using modelling. The Cd concentration ratio (CCR) is the ratio of Cd concentration in diet [µg/kg dw] to seawater [µg/L].

Most studies comparing the relative importance of dietary and aqueous uptake, assume steady state conditions (Lee & Fisher, 2016; Thomann, 1981). However, our modelling approach showed that the prediction of relative importance (equation 20) is the same regardless of whether it is derived considering steady state conditions or not (k_e being zero). The assumption being that k_e is the same for both uptake routes, which is reasonable in crab, as Cd accumulated in HP will be tightly bound to MT regardless origin (Pedersen, et al., 2014; Pedersen, et al., 1994).

When interpreting model outputs, uncertainty connected to the used input parameters and underlying assumptions has to be taken into account. The used ingestion rate was determined for crabs in captivity and feeding *ad libitum* on constantly present feed, not necessarily being representative *in situ* and also other factors like physiological state and temperature can influence I (Woll, et al., 2006). For fish, there is evidence that dietary Cd uptake is regulated and the increase in uptake non-proportional to feed concentrations with a saturation at high concentrations (Douben, 1989; Reinfelder, et al., 1998). The importance of the dietary route will then be over-estimated with increasing concentrations (Reinfelder, et al., 1998). However, in green crab no sign of saturation in uptake was seen at Cd concentrations up to 5.1 mg/kg ww

at a high feeding rate (Pedersen, et al., 2014) and the aqueous uptake was increasing proportional over a wide range of exposure concentrations (Bjerregaard, et al., 2005). Also developmental stage and organism size were suggested to influence metal accumulation (Reinfelder, et al., 1998). In the present study, the size of the experimental animals was determined by practical issues such as availability, demand of space and large enough size for gavage feeding. However, no clear relationship between size and Cd concentration was found in crabs of commercial size (Julshamn, et al., 2012). Further accumulation might be influenced by other environmental and also physiological conditions of the crab.

4. Conclusion

Tracing stable isotopes is a suitable method to investigate the accumulation of trace metals in the same organism at the same time. Analytical challenges with background concentrations of natural isotope distribution and polyatomic interferences in the different matrices can be overcome with the right analytical setup and modern mathematical corrections using a computer software helping to solve equations .

For the brown crab, we have shown that the dietary route is more important for the uptake of Cd in HP. The accumulation parameters, uptake rate constant from feed k_f and water k_w and assimilation efficiency α from feed were determined from the data of the conducted laboratory study using non-linear regression modelling. We applied the estimated parameters in a further modelling approach combined with naturally relevant concentrations in diet and seawater to determine the importance of the uptake routes. Considering naturally relevant concentrations, it is clear that the dietary pathway is far more important for the uptake of Cd into HP and thereby the whole body burden of brown crab.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Paper 3

Paper 4

Cadmium in the shore crab *Carcinus maenas* along the Norwegian Coast: geographical and seasonal variation and correlation to physiological parameters

Heidi Knutsen[1], Martin Wiech[1], Arne Duinker[1] and Amund Maage[1,2]

[1]National Institute of Nutrition and Seafood Research, 5002 Bergen, Norway

[2]University of Bergen, Allegt. 41, 2020 Bergen

Abstract

Previously, high concentrations of cadmium have been found in the hepatopancreas of the edible or brown crab (*Cancer pagurus*) sampled from positions north of about 67 °N, compared to regions further south along the Norwegian coast, with no clear understanding why. In order to study a similar organism in the same ecosystem, the present study analyzed 210 shore crabs (*Carcinus maenas*) from four different locations along the Norwegian coast, two in the North and two in the South. The physiological variables size, sex, moulting stage, hepatosomatic index, carapace color and gonad maturation were registered, in attempt to explain the high inter-individual variation in cadmium levels in hepatopancreas. In contrast to the brown crabs, the shore crabs showed no clear geographical differences in cadmium concentrations. This indicates physiological differences between the two crab species. No clear and consistent correlations were found between cadmium levels and physiological parameters, except for sex, where cadmium concentration in hepatopancreas was twice as high in males compared to females. The cadmium levels also varied with season, with approximately 40 and 60 % lower cadmium concentration in April than August for male and female shore crabs, respectively. None of the analyzed cadmium concentrations in muscle meat from claws exceeded EUs food safety limit, and low cadmium levels in soup prepared from shore crabs clearly indicated that this dish is not problematic regarding food safety.

Corresponding author: Heidi Knutsen, phone: 47851733, e-mail: hknutsen10@gmail.com

1. Introduction

It is well established that marine invertebrates such as crustaceans and mollusks can accumulate cadmium (Jennings and Rainbow, 1979; Ray, 1984; Wright, 1976). A comparison of typical European foodstuffs revealed particularly high cadmium levels in crustaceans (EFSA, 2012a). As such, cadmium in crustaceans is of considerable interest both regarding toxic effects on the organisms itself (Weis, 2012) as well as the suitability as food for humans. To ensure food safety, the European Commission has established upper limits for cadmium in several foods, where the limit for cadmium in claw meat of crustaceans is set at 0.5 mg Cd/kg wet weight (ww) (EU, 2006). There is at present no legal limit for cadmium in the brown body meat, commonly consumed from crabs (Maulvault *et al.,* 2013), although it mainly consists of hepatopancreas, where the majority of cadmium is accumulated (Bjerregaard, 1990; Bjerregaard *et al.,* 2005; Davies *et al.*, 1981; Hutcheston, 1974; Wiech *et al.,* 2017) and gonad. The brown crab *Cancer pagurus* is commercially important, with an annual catch volume in Europe of approximately 50 000 tons in total (Bakketeig *et al.,* 2016) and rising to 10 000 tons in Norway alone (Norwegian Directorate of Fisheries, 2017). Findings of cadmium levels above the legal limit for claw meat in the brown crab from Northern Norway has had a crucial negative impact on the crab fisheries in this area. To investigate the cadmium levels in brown crabs along the whole Norwegian coast, cadmium levels in brown meat and muscle meat from claws were measured of in a total of 475 frozen and cooked brown crabs sampled at 47 different sites along the Norwegian coast between July 2011 and January 2012. A pattern with significantly higher cadmium levels in brown crabs sampled from positions north of about 67 °N (around Saltfjorden) was found compared to regions further south (Julshamn et al., 2012. The average cadmium concentrations in brown meat in frozen and cooked brown crabs sampled from positions north of Saltfjorden varied from 6.7 to 25 mg/kg wet weight and from 0.55 to 4.8 mg/kg wet weight in crabs sampled at positions south of Saltfjorden. In another survey, cadmium was measured in brown crabs sampled at 20 locations from Salten and further north to Vesterålen (Frantzen *et al*., 2015). In agreement with the survey from 2011 and 2012, high levels of cadmium were found varying from 2.4 mg/kg wet weight to 17 mg/kg wet weight in brown meat. No difference was seen between samples from inner fjord and outer coast localities.

Several follow-up studies have been performed in attempt to explain the elevated cadmium levels in brown crabs from Northern Norway compared to the rest of the Norwegian coast. However, no obvious point source from industry has been determined responsible for the high

cadmium levels found in the brown crabs (Falk 2012). Further, measurements of cadmium in surface water, groundwater, soil and bedrock have not displayed elevated cadmium levels in the Salten region (Finne, 2013). Surveys have shown relatively low cadmium levels in fish species and blue mussels from Northern Norway (Julshamn *et al.*, 2013a; Ørnsrud and Måge, 2012; Foldøy Tverdal, 2012), and no correlation to the elevated values in the brown crabs were found.

As reported for the brown crab (Wiech *et al.,* 2017), also the shore crab *Carcinus maenas* is able to accumulate high levels of cadmium in heptaopancreas (Rainbow *et al.,* 1995). As these species are also sharing parts of the same ecological niche, a comparison of their cadmium levels is of considerable interest. The smaller shore crab is found subtidally as well as intertidally on all shores (Crothers, 1968), while the brown crab is abundant from the shallow sublittoral to depths of about 100 meters (Neal and Wilson, 2008). Shore crab is considered a delicacy in Spain and Portugal, with commercial fisheries yield of up to 900 tons per year for France, Portugal and Spain together (Klassen and Locke, 2007). The culinary popularity is also increasing in Norway, especially as a base for shore crab soup. In terms of food safety, it is therefore important to study the cadmium levels in shore crabs. Comparison of brown and shore crabs geographical cadmium pattern would contribute to explaining the high cadmium levels in brown crabs north of 67 °N.

In brown crabs, concluding studies are hampered by large inter-individual variability in cadmium between brown crabs from the same geographical areas, with especially high variation in the hepatopancreas (Davies *et al.*, 1981; Maulvault *et al.*, 2012; Wiech *et al.*, 2017). Shore crabs have also shown to display large variability in their cadmium levels (Bjerregaard 1982, 1990, 1991; Bjerregaard and Depledge 2002; Bondgaard *et al.* 2000; Nørum *et al.* 2003). The high variation could be caused by biological factors. Laboratory studies have shown relationships between cadmium levels and physiological variables such as sex (Bjerregaard *et al.* 2005), size (Bjerregaard and Depledge, 2002), moult stage (Bondgaard *et al.* 2000; Bondgaard and Bjerregaard 2005; Nørum *et al.* 2005), ovarian maturation (Bondgaard *et al.,* 2000) and variables indicating the condition of the crab (Bjerregaard, 1991), like water content in tissues (Bjerregaard and Depledge 2002). As observed by Bjerregaard *et al.* (2005), the tissue cadmium content may also vary seasonally. Cadmium bioaccumulation increases with increasing temperature (Ray 1984), and reduced salinity stimulate uptake of anionic cadmium species in brachyuran crabs (Wright 1977; Burke 2003).

In the present study, cadmium levels in shore crabs were investigated for comparison to the problematic high levels in brown crabs, which have many parallels in physiology as well as a similar ecological niche as the shore crab. Also, the cadmium levels in shore crabs were investigated due to food safety reasons. This paper describes geographical (investigation 1) and seasonal (investigation 2) variations in cadmium levels in shore crabs sampled from four different sites along the Norwegian coast. In addition, the study examined effects of different physiological parameters on individual cadmium levels (investigation 3). Lastly, cadmium concentrations in shore crab soup (investigation 4) are described for an evaluation of food safety.

2. Materials and methods

2.1 Sampling of biological material

Male and female shore crabs *Carcinus maenas* with carapace width (CW) varying from 29 to 88 mm were caught along the Norwegian coast in baited pots at approximately 1-5 m water depth between March and August 2016. The sampling locations (figure 1) were chosen according to earlier studies on cadmium in the brown crab (Julshamn *et al.*, 2012; Wiech *et al.*, 2017).

Figure 1. The Norwegian coastline showing the four sampling areas Kvitsøy, Sotra, Fleinvær and Vesterålen

2.2 Investigation 1: Geographical variation in cadmium

Shore crabs of similar size (CW 62 ± 7 mm (mean ± SD)) were collected from Kvitsøy (59 °N), Sotra (60 °N), Fleinvær (67 °N) and Vesterålen (68 °N) during the spring of 2016 (March-May). From each site, 30 shore crabs were collected with equal sex distribution, except from Fleinvær, with 27 male and three female crabs. Although an effort was made to keep experimental groups as uniform as possible, there was some variation in sizes of specimens, with the male shore crabs from Fleinvær being significantly larger (70 ± 3 mm) than the other males (64 ± 4 mm), and the females from Kvitsøy being significantly smaller (50 ± 3 mm) than the other females (58 ± 4 mm).

2.3 Investigation 2: Seasonal variation in cadmium

To investigate whether the cadmium content in shore crabs varies with season, 30 shore crabs (61 ± 5 mm) were collected in the end of August 2016 (Sotra-August) in addition to the 30 shore crabs (61 ± 5 mm) collected earlier from Sotra in the middle of April 2016 (Sotra-April). The sex distribution was equal in both groups.

2.4 Investigation 3: Physiological variables and their effect on cadmium

To examine the potential effect of size on cadmium levels, in addition to the 30 crabs from investigation 1, 30 shore crabs were collected from Sotra and Vesterålen during April-May 2016 to obtain the largest size range possible (CW from 29 to 88 mm and whole body weight from 6 to 170 gram).

To investigate the correlation of cadmium levels to further physiological variables, several individual physiological variables were measured in all of the sampled shore crabs:

After arrival at the National Institute of Nutrition and Seafood Research (NIFES), the crabs were sacrificed following the guidelines in WHO/FAO (2012), by piercing the nerve ganglia as described by Baker (1955) and dissected freshly, as freezing and boiling may affect cadmium levels in crab tissues (Wiech *et al.*, 2017). The same observer recorded all visually examined measures to minimize bias.

For each individual, carapace width, whole body wet weight, sex, damage on the exoskeleton, missing legs and/or claws were recorded. One of the following carapace colors was assigned: green, brown, blue/black, orange or red. For female shore crabs the presence of sperm plug was noted.Crabs were assigned to one of four different moulting stages (early post moult, recent moult, inter-moult or degraded) by examination of carapace hardness, levels of biofouling and visual indices according to Haig *et al.* (2016).

To determine the hepatosomatic index (HSI), an indication of lipid stores, the hepatopancreas was removed and weighed, and HSI was calculated:

$$HSI = \frac{m_{HP}}{m_{whole\,body} - m_{HP}} * 100\% \qquad (1)$$

Where m_{HP} and $m_{whole\,body}$ are individual hepatopancreas and whole body wet weights, respectively. Hepatopancreas samples were individually homogenized and kept for analysis.

Gonads were staged as described in Haig *et al.* (2016) and removed from mature female crabs and pooled for analysis for each location separately. Pooled samples were also prepared for muscle meat for each sampling area and sex. After the wet weight was obtained for all samples, they were frozen and subsequently freeze-dried (Freezone 18 liter by Labconco, Kansas, USA) to determine the dry weight content. The water content (WW%) was obtained for all samples:

$$WW\% = 100\% - \frac{wet\,weight}{dry\,weight} * 100\% \qquad (2)$$

Where $wet\,weight$ and $dry\,weight$ are sample weight before and after freeze drying, respectively.

2.5 Investigation 4: Cadmium in shore crab soup

To measure possible cadmium exposure from shore crab soup, triplicates of soups were made using crabs from Sotra and Vesterålen, separately. From Sotra, 30 shore crabs were collected, with equal sex distribution (CW = 62 ± 6 mm and 58 ± 4 mm for male and female crabs, respectively). From Vesterålen, the selection was limited to 15 male crabs (CW = 74 ± 2 mm). Approximately the same weight of shore crabs were used in each triplicate (397 ± 13 g and 457 ± 1.0 g for Vesterålen and Sotra, respectively).

After the crabs were sacrificed, they were cut in half and fried while crushing with a solid kitchen spoon in a saucepan with heated vegetable oil with no salt. After about 5 minutes, the crabs turned red, and water was added to cover the crabs (4-5 dl). After 30 minutes of boiling, the soup was sifted off and cooled for freeze-drying and homogenization.

2.6 Chemical analysis

Freeze-dried tissue and soup samples were homogenized and prepared for metal analysis using ICP-MS (iCAP Q) as described by Julshamn et al (2007). The method was accredited according to NS-EN 17025, and the quality of the metal measurements was assured by the use of the certified reference materials (CRM) Tort-3 (Lobster Hepatopancreas, National Research Council, Canada) and 1566b-O.T. (Oyster Tissue, National Institute of Standards and

Technology, Gaithersburg, USA). Average values for all metals were within 20 % of the certified values, and the dry weight (dw) based quantification limit (LOQ_{dw}) for cadmium was set to 0,005 mg/kg with standard sample size (0.2 g). All individual samples were over the wet weight based quantification limit (LOQ_{ww}), calculated as:

$$LOQ_{ww} = LOQ_{dw} * \frac{wet\ weight_{sample}}{dry\ weight_{sample}} \tag{3}$$

2.7 Statistical analysis

When necessary, the data was box-cox transformed to obtain normality and homogeneity of variances, tested for by normal plots and Levene's F test, respectively. Results were evaluated using analysis of variance (ANOVA) followed by Tukey HSD post hoc test as the multiple comparison procedure. The significance level was 0.05. Simple linear regression analysis was performed by using Pearson's linear correlation (STATISTICA v. 13.1, ©1984-2016 by Statsoft, Tulsa, USA).

3. Results

The mean cadmium concentrations in hepatopancreas was 1.1 ± 1.2 mg/kg ww (mean $\pm$ SD) corresponding to 3.4 ± 4.1 mg/kg dw (mean $\pm$ SD), and ranged from 0.046 to 11 mg/kg ww corresponding to 0.13 to 39 mg/kg dw, underlining the high individual variability. Furthermore, the cadmium concentrations were higher for male than female shore crabs for all locations with 1.3 ± 1.3 mg/kg ww (mean $\pm$ SD) corresponding to 4.3 ± 4.6 mg/kg dw (mean $\pm$ SD) and 0.61 ± 0.79 mg/kg ww (mean $\pm$ SD) corresponding to 2.0 ± 2.5 mg/kg dw (mean $\pm$ SD) for male and female shore crabs, respectively (table 1).

Table 1 Cadmium concentrations (mg/kg) based on wet weight (ww) and dry weight (dw) in hepatopancreas of shore crabs (Carcinus maenas) from the Norwegian coast. Mean ± standard deviation (SD) and concentration ranges are given for each group

Area	Male					Female				
	N	Mean ± SD (ww)	Range (ww)	Mean ± SD (dw)	Range (dw)	N	Mean ± SD (ww)	Range (ww)	Mean ± SD (dw)	Range (dw)
Kvitsøy	15	0.98 ± 0.68	0.11-2.7	3.0 ± 2.1	0.38-7.6	15	0.45 ± 0.57	0.056-2.3	1.4 ± 1.7	0.16-6.6
Sotra-April	33	1.0 ± 0.59	0.14-2.4	3.3 ± 2.0	0.36-7.4	27	0.91 ± 1.1	0.059-4.0	2.8 ± 3.2	0.19-12
Fleinvær	27	2.4 ± 2.2	0.37-11	7.7 ± 7.7	1.3-39	3	0.58 ± 0.35	0.20-0.87	2.0 ± 1.0	0.90-2.7
Vesterålen	42	1.1 ± 1.0	0.16-4.1	3.9 ± 3.6	0.37-14.9	18	0.47 ± 0.54	0.048-2.4	1.6 ± 1.7	0.13-7.5
Sotra-August	15	0.90 ± 0.78	0.093-3.4	3.2 ± 3.0	0.44-12.4	15	0.39 ± 0.60	0.046-2.3	1.5 ± 2.3	0.18-9.1
All areas	132	1.3 ± 1.3	0.093-11	4.3 ± 4.6	0.36-39	78	0.61 ± 0.79	0.046-4.0	2.0 ± 2.5	0.13-12

Cadmium concentrations in muscle meat and gonads were significantly lower than in hepatopancreas for both sexes ($p < 0.0001$) with 0.0027 ± 0.0017 mg/kg ww (mean $\pm$ SD) corresponding to 0.0112 ± 0.0069 mg/kg dw (mean $\pm$ SD) for muscle meat in claws, and 0.0149 ± 0.0055 mg/kg ww (mean $\pm$ SD) corresponding to 0.036 ± 0.014 mg/kg dw (mean $\pm$ SD) for gonads, respectively (supplementary table 1 and 2). In muscle meat, there was no significant difference between males and females. For the analyzed tissues, the cadmium distribution was 99.7 % in hepatopancreas and 0.3 % in muscle meat from claws for the male crabs. For the female shore crabs the cadmium distribution was 92 % in hepatopancreas, 0.1 % in muscle meat from claws and 7.9 % in gonads. The water content in these tissues was 32 ± 5.3 % and 24 ± 2.1 % for hepatopancreas and muscle meat for male shore crabs, and 31 ± 4.3 %, 24 ± 1.4 % and 42 ± 5.4 % for for hepatopancreas, muscle and gonads for the female shore crabs. More detailed values on weight, carapace width, dry matter in hepatopancreas and hepatosomatic index are presented in table 2.

Area	Male				Female			
	Weight (g)	CW (mm)	DM (%)	HSI (%)	Weight (g)	CW (mm)	DM (%)	HSI (%)
Kvitsøy	58 ± 11	6.2 ± 0.35	32 ± 5.1	5.2 ± 1.2	26 ± 4.5	5.0 ± 0.34	33 ± 5.5	5.4 ± 0.99
Sotra-April	73 ± 26	6.7 ± 0.96	31 ± 5.5	6.1 ± 1.3	30 ± 12	5.2 ± 0.79	31 ± 4.3	7.9 ± 2.4
Fleinvær	86 ± 14	7.0 ± 0.33	31 ± 3.9	7.0 ± 1.2	59 ± 17	6.4 ± 0.56	29 ± 8.3	8.0 ± 0.89
Vesterålen	74 ± 44	6.4 ± 1.4	32 ± 6.7	7.8 ± 2.5	39 ± 11	5.6 ± 0.52	31 ± 5.8	7.7 ± 1.6
Sotra-August	54 ± 15	6.3 ± 0.56	28 ± 4.9	7.5 ± 1.4	42 ± 6.5	5.8 ± 0.32	27 ± 4.3	9.4 ± 1.0
All areas	72 ± 31	6.6 ± 1.0	31 ± 5.6	6.9 ± 2.0	35 ± 12	5.4 ± 0.67	31 ± 5.4	7.7 ± 2.1

3.1 Investigation 1: Geographical variation in cadmium

Cadmium concentrations in hepatopancreas based on dry weight and wet weight (table 1) did not vary significantly between the different sampling areas for the female shore crabs (figure 2). Male crabs from Fleinvær however, had significantly higher wet weight based cadmium concentrations in hepatopancreas compared to the male crabs from Kvitsøy in Southern Norway ($p < 0.01$), and Vesterålen in Northern Norway ($p < 0.001$). On dry weight basis cadmium concentrations found in male crabs from Fleinvær were in addition higher than in male crabs from Sotra ($p < 0.02$). The male crabs from Vesterålen did not have higher cadmium levels compared to the crabs sampled further south (figure 2). There was no significant geographical difference in cadmium levels in muscle and gonads for neither males nor females ($p > 0.05$) (supplementary table 1 and 2).
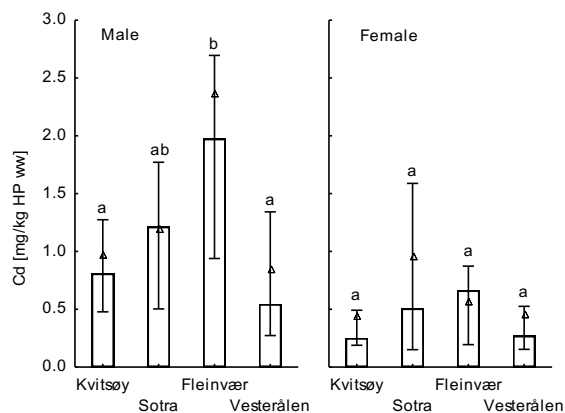

Figure 2. Geographical variation in cadmium concentrations in hepatopancreas (mg/kg wet weight) of male (left panel, n = 72) and female (right panel, n = 48) crabs collected from Kvitsøy and Sotra in south, and Fleinvær and Vesterålen in north (median ± 25 % percentile is given and triangle symbols shows the mean for each location, sampling points with no letters in common show statistically significant differences)

3.2 Investigation 2: Seasonal variation in cadmium

Crabs sampled in August had lower wet and dry weight based cadmium concentrations in hepatopancreas than in April, and the difference was significant for the female crabs ($p < 0.03$), while not significant for the male crabs ($p > 0.05$) (figure 3). The total cadmium content in hepatopancreas was not statistically significantly different between August and April ($p > 0.1$). However, there was a clear trend in measured cadmium concentration, with about two times lower concentrations for both sexes in August than in April. Statistically, the difference was probably covered by the large variation between individuals. There was no significant seasonal variation in cadmium levels in muscle and gonads ($p > 0.7$).

3.3 Investigation 3: Physiological variables and their effect on cadmium

The size parameters carapace width and whole body weight were strongly correlated for both sexes ($r^2 = 0.90$, $p < 0.001$ and $r^2 = 0.88$, $p < 0.001$ for male and female shore crabs, respectively (Supplementary table 3). Carapace width was chosen as the main size parameter for further examination, and it was positively correlated with water content in hepatopancreas for both male ($r^2 = 0.25$, $p < 0.0001$) and female ($r^2 = 0.32$, $p < 0.0001$) shore crabs. The hepatosomatic index was negatively correlated with carapace width for the males ($r^2 = -0.35$, $p < 0.0001$), though not for females ($r^2 = 0.026$, $p < 0.2$).
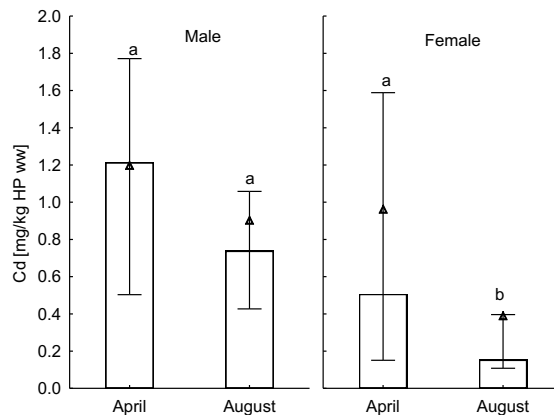

Figure 3. Cadmium concentrations in hepatopancreas (mg/kg wet weight) of male (left panel, n = 30) and female (right panel, n = 30) crabs collected from Sotra in April and August (median ± 25 % percentile is given and triangle symbols shows the mean for each location, sampling points with no letters in common show statistically significant differences)

Overall, size was not correlated with cadmium concentrations in hepatopancreas despite the relatively large size variation for the shore crabs from Sotra and Vesterålen (figure 4) – a difference of about three times between maximum and minimum carapace width. For the male crabs from Vesterålen there was a weak significantly positive correlation between carapace width and cadmium concentrations ($r^2 = 0.15$, $p < 0.01$ on wet weight basis and $r^2 = 0.29$, $p < 0.001$ on dry weight basis). The correlation was pronounced for carapace width and total cadmium content in hepatopancreas ($r^2 = 0.47$, $p < 0.0001$). For male crabs from Sotra, cadmium concentrations were not significantly correlated with carapace width, but the total cadmium content in hepatopancreas showed a weak correlation with carapace width ($r^2 = 0.13$, $p < 0.05$). The cadmium concentrations were not significantly correlated with carapace width for female crabs from neither Vesterålen nor Sotra. However, the cadmium content was weakly correlated with carapace width for the females from Sotra ($r^2 = 0.15$, $p < 0.05$).
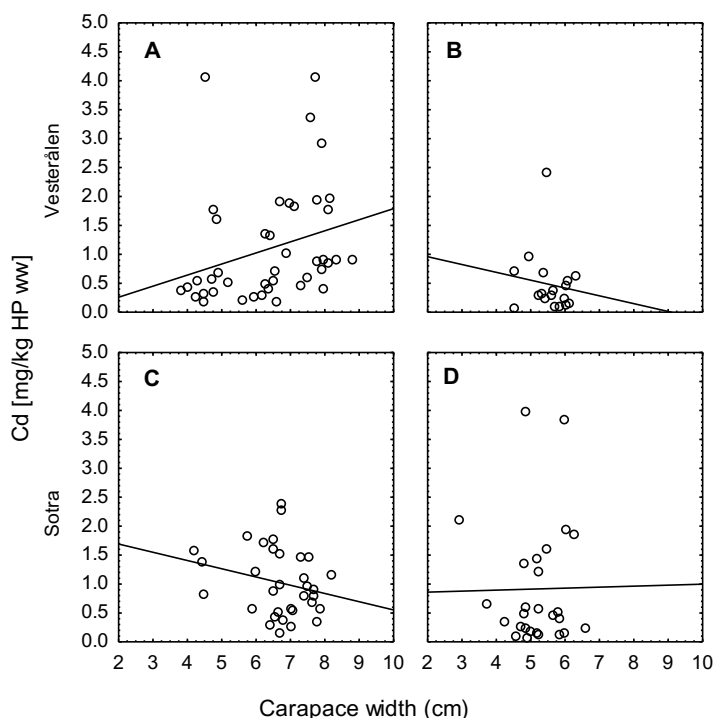

Figure 4. Relationship between cadmium concentration in hepatopancreas (mg/kg wet weight) and carapace with of male (n = 75) (A and C) and female (n = 45) (B and D) shore crabs from Vesterålen in north (upper panel) and Sotra in south (lower panel)

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Overall, there was no clear correlation between cadmium concentration in hepatopancreas (mg/kg ww) and the physiological variable water content. Only for male shore crabs from Vesterålen a weak significantly positive correlation ($r^2 = 0.20$, $p < 0.05$) was observed. On dry weight basis (mg Cd/kg dw), the correlation was pronounced ($r^2 = 0.39$, $p < 0.001$). In addition, there was a significantly positive correlation between water- and cadmium content in hepatopancreas for the male shore crabs from Vesterålen ($r^2 = 0.30$, $p < 0.001$).

Male shore crabs had higher cadmium concentrations and a higher total amount of cadmium in hepatopancreas in all experimental groups ($p < 0.003$). Physiological variables that were significantly different between male and female shore crabs might be of importance for the different cadmium levels. The male crabs were significantly larger than females in both CW and body weight ($p < 0.0001$) and the females had a significantly lower hepatosomatic index ($p < 0.02$) (Table 2). However, there was no clear correlation between HSI and cadmium levels when sexes were segregated, except for a significant negative correlation between cadmium concentration on dry weight basis and HSI for the male shore crabs from Vesterålen: $r^2 = 0.27$, $p < 0.001$). However, a relationship between cadmium concentration and HSI was indicated, as the females had significantly higher HSI and lower cadmium levels in hepatopancreas. Additionally, both sexes had significantly higher HSI ($p < 0.0003$) and lower cadmium concentration in August than April at Sotra. For the females from Sotra-April and Sotra-August in total, the correlation between cadmium concentration and HSI was significantly negative ($r^2 = 0.24$, $p < 0.006$), whereas there was no significant correlation between total cadmium content in hepatopancreas and HSI ($p > 0.05$). Female crabs showed a seasonal trend in dry matter content in Sotra with a lower dry matter content in August ($p = 0.066$), while this was not observed in male crabs. In male crabs, the dry matter content increased significantly from recent moult to inter-moult crabs, while this was only visible as a trend in females (supplementary figure 1).

The proportion of red females was larger than for males (approximately 65 and 50 %, respectively), but no significant correlation was found between carapace color and cadmium levels ($p > 0.05$) (supplementary figure 2). There were no clear and consistent relationships between cadmium and other physiological variables such as moult stage (supplementary figure 3), gonad maturation stage (supplementary figure 4 and 5), and the presence of sperm plugs, probably linked to high variation in Cd values and low variation in the measurement of the parameters.

3.4 Investigation 4: Cadmium in shore crab soup

The cadmium concentrations in shore crab soups from Sotra and Vesterålen ranged from 17 to 79 µg/kg ww, with average values of 28 ± 11 and 63 ± 14 µg/kg ww, respectively. In total, the average cadmium concentration was 44 ± 22 µg/kg ww soup. Based on cadmium levels in hepatopancreas in shore crabs from Sotra and Vesterålen (table 1), it was calculated that approximately 62 % of the crabs cadmium levels were extracted into the soup during the cooking process.

4. Discussion

4.1   Geographical variation in cadmium

The main objective in the present study was to investigate whether the shore crab shows as large differences in cadmium concentrations in hepatopancreas as have been found earlier in brown crabs from northern and southern sites along the Norwegian coast. The cadmium concentrations in the shore crabs in this study did not follow such a clear geographical pattern. Only males from one of the two locations in the North showed significantly higher concentrations. This is in contrast to the brown crab, where concentrations in the brown meat were significantly higher in Northern Norway, compared to Southern Norway (Julshamn *et al.,* 2012). Except for the relatively high cadmium concentrations in male shore crabs from Fleinvær, the cadmium levels in the analyzed tissues in the present study correspond fairly well to earlier published values for shore crabs from Denmark and Scotland (Depledge and Bjerregarrd, 2002; Bjerregaard, 1982, 1990; Rainbow, 1985). The male shore crabs from Fleinvær were larger than the other males, and one reason for their high cadmium levels may be that they have foraged on different and potentially larger and older prey, with potentially higher cadmium levels. It is also possible that the cadmium levels are higher in this area, although the cadmium concentrations in the females from Fleinvær were not correspondingly high. However, this may be due to the low sampling size of female shore crabs from this area, with only three females from Fleinvær. Brown crabs from the same area did not show elevated cadmium levels compared to other samples in the North (Julshamn *et al.*, 2013b; Julshamn *et al.*, 2012).

The differences in geographical cadmium pattern between shore crabs and brown crabs from the Norwegian coast may be explained by several causes. As food presumably is the most important cadmium source for crabs (Davies *et al.,* 1981; Bjerregaard *et al.,* 2005), differences in diet might be of importance for the different cadmium levels between shore crabs and brown crabs sampled along the Norwegian coast. Compared to the cadmium levels in the brown crab (Julshamn *et al.,* 2012), the levels in the shore crab in this study were low along the whole Norwegian coastline. The brown crab generally consumes larger organisms (Mascar and Seed, 2001) with potentially higher cadmium levels. Further, differences in diet might be a result of their different distribution in the water column, as the shore crab generally lives in shallower waters (Crothers, 1968; Neal and Wilson, 2008), where different prey species might be abundant.

Crabs also accumulate cadmium from the water phase (Jennings and Rainbow, 1979; Weis, 2012; Davies *et al.,* 1981) and it is possible that differences in cadmium accumulation from the water is associated with the higher cadmium levels in brown crabs compared to shore crabs. As the brown crab is more abundant in deeper water, it is possible that it is more exposed to the cadmium rich deep-water (Falk and Nøst, 2013, Janssen *et al.* , 2014) than the shore crab. However, the cadmium levels in brown meat in brown crabs from Northern Norway are shown to be higher in males (Frantzen *et al.,* 2015), even though females generally migrates to a greater extent to deeper waters where the cadmium concentrations potentially are higher (Ungfors *et al.,* 2007; Falk and Nøst, 2013). Further, several studies indicate that the rates of cadmium accumulation from the water phase to the hepatopancreas are too low to explain the high cadmium levels in hepatopancreas and/or brown meat (Bjerregaard *et al.,* 2005; Davies *et al.,* 1981; Jennings and Rainbow, 1979; Nørum *et al.,* 2005).

It might be an explanatory feature that the shore crab could probably be better adapted to the cold climate in Northern Norway compared to the brown crab. The water temperature along the Norwegian coast generally decreases with increasing latitude and the mean temperature for 2015 to 2017 at Sognesjøen (61 °N), a station close to our sampling sites in Southern Norway, was 10.0 °C, ranging from 5.8° to 15.6 °C at a depth of 5 m. At a station in the proximity of our northernmost site, Eggum (68 °N), a mean temperature of 8.3 °C with, ranging from 4.7 ° to 12.3 °C was measured for 2015 to 2017 at a depth of 5 m (IMR, Permanent hydrographic stations). The shore crab is known to be very robust and survives a wide range of temperatures from approximately 0 to 35 °C and tolerates salinities from 4 to 52 ‰ (Klassen and Locke, 2007). This suggests that the shore crab grows equally good in northern and Southern Norway. There is evidence that brown crabs do not feed at all at temperatures below 5 °C, as well as migration is limited (Karlsson and Christiansen, 1996). Further, a survey of brown crabs has shown that they moult less frequently in northern compared to Southern Norway (Snorre Bakke, personal communication, January 23, 2017). Consequently, the growth rate will be relatively lower for brown crabs from Northern Norway. As such, a brown crab of a given size from Northern Norway might have had longer time to accumulate metals such as cadmium, and will consequently have higher cadmium levels compared to a brown crab of similar size sampled further south. This will probably not apply for the shore crab, under the assumption that shore crabs have similar growth rates in the north and south. Further, Bergey and Weis (2007) has suggested moulting as a mechanism for depuration of lead for the fiddler crab *Uca pugnax.* If moulting is a feasible mechanism for crabs to also departure cadmium, it is possible that lower

moulting frequency for brown crabs from Northern Norway result in less cadmium excretion and thereby higher cadmium concentrations compared to brown crabs from Southern Norway.

4.2   Seasonal variation in cadmium

In agreement with findings from Bjerregaard *et al.* (2005), the results in the present study showed that the cadmium concentrations in hepatopancreas varies with season. The concentration was significantly lower for female shore crabs sampled in August than April. The seasonal variation might be explained in terms of changing bioavailability of the metal due to changing physicochemical conditions of the environment as well as changing physiological state of the individuals. With rising water temperature, the crabs activity will probably increase (Klassen and Locke, 2007; Griffen *et al.*, 2012), which may lead to increased food intake. This is indicated by significantly higher hepatosomatic index for both sexes in August than April. With higher HSI, the lipid stores consequently increase, which seems to lower the cadmium concentration by dilution, as indicated by the significantly negative correlation between HSI and cadmium concentration for the females sampled in August and April in total. Further, the higher concentrations of cadmium in hepatopancreas in April could be explained by the tendency to higher water content in hepatopancreas in the crabs sampled in August. This is consistent with the total content of cadmium not differing between the two months. In addition, the lower cadmium levels in August might be a consequence of a potentially shorter biological half-life of cadmium as the temperature increases during summer, with subsequently higher activity among the crabs, as discussed by Bjerregaard *et al.* (2005).

Seasonal changes in physiological variables such as ovarian maturation and moult stage (Bondgaard *et al.,* 2000: Bondgaard and Bjerregaard, 2000; Nørum *et al.*, 2005) might also influence the cadmium levels, but the span in variation for these parameters was too low to reveal any effects. In agreement with the results in the present study, elevated cadmium levels are reported for the American oyster (*Crassostrea virginica*) in April with a decline throughout the summer (Frazier, 1979).

As the bioavailability of cadmium increase with temperature (Klassen and Locke, 2007; Ray, 1984; Rainbow, 1997; Burke *et al.,* 2003), the cadmium accumulation from the water phase would probably be higher for the shore crabs in August than April. However, the cadmium levels were not elevated in August for the shore crabs in this study. Therefore, cadmium uptake from the water phase does not explain the observed variations.

4.3 Physiological variables and their effect on cadmium

For both sexes, the size parameters were positively correlated with each other and with water content in hepatopancreas, in agreement with other studies (Bjerregaard and Depledge, 2002; Nissen *et al.,* 2005). For the male shore crabs, the HSI was negatively correlated with size, which indicates that the relative energy reserves decrease with increasing size. In concordance to Nørrum et al. (2013) we found a trend towards a higher dry matter content in hepatopancreas in inter-moult crabs in comparison to recent moult crabs. This is most likely a result of active foraging.

We found an indication of cadmium accumulation over the crab's lifetime, as the total amount of cadmium was correlated with size. However there was no consistent effect of size on cadmium concentrations even though the range in size was relatively large. The increase of water content with size might mask the increase of the wet weight based concentration. Little or no correlation between size and cadmium concentrations has been found for shore crabs from Denmark (Bjerregaard and Depledge, 2002; Nissen *et al.,* 2005), king crabs (*Pseudocarcinus gigas)* from Australia (Turoczy *et al.,* 2001) and brown crabs from Norway (Julshamn *et al.,* 2012). It is possible that crabs excrete cadmium during moulting (Bergey and Weis, 2007), which could explain why the cadmium concentrations do not increase considerably with size. However, this needs to be elucidated further.

There was a clear difference between the sexes regarding cadmium concentrations in hepatopancreas. The cadmium concentration was more than twice as high for the male shore crabs. In correspondence with these results, Bjerregaard *et al.* (2005) also found generally lower cadmium concentration in female hepatpancreas, compared to male shore crabs. As food presumably is the most important cadmium source for shore crabs (Bjerregaard *et al*., 2005; Pedersen *et al*., 2014), it is possible that differences in foraging strategy between male and female shore crabs may lead to differences in accumulation of cadmium. The sexes behave differently in the coastal zone, where the females generally stay at deeper waters than the males (Reid *et al.,* 1997). Furthermore, the males are generally larger, with bigger and presumably stronger claws, which enables foraging on larger organisms (Kaiser et al., 1990) with potentially higher cadmium levels.

The cadmium concentration in muscle meat from claws was not significantly different between male and female shore crabs. The majority of the accumulated cadmium was measured in hepatopancreas, and the whole body cadmium distribution was approximately 92, 7.9 and 0.10 % in hepatopancreas, female gonads and muscle meat from claws, respectively for the analyzed

tissues. The cadmium distribution in the analyzed tissues was similar to other studies on both shore crabs and brown crabs (Bondgaard and Bjerregaard, 2005; Bjerregaard *et al.,* 2005; Weis, 2012; Frantzen *et al.,* 2015; Wiech *et al.,* 2017).

Except for a clear relationship between cadmium concentrations and sex, and weak correlations between hepatosomatic index and carapace width and cadmium, there was no correlation between cadmium concentrations and the registered physiological variables. Limited range in visually assessed parameters such as carapace colour, number of legs and claws, moulting stage, presence of sperm plugs and gonad maturation might be the reason why no relationships between cadmium levels and these individual parameters were found. Other studies have shown higher cadmium accumulation rates for crabs in early post-moult and early ovarian maturation stages when exposed to cadmium in water (Bondgaard *et al.,* 2000; Bondgaard and Bjerregaard, 2005; Nørum *et al.,* 2005), and green shore crabs seems to accumulate more cadmium than red shore crabs (Nissen *et al.,* 2005; Styrishave *et al.,* 2000).

4.4 Cadmium in shore crab soup

Even though the amount of cadmium extracted from the crabs to the soup was relatively high, the cadmium concentrations in the prepared shore crab soup were low. Therefore, shore crab soup was considered to be safe regarding cadmium exposure. Based on the highest measured cadmium concentration of 79 µg Cd/kg ww, a portion size of 100 g would constitute approximately 5 % of the tolerable weekly intake (TWI) for a person weighing 70 kg, based on the TWI of 2.5 µg Cd/kg body weight (EFSA, 2009), set by the European Food Safety Authority (EFSA). However, it is estimated that the average cadmium exposure from food is approximately 1.7 µg/kg body weight per week for an adult Norwegian person (VKM, 2015). Taking this into consideration, the additional dietary cadmium exposure allocated to other dietary sources is 56 µg per week given a body weight of 70 kg, which corresponds to approximately seven portions of shore crab soup per week. As such, shore crab soup is not considered problematic regarding food safety. Furthermore, a cooking time of 30 minutes may be excessive as the soup may become bitter during the long cooking process (personal observation). However, it was chosen as worst-case scenario to ensure sufficient cadmium extraction. In addition, all the pooled samples were under the legal limit of 0.5 mg Cd/kg ww in claw meat for humane consume, set by EU (EU, 2006).

5. Conclusion

The cadmium concentrations in shore crabs in this study were very low in muscle meat from claws, and between 0.046 mg Cd/kg ww and 11 mg/kg ww in hepatopancreas. There was no clear geographical difference with latitude as opposed to earlier findings in the brown crab. Possible explanations for this may be that these species have different feeding habits or that the shore crab is better adapted to the colder climate in Northern Norway. Sex had a clear impact on the cadmium levels in hepatopancreas, as the male shore crabs had approximately twice as high cadmium levels compared to the females. No clear and consistent correlations were found between cadmium and other registered individual variables, but some minor relationships were seen with an indication of cadmium accumulation over time as well as a weak relationship between cadmium concentrations and fluctuating water contents of tissues. Cadmium concentrations were lower in August than in April. Most of the total amount of cadmium was allocated in the hepatopancreas while muscle meat and gonads of females contributed together with less than 10 %. None of the measured cadmium levels exceeded EUs legal limit of 0.5 mg Cd/kg ww set for claw meat for human consumption.

Low cadmium levels in shore crab soup make it a safe food item regarding cadmium and food safety.

Acknowledgments

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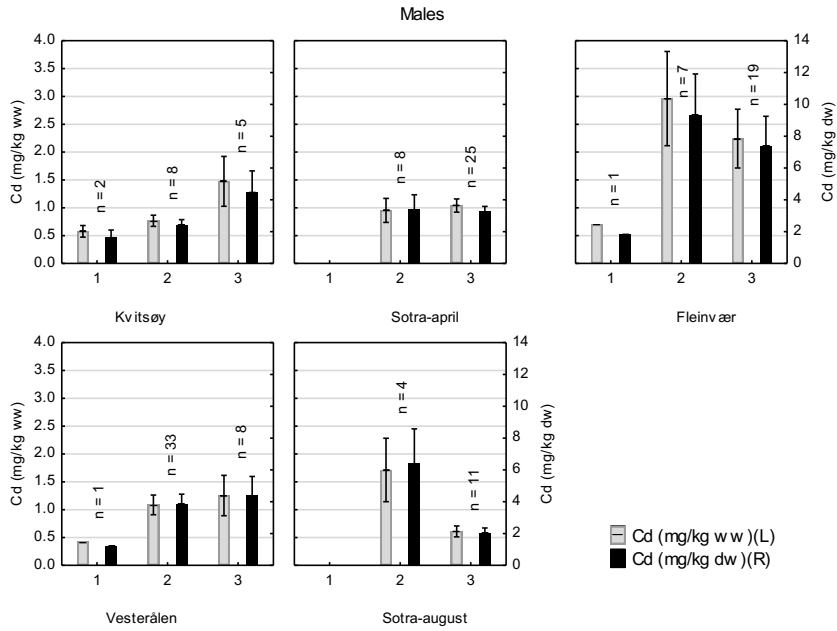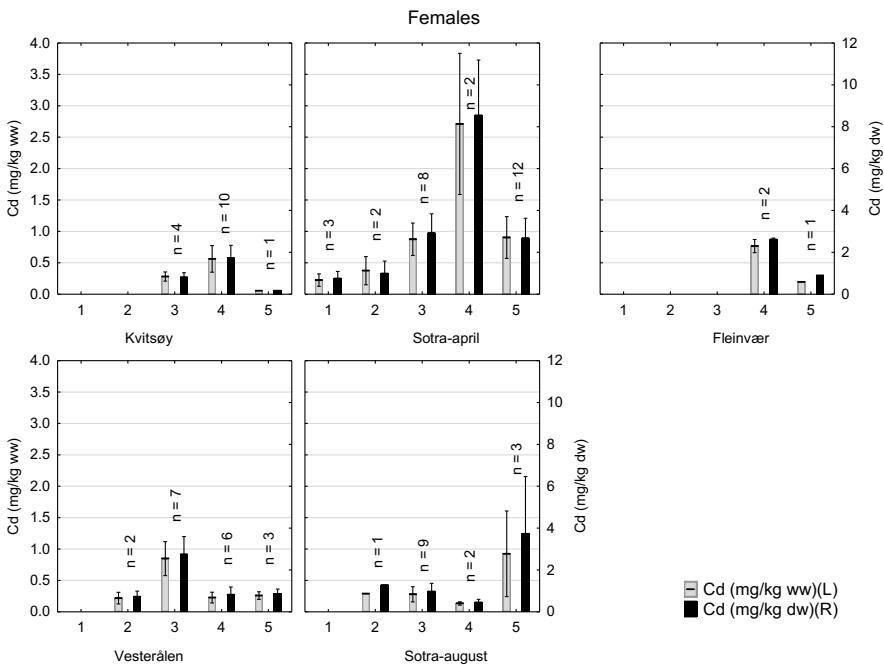
Supplementary Material

Figures
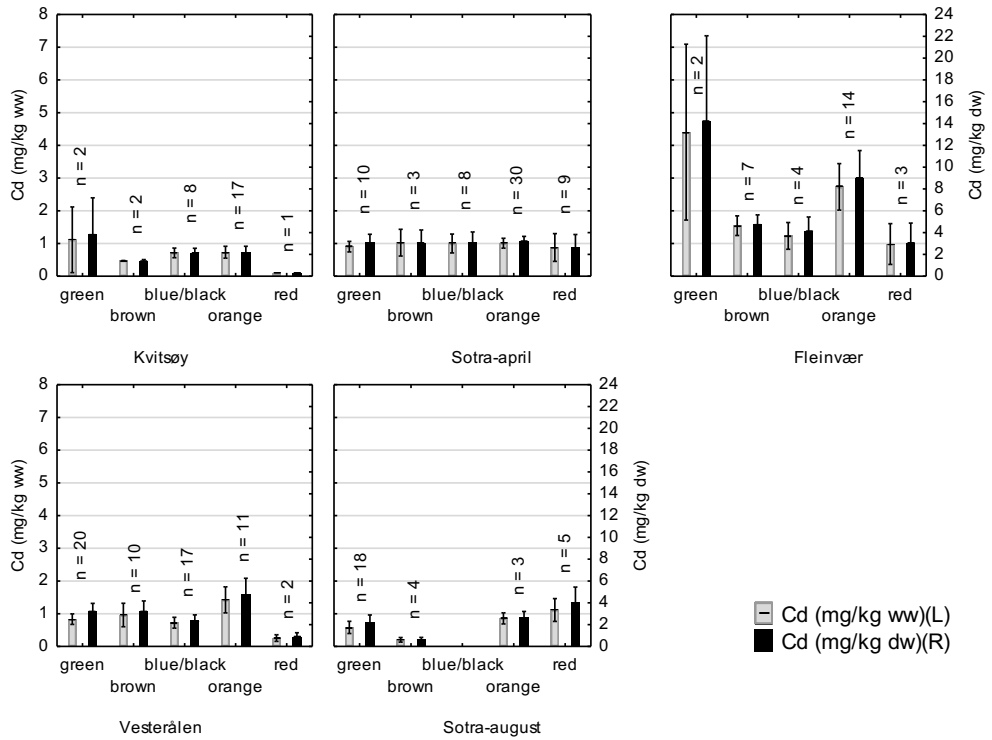
Supplementary figure 1. Wet and dry weight based concentrations of cadmium in hepatopancreas of shore crabs (Carcinus maenas) from different sites along the Norwegian coast with different carapace color. Bars denote the mean concentration and whiskers the standard error. The number of crabs within each category is given.
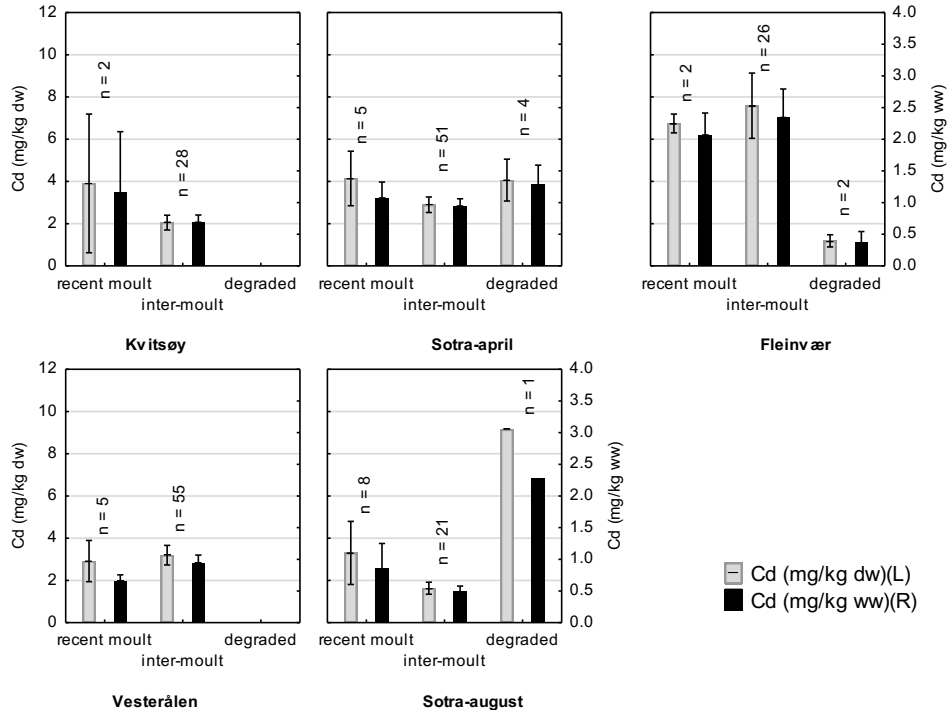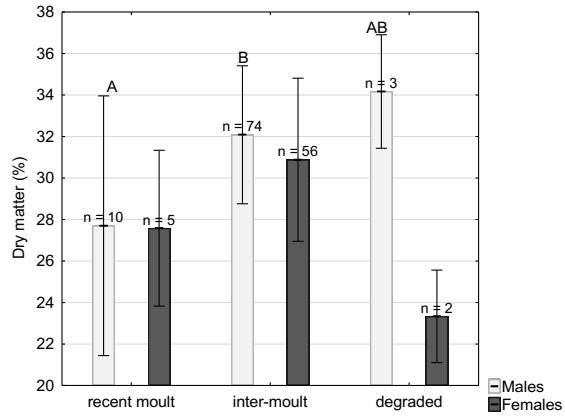
Supplementary figure 2. Wet and dry weight based concentrations of cadmium in hepatopancreas of shore crabs (Carcinus maenas) from different sites along the Norwegian coast at different moulting stages. Bars denote the mean concentration and whiskers the standard error. The number of crabs within each category is given.

Supplementary figure 3. Wet and dry weight based concentrations of Cd in hepatopancreas of male shore crabs (Carcinus maenas) from different sites along the Norwegian coast at different gonad maturation stages. Bars denote the mean concentration and whiskers the standard error. The number of crabs within each category is given.

Supplementary figure 4. Wet and dry weight based concentrations of Cd in hepatopancreas of female shore crabs (Carcinus maenas) from different sites along the Norwegian coast at different gonad maturation stages. Bars denote the mean concentration and whiskers the standard error. The number of crabs within each category is given.

Supplementary figure 5 Dry matter content of hepatopancreas of male and female shore crabs at different moulting stages. Bars denote the mean concentration and whiskers one standard deviation. The number of crabs within each category is given. Different letters indicate significant differences.

Tables

Supplementary table 1. Cadmium concentrations (mg/kg wet weight) in muscle meat from claws and female gonads of shore crabs (Carcinus maenas) from the Norwegian coast. Mean ± SD and concentration ranges are given for each group, except for the measured concentration levels in female gonads, as these results are based on measurements of only one pooled sample (N = 1).

Area	Tissue	Male			Female		
		N	Mean ± SD (mg/kg ww)	Range (mg/kg ww)	N	Mean ± SD (mg/kg ww)	Range (mg/kg ww)
Kvitsøy	Muscle	3	0.0053 ± 0.0042	0.0028 – 0.010	3	0.0028 ± 0.00058	0.0021 – 0.0032
	Gonad	-	-	-	1	0.023	-
Sotra-April	Muscle	3	0.0022 ± 0.00063	0.0015 – 0.0027	3	0.0024 ± 0.00033	0.0020 – 0.0027
	Gonad	-	-	-	1	0.017	-
Fleinvær	Muscle	3	0.0027 ± 0.0015	0.0017 – 0.0044	3	0.0037 ± 0.0015	0.0020 – 0.0051
	Gonad	-	-	-	1	0.0092	-
Vesterålen	Muscle	3	0.0018 ± 0.00037	0.0015 – 0.0022	3	0.0020 ± 0.00023	0.0018 – 0.0022
	Gonad	-	-	-	1	0.015	-
Sotra-August	Muscle	3	0.0017 ± 0.0012	0.0030 – 0.0012	3	0.0020 ± 0.0	0.0020 – 0.0020
	Gonad	-	-	-	1	0.015	-
All areas	Muscle	15	0.0027 ± 0.0022	0.0010 – 0.010	15	0.0025 ± 0.00090	0.0018 – 0.0051
	Gonad	-	-	-	5	0.015 ± 0.0055	0.0092 – 0.023

Supplementary table 2. Cadmium concentrations (mg/kg dry weight) in muscle meat from claws and female gonads of shore crabs (Carcinus maenas) from the Norwegian coast. Mean ± SD and concentration ranges are given for each group, except for the measured concentration levels in female gonads, as these results are based on measurements of only one pooled sample (N = 1).

| Area	Tissue	Male			Female		
		N	Mean ± SD (mg/kg dw)	Range (mg/kg dw)	N	Mean ± SD (mg/kg dw)	Range (mg/kg dw)
Kvitsøy	Muscle	3	0.022 ± 0.016	0.013 – 0.041	3	0.012 ± 0.0013	0.010 – 0.013
	Gonad	-	-	-	1	0.050	-
Sotra-April	Muscle	3	0.0087 ± 0.0026	0.0059 – 0.011	3	0.0095 ± 0.0010	0.0083 – 0.010
	Gonad	-	-	-	1	0.044	-
Fleinvær	Muscle	3	0.013 ± 0.0083	0.0062 – 0.022	3	0.015 ± 0.0067	0.0080 – 0.021
	Gonad	-	-	-	1	0.020	-
Vesterålen	Muscle	3	0.0069 ± 0.0015	0.0058 – 0.0086	3	0.0086 ± 0.0010	0.0074 – 0.0094
	Gonad	-	-	-	1	0.022	-
Sotra-August	Muscle	3	0.0072 ± 0.0045	0.0044 – 0.012	3	0.0085 ± 0.00036	0.0084 – 0.0085
	Gonad	-	-	-	1	0.044	-
All areas	Muscle	15	0.012 ± 0.0093	0.0044 – 0.041	15	0.011 ± 0.0036	0.0074 – 0.021
	Gonad	-	-	-	5	0.036 ± 0.014	0.020 – 0.050

Supplementary table 3. Significant pearson correlations between carapace width (cm), water content in hepatopancres (%), hepatosomatic index (%) and cadmium concentrations (mg/kg) based on dry weigth and wet weight and total cadmium content (mg).

Variables	Male shore crabs	Female shore crabs
Carapace with (cm) and whole wet weight (g)	r = 0.9498***, r^2 = 0.90 for all locations	r = 0.9404***, r^2 = 0.88 for all locations
Carapace width (cm) and water content in HP (%)	r = 0.4989***, r^2 = 0.25 for all locations	r = 0,5633*** , r^2 = 0.32 for all locations
Carapace width (cm) and HSI (%)	r = -0.5954*** , r^2 = -0.35 for all locations	-
Carapace width (cm) and Cd (mg/kg ww)	r = 0.2233**, r^2 = 0.050 for all locations r = 0.3983**, r^2 = 0.15 for Vesterålen	-
Carapace width (cm) and Cd (mg/kg dw)	r = 0.3030***, r^2 = 0.092 for all locations r = 0.5344***, r^2 = 0.29 for Vesterålen	-
Carapace width (mm) and Cd content (mg) in HP	r = 0.5312***, r^2 = 0.28 for all locations r = 0.3594*, r^2 = 0.13 for Sotra-April r = 0.6872***, r^2 = 0.47 for Vesterålen	r = 0.2824*, r^2 = 0.080 for all locations r = 0.3835*, r^2 = 0.15 for Sotra-April
Water content in HP (%) and Cd (mg/kg ww)	r = 0.1713* r^2 = 0.029 for all locations r = 0.4424*, r^2 = 0.20 for Vesterålen	-
Water content in HP (%) and Cd (mg/kg dw)	r = 0.3574*** r^2 = 0.13 for all locations r = 0.6260***, r^2 = 0.39 for Vesterålen	-
Water content in HP (%) and Cd content (mg)	r = 0.2074* r^2 = 0.043 for all locations r = 0.5493***, r^2 = 0.30 for Vesterålen	-
HSI (%) and Cd (mg/kg ww)	r = -0.3511* , r^2 = 0.12 for Vesterålen	r = -0.2244* , r^2 = 0.050 for all locations r = -0.4529*, r^2 = -0.21 for Sotra-April
HSI (%) and Cd (mg/kg dw)	r = -0.2549*, r^2 = -0.065 for all locations r = -0.5163***, r^2 = 0.27 for Vesterålen	r = -0.2249*, r^2 = -0.051 for all locations r = -0.5078**, r^2 = -0.25 for Vesterålen
HSI (%) and Cd (mg)	r = -0.3548*, r^2 = 0.13 for Vesterålen	

Errata

Errata for

Cadmium in Brown Crab *Cancer pagurus* in Norwegian Waters

Martin Wiech
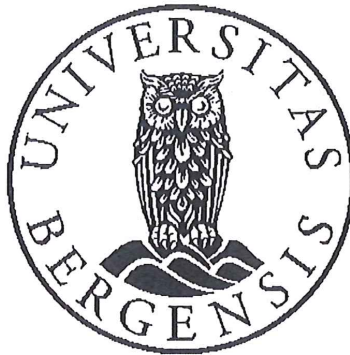
Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

10 May 2018

(date and sign. of candidate)   (date and sign. of faculty)

Errata

Page IV: "brown" was added before "crab"

Page 1: "a" was deleted before "planktonic"

Page 2: "indicates" was changed to "indicated"

Page 3: "the" was deleted after "The"

Page 4: "and fuel combustion" deleted after "and"

Page 4: "heavy" added before "industrial"

Page 5: "nutrient" was changed to "nutrients"

Page 7: "the" inserted after "indicate" and "in"

Page 7: "assessments" changed to "assessment"

Page 9: "was" was changed to "were"

Page 9: "brown" was added before "crab"

Page 10: "of" inserted after "catch"

Page 11: "the brown" deleted before "crab"

Page 11: "described" was changed to "described"

Page 16: "(Bjerregaard and Depledge, 1994)" was deleted after "(Bjerregaard and Depledge, 1994)"

Page 16: "brown" was added before "crabs

Page 17 : "foyr" was changed to "four"

Page 23: "and shore" was deleted after "brown"

Page 25: "levels" was changed to "level"

Page 28: "in" was changed to "is"

Page 28: "with, ranging from 4.7 ° to 12.3 °C during the same time and at the same depth" was changed to ", ranging from 4.7 ° to 12.3 °C during the same time and at the same depth, was measured"

Page 28: "and" added after "spring"

Page 29: "in" added after "found"

Page 31: "in" deleted after "species"

Page 19: "to" inserted after "compared"

Page 37: "are" to "area"

Page 37: "compared" was changed to "comparable"

Page 37: "could be at risk" changed to "are at risk"

Page 38: "not" was changes to "no"

Page 41: "in" deleted after "in a"

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