

Responses to Toxic Chemicals at the Molecular, Cellular, Tissue, and Organismal Level

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INTRODUCTION

A variety of anthropogenic chemicals, referred to as organic xenobiotics, and trace metals can be present in the blue crab's estuarine environment. Organic xenobiotics include aromatic hydrocarbons, organometallics, organohalogens, and various pesticides, all of which have the potential to affect crab growth, reproduction, and development. Although trace metals such as cobalt, copper, and selenium are natural components of aquatic systems and essential for a number of physiological functions, in excess they can become toxic. This chapter focuses on how blue crabs respond to and are affected by exposure to organic xenobiotics and trace metals.

ORGANIC CONTAMINANTS

Blue crabs collected from a number of contaminated sites have been found to contain elevated concentrations of each site's contaminants (Roberts 1981; Marcus and Mathews 1987; Hale 1988; Mothershead et al. 1991; Murray et al. 1992). Xenobiotics enter crabs from water, sediment, or food via the gill or stomach and accumulate in the lipid-rich hepatopancreas (Fig. 1). The crustacean hepatopancreas has many of the functions associated with the

vertebrate liver, pancreas, and small intestine. These include synthesis and secretion of digestive enzymes, uptake of nutrients, and accumulation of nutrient reserves (Gibson and Barker 1979; Wright and Ahearn 1997). The hemolymph functions as an important avenue for transporting xenobiotics and xenobiotic metabolites. After xenobiotics enter the crab, their fate is determined by the processes of accumulation, biotransformation, and elimination. The relative importance of these different processes for a particular xenobiotic depends on a number of factors including the physical-chemical properties of the xenobiotic, the ability of the crab enzyme systems to metabolize the compound, and the amount of storage lipid in the crab. Known biotransformation pathways of xenobiotics and their effects in blue crabs are shown schematically in Fig. 2.

Although our focus is the blue crab, we refer to studies of other crustacean groups, assuming that most crustaceans respond to xenobiotics in a similar manner. First we summarize our present knowledge of protein enzyme systems in blue crabs that bind and metabolize organic xenobiotics. Next we address the location of these systems within tissues and particular cell types. We then follow with a review of the effects of various contaminant groups and their metabolites on blue crabs. For additional

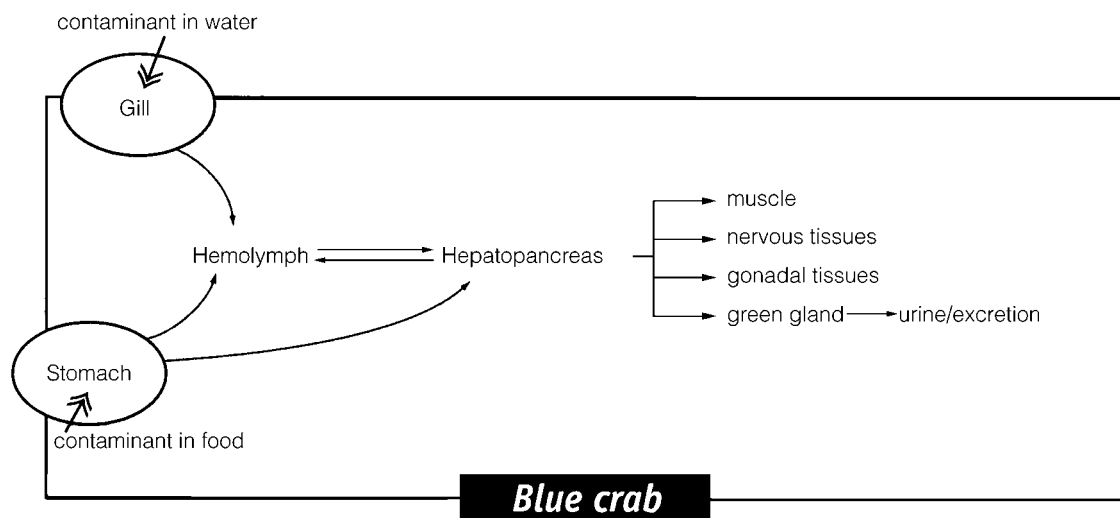


Figure 1. Uptake and bioaccumulation of organic contaminants by blue crabs.

information, the reader is referred to a number of excellent reviews that discuss xenobiotic metabolism by crustaceans (James 1989a; Livingstone 1991; Kleinow et al. 1992).

Molecular Responses

Metabolism of Xenobiotics

Most organic contaminants of concern are hydrophobic. A number of enzyme systems can add polar groups to these compounds, which increase their water solubility and thus facilitate their elimination. However, for some compounds the metabolites are more toxic than the parent compound. For example, the binding of certain reactive benzo(a)pyrene metabolites, i.e., arene oxides, to DNA in liver cells of mammals initiates carcinogenesis (Ames et al. 1972; Weisburger 1978; Brookes et al. 1979; Miller and Miller 1979). The reactions carried out by biotransformation enzyme systems can be broadly divided into two groups: phase 1 reactions which include oxidation, reduction, and hydrolysis, and phase 2 reactions which involve conjugation of sulfate, sugars, or peptides to polar groups such as $-\text{COOH}$, $-\text{OH}$, or $-\text{NH}_2$ groups which, in some cases, were added to the xenobiotic during phase 1 reactions. Phase 2 metabolites tend to be highly water soluble and can be more rapidly eliminated from crabs. Some contaminants already contain a

polar group. e.g., phenols, and only phase 2 reactions take place with these compounds.

Phase 1 Reactions. One of the most investigated of phase 1 enzyme systems is the cytochrome P-450 dependent monooxygenase (also called mixed function oxidase or MFO) system which oxidizes xenobiotics by hydroxylation, O-dealkylation, N-dealkylation, or epoxidation. A carbon monoxide difference spectrum showing the presence of cytochrome P-450 isolated from hepatopancreas microsomes of the blue crab, is shown in Fig. 3. Inhibition of cytochrome P-450 by detergents and by treatment with phospholipase C indicates that phospholipids are necessary for MFO activity in blue crabs (Singer and Lee 1977; Lee and Quattrochi 1987). Thus, the MFO system in crabs, as in other animals, is a multi-component system composed of phospholipid, cytochrome P-450, and NADPH cytochrome P-450 reductase (Lu 1976; Philpot et al. 1976; Porter and Coon 1991). Partial purification of cytochrome P-450 from blue crab hepatopancreas has been achieved by using sodium cholate solubilization of microsomes, affinity chromatography (Octylamine-Sepharose 4B), and hydroxyapatite chromatography (Conner and Singer 1981).

Examples of substrates metabolized by the MFO system in blue crabs are shown in Fig. 4. Figure 5 diagrams the steps involved in the hydroxylation of the

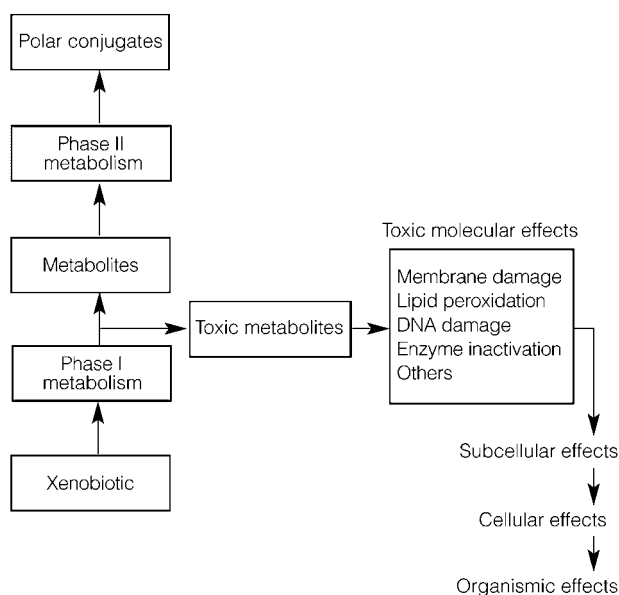


Figure 2. Biotransformation and effects of xenobiotics in blue crabs.

polycyclic aromatic hydrocarbon (PAH), benzo(a)pyrene, by the MFO system. The steps shown here are based primarily on studies with the vertebrate MFO system (Ahbad 1979; Hodgson 1979; Nebert and Jensen 1979; Guengerich 1990, 1993). In summary, the benzo(a)pyrene binds to the oxidized cytochrome P-450(Fe^{3+}) and the complex undergoes reduction to cytochrome P-450(Fe^{2+}) which then interacts with oxygen. A hydroxylated substrate, e.g. 3-hydroxybenzo(a)pyrene, and a molecule of water leave the now reoxidized cytochrome P-450. The substrate-oxidized P-450 complex is reduced by two electrons from NADPH carried by NADPH cytochrome P-450 reductase. The superoxide anion (O_2^-) is thought to be formed during the reaction and participates in the hydroxylation of the substrates.

Important intermediates in the oxidative metabolism of hydrocarbons and other xenobiotics are alkene and arene oxides, many of which are very reactive electrophiles capable of interactions with cellular macromolecules such as DNA and proteins. Epoxide hydrolase, another phase 1 enzyme that metabolizes these epoxides to dihydrodiols, has been found in the blue crab hepatopancreas (James et al. 1979a).

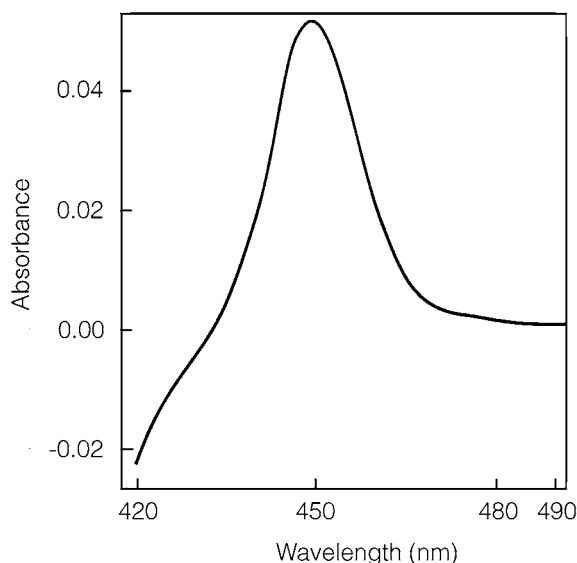


Figure 3. Carbon monoxide-difference spectrum of dithionite-reduced hepatopancreas microsomes from blue crabs.

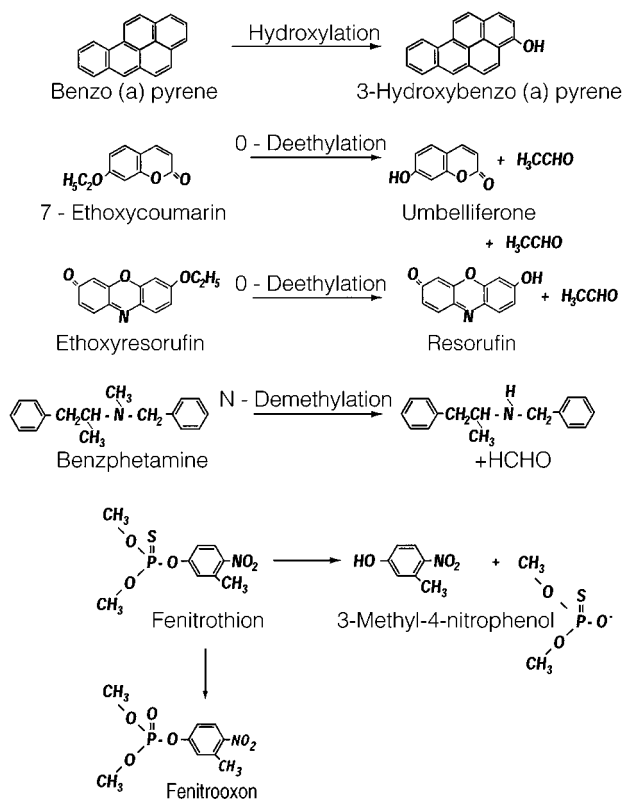


Figure 4. Mixed function oxidase reactions reported in blue crabs.

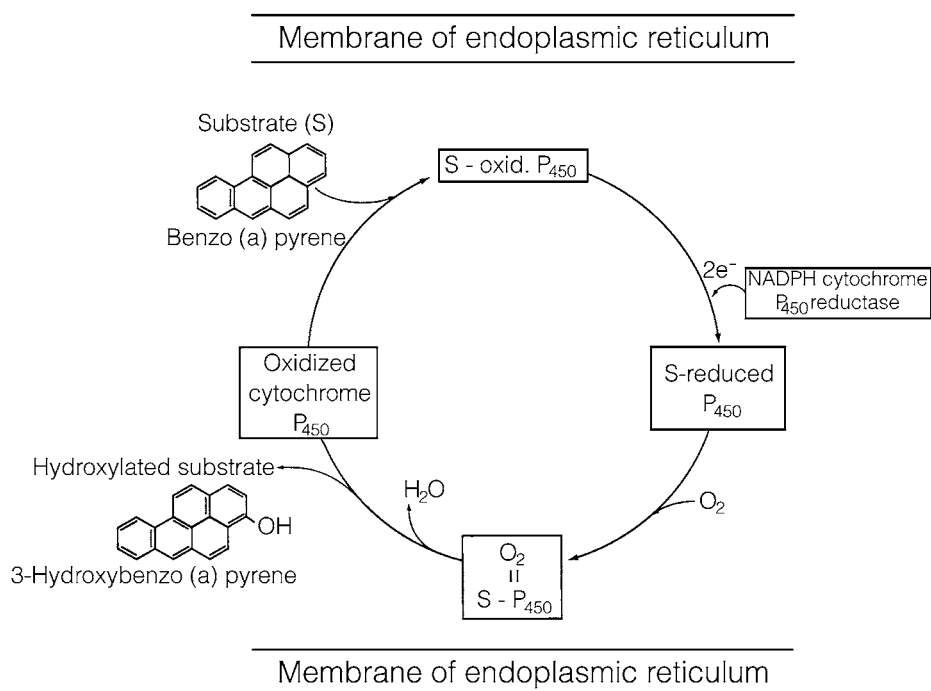


Figure 5. Reactions involved in the metabolism of benzo(a)pyrene by the mixed function oxidase system.

Phase 2 Reactions. Phase 2 reactions involve conjugation of phase 1 products with a polar or ionic moiety. The most common moieties involved in these conjugation reactions are glucose, glucuronic acid, sulfate, and glutathione. In general these conjugation products are water soluble so they are more easily eliminated from the animal than the parent compound. Many of the phase 1 products are electrophiles or nucleophiles. Electrophiles are molecules containing electron-deficient atoms with a partial or full positive charge that allows them to react by sharing electron pairs with electron-rich atoms in nucleophiles. Glutathione-S-transferase catalyzes the conjugation of the nucleophilic tripeptide, glutathione (GSH, γ -glutamylcysteinylglycine), to electrophiles that are produced by P-450 systems acting on various xenobiotics (Armstrong 1991). Electrophilic substrates shown to be conjugated to glutathione by blue crab glutathione-S-transferase include 1-chloro-2, 4-dinitrobenzene; 1,2-dichloro-4-nitrobenzene (Fig. 6); 1,2-epoxy-3(p-nitrophenoxy) propane; styrene 7,8-oxide; p-nitrophenyl

acetate; bromosulphophthalin; and benzo(a)pyrene-4,5-oxide (Tate and Herf 1978; James et al. 1979a; Johnston and Corbett 1986a; Keeran and Lee 1987).

Nucleophiles formed by phase 1 reactions are conjugated at the nucleophilic functional groups. For example, hydroxylated compounds are conjugated to sulfate or carbohydrates. *In vivo* studies with several crustacean species have shown formation of sulfate and glycoside conjugates after exposure to various polycyclic aromatic hydrocarbons (Corner et al. 1973; Sanborn and Malins 1980; Little et al. 1985; Reichert et al. 1985). Sulfo-

transferases catalyze the transfer of the sulfuryl group, SO_3^- , from phosphoadenosyl phosphosulfate (PAPS) to a nucleophilic acceptor, such as a hydroxyl or amino group. For example, pentachlorophenol is conjugated by sulfotransferases to form pentachlorophenol sulfate in blue crabs (Fig. 6). Phase 1 metabolites containing phenolic or carboxylic acid groups or other nucleophilic centers can undergo glycosylation (Fig. 6), where UDPG = uridine diphospho-D-glucuronic acid or uridine diphospho-D-glucose. In crustaceans the sugar moiety is more often glucose than glucuronic acid (Kleinow et al. 1992). In blue crabs an example is the glycoside conjugated to 3-methyl-4-nitrophenol, a metabolite of the organophosphate insecticide fenitrothion (Figs. 4, 6; Johnston and Corbett 1986b). *In vivo* studies have shown that blue crabs form various conjugates of the metabolites of tributyltin oxide, benzo(a)pyrene and chlorobenzoic acid. A significant amount of these metabolites is bound to macromolecules, including GSH-S-transferase (Lee 1989).

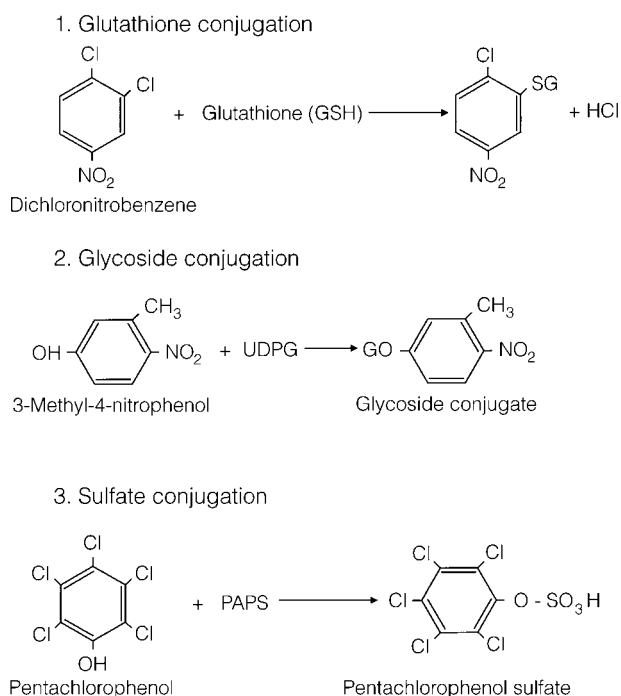


Figure 6. Phase 2 conjugation reactions reported in blue crabs.

Cellular and Tissues Responses

The hepatopancreas has many of the functions associated with the vertebrate liver, pancreas, and small intestine (Gibson and Barker 1979; Wright and Ahearn 1997). *In vivo* and *in vitro* studies have shown that blue crab hepatopancreas plays a major role in xenobiotic metabolism (Sheridan 1975; Lee et al. 1976; Bend et al. 1977; Johnston and Corbett 1986a,b; James 1989b; Rice et al. 1989; Oberdörster et al. 1998). Cytochrome P-450 and glutathione-S-transferase and other phase 2 enzyme systems have been found in crustacean hepatopancreas (James et al. 1979a,b; Lindstrom-Seppa and Hanninen 1986; James 1986, 1987, 1990; Keeran and Lee 1987; Almar et al. 1988; James and Boyle 1998). In the blue crab, while the hepatopancreas has high specific content of cytochrome P-450, the cytochrome P-450 dependent MFO activity of this tissue is low due to inhibitors released when the hepatopancreas is homogenized (James et al. 1979b; Lee 1981, 1986). Activity of MFO is high in the blue crab stomach and green gland (Singer et al. 1980), but

low in blood, gill, reproductive tissues, eyestalk, and cardiac muscle (Singer and Lee 1977). Glutathione S-transferase activity is high in blue crab hepatopancreas and gills (Lee et al. 1988).

Different cells types found in the crustacean hepatopancreas include E-, F-, R-, and B-cells (Fig. 7; Robinson and Dillaman 1985; Al-Mohanna and Nott 1986; Wright and Ahearn 1997). The F-, R-, and B-cells are derived from embryonic or E-cells (Vogt 1994; Biesiot and McDowell 1995). The R-cells are storage cells with large amounts of lipid (Fig. 7), while the F- and B-cells are thought to be important in protein synthesis. The F-cells have a fibrillar appearance due to the presence of abundant rough endoplasmic reticulum and Golgi network (Fig. 7; Al-Mohanna et al. 1985; Robinson and Dillaman 1985; Ahearn 1988).

Lee (1989) introduced a number of ^{14}C - xenobiotics into blue crab food and determined the distribution of the xenobiotics and their metabolites within the hepatopancreatic cells. For compounds not readily metabolized, i.e. hexachlorobiphenyl, Mirex, and DDE, radioactivity is primarily in the storage lipid in the R-cells (Table 1). For compounds more extensively metabolized, i.e., benzo(a)-pyrene, tributyltin, pentachlorophenol, 1-chloro-2,4-dinitrobenzene, 1,2,4,5-tetrachlorobenzene,

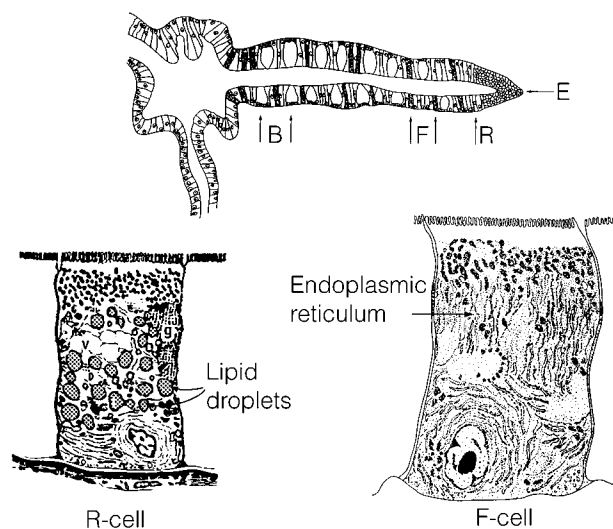


Figure 7. Diagram of cross-section of blue crab hepatopancreas tubule showing location of E-, F-, R- and B-cells, as well as diagrams of R- and F-cells.

Table 1. Distribution of xenobiotics and their metabolites within different cells of blue crab hepatopancreas.

¹⁴ C-compound	R-Cells (%)	F-Cells (%)	B-Cells (%)
Hexachlorobiphenyl	98	2	2
Benzo(a)pyrene	22	51	31
2,4-Dinitrochlorobenzene	12	68	27
Tributyltin chloride	10	72	14

¹⁴C-labeled compounds (8×10^5 disintegrations per minute) were added to the food and fed to juvenile male blue crabs. The animals were sacrificed after 2 d and the hepatopancreas removed and treated with collagenase. The different cell types of the hepatopancreas were separated by Percoll density gradients. Data from Lee (1989).

chlorobenzene, 2,3,6-trichlorophenol, 2,4-dichloro-6-nitrobenzene, 2,4-dichloro-6-nitroaniline, fluorene, and p-chlorobenzoic acid, much of the radioactivity is in the cytosol of the F-cells (Lee 1989; Table 1). It appears that initially these compounds enter R-cell lipids and are then transferred to and metabolized by F-cells, with the principal product being water-soluble conjugates that are eliminated from the crab. The cytochrome P-450 in blue crabs is associated with the endoplasmic reticulum of F-cells (Lee 1986). The highest activity of glutathione-S-transferase in blue crabs is in F-cell cytosol, with significantly lower activity in B-cells and barely detectable activity in R-cells (Keeran and Lee 1987).

Binding of Xenobiotics to Cellular Macromolecules

After entrance of a xenobiotic into cells, the compound and its metabolites are distributed among the cytosol, outer membrane, lipid droplets, and different organelles. The compounds and their metabolites can be "dissolved" in the lipid droplets or the hydrophobic component of the membrane or organelle, or covalently bound to cellular macromolecules such as DNA, RNA, or proteins. For some compounds, cellular damage has been found only in organs where there is covalent binding of metabolites to macromolecules (Bartolone et al. 1987;

Hodgson and Levi 1987). In mammals the binding of xenobiotics to DNA has been used as a measure of their carcinogenic potential (Lutz 1979). Studies with fish have shown that PAH metabolites form DNA adducts (Reichert et al. 1985; Varanasi et al. 1986; Von Hofe and Puffe 1986; Sikka et al. 1991). DNA adducts of benzo(a)pyrene have been found in two species of lobsters, *Panulirus argus* and *Homarus americanus* (James et al. 1992, 1995), and would be expected to be formed in blue crabs. In the blue crab, introduction of ¹⁴C-benzo(a)pyrene, tributyltin, bromobenzene, and fluorene via food leads to binding of their metabolites to macromolecules in hepatopancreas cells (Lee 1989). A portion of these metabolites is bound to cellular lipoproteins and glutathione-S-transferases, one of the major proteins in the cytosol of blue crab hepatopancreas (Keeran and Lee 1987). At present, little is known about the relation between binding of contaminants to cellular macromolecules and their effects on blue crabs.

Organismal Responses to Different Classes of Xenobiotics

Blue crabs are exposed in estuaries to various classes of contaminants including polycyclic aromatic hydrocarbons, organophosphorus pesticides, organochlorine compounds, and organometallics. Each of these classes has very different effects on

blue crabs depending on the physical-chemical properties of the compounds as well as the relative importance of the processes of accumulation, biotransformation, and elimination.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons present in estuaries and nearshore coastal waters as a consequence of crude oil production, shipping, and use of petroleum hydrocarbons are metabolized by crustacean cytochrome P-450 systems (Lee et al. 1976; Lee 1981; James 1989a,b). The polar metabolites produced by the P-450 system can be conjugated to glutathione, glucose, or sulfate (Corner et al. 1973; Sanborn and Malins 1980; Little et al. 1985; Reichert et al. 1985). A variety of PAHs taken up from food and water by blue crabs are oxidized, conjugated, and eliminated (Lee et al. 1976; Moese and O'Connor 1985).

Exposure of fish to PAH is correlated with reduced plasma estradiol concentrations, disruption of vitellogenesis, and decreased egg production (Hall and Oris 1991; Johnson et al. 1993). Exposure of juvenile blue crabs to benzene, dimethylnaphthalene, and the water-soluble fraction of South Louisiana crude oil inhibits growth and molting (Cantelmo et al. 1981, 1982; Wang and Stickle 1987). Recent studies show that several PAHs enhance ecdysone (molt hormone)-dependent gene transcription and ecdysone-dependent cell proliferation (Oberdörster et al. 1999). Thus, PAHs can be considered to be endocrine-disrupting chemicals in fish and crabs.

Organophosphorus Pesticides

Organophosphate and organochlorine compounds are designed to be arthropod (insect) poisons and, therefore, are inherently toxic to blue crabs and other crustaceans. Organophosphate pesticides impair the nervous system by binding to and inhibiting acetylcholinesterase (Hodgson and Levi 1987). Crustaceans are more sensitive to these pesticides than marine fishes by several orders of magnitude (Eisler 1969; Odenkirchen and Eisler 1988), suggesting that the esterases of crustaceans and fish have different sensitivities to cholinesterase

inhibitors. Blue crabs are also sensitive to a number of organophosphate insecticides (Butler 1965; Schimmel et al. 1983). Johnston and Corbett (1985, 1986a,b) have reported on the effects and metabolism of the widely used organophosphate insecticide, fenitrothion, by blue crabs. Fenitrothion is oxidized by the crab's MFO system to fenitrooxon (Fig. 4) which is a well known cholinesterase inhibitor. In addition to fenitrooxon, methylnitrophenols are formed which in turn form sulfate and glycoside conjugates (Figs. 4, 6). Other crustacean species have fenitrothion metabolic pathways similar to those found for blue crabs (Kobayashi et al. 1985; Escartin and Porte 1996). An important effect of the organophosphate, parathion, is due to the action of the MFO to produce paraoxon, a cholinesterase inhibitor (Fukuto 1990). Presumably blue crabs can form paraoxon using the MFO system via a mechanism similar to the formation of fenitrooxon from fenitrothion.

Organochlorine Compounds

Blue crabs can be exposed to a variety of organochlorine compounds in contaminated estuaries, with effects on growth, reproduction, and development (Nimmo et al. 1975; Bookhout et al. 1976, 1980; Koenig et al. 1976; Schimmel et al. 1979). Several organochlorine compounds, including polychlorobiphenyls, tetrachlorophenol and pentachlorophenol inhibit limb regeneration in fiddler crabs *Uca pugilator* and grass shrimp *Palaemonetes pugio*, whereas DDT accelerates regeneration (Weis et al. 1992). Sheridan (1975) showed that DDT was metabolized to DDE and DDD (dechlorination products) when blue crabs were exposed to DDT in the water. Storage and metabolism of DDT takes place in the hepatopancreas. Highly chlorinated compounds such as Mirex and hexachlorinated biphenyls are metabolized at a very slow rate, if at all, and these compounds accumulate in the hepatopancreas of the blue crab (Schoor 1974; Lee 1989, 1993). Mirex is toxic to blue crabs at concentrations as low as 0.5 ppb (Tagatz et al. 1975). Less chlorinated compounds including pentachlorophenol, p-chlorobenzoic acid, 1,2,4,5-tetrachlorobenzene, chlorobenzene, 2,3,6-trichlorophenol, 2,4-dichloro-

6-nitroaniline, and 1-chloro-2,4-dinitrochlorobenzene are rapidly metabolized, conjugated, and eliminated by the blue crab (Lee 1989). The metabolites of the different compounds form sulfate, glycoside, or glutathione conjugates with the relative proportion of each type of conjugates depending on the properties of the metabolite. 1-chloro-2,4-dinitrochlorobenzene is conjugated primarily to glutathione and is rapidly eliminated from the blue crab. Figure 8 shows the subcellular distribution of ^{14}C -1-chloro-2,4-dinitrobenzene given in the blue crab's food. After 3 d, most of the radioactivity found in the crab is in the hepatopancreas cytosol. This radioactivity is associated with glutathione conjugates that are rapidly eliminated from the crab. In contrast, blue crabs fed food containing ^{14}C -1,2,4,5-tetrachlorobenzene have much of the radioactivity in lipid droplets of hepatopancreas R-cells (Fig. 8). The lipid droplet radioactivity is associated with unmetabolized tetrachlorobenzene. Radioactivity found in the cytosol of F-cells is associated with sulfate or glucose conjugates.

Organometallics

The most common organometallic compounds in the blue crab's environment are probably methylmercury and tributyltin. Marine animals accumulate and only slowly eliminate methylmercury which is taken up primarily from the food (Riisgard and Famme 1986; Schultz and Newman 1997). The high toxicity of methylmercury may be due to its affinity for sulhydryl groups that can affect a variety of cellular functions including DNA repair (Massaro 1992, 1997). The formation of conjugates of methylmercury with glutathione and cysteinylglycine has been reported in mammals (Urano et al. 1988). Inorganic mercury and methylmercury reduce the rate of limb regeneration in the fiddler crab (Weis et al. 1992). However, the concentrations used in these studies are much higher than exist in even contaminated environments. Other than these data, little information is available on the effects of inorganic mercury and methylmercury on crustaceans.

Tributyltin (TBT), which has been used as an anti-foulant in marine paints, is toxic to most marine

invertebrates. It is an ionic, polar compound that enters organisms through the lipid membranes and is metabolized by phase 1 and 2 reactions. Blue crabs exposed to TBT upregulate P-450 isozymes (Oberdörster et al. 1998). The cytochrome P-450 systems in the hepatopancreas oxidize TBT to a series of hydroxylated derivatives followed by conjugation of the hydroxybutyldibutyltin to glucose or sulfate

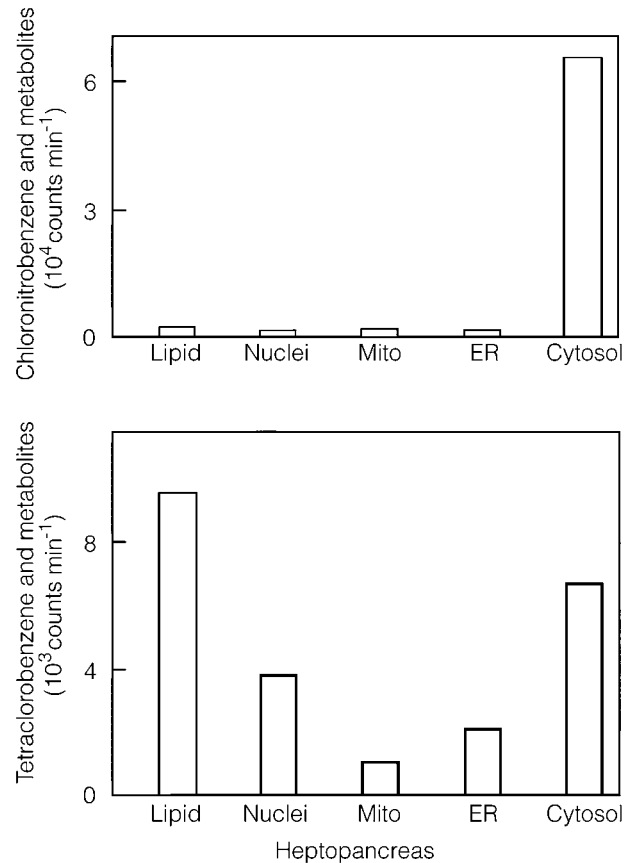


Figure 8. Upper. Distribution of radioactivity in different subcellular fractions 3 d after feeding a male blue crab a diet containing ^{14}C 1-chloro-2,4-dinitrobenzene (6×10^5 counts per minute). Cell fractions were prepared from tissue homogenates by differential centrifugation. Radioactivity determined with scintillation counter. ER=endoplasmic reticulum, mito=mitochondria, lipid=free lipids that float to the surface after cell homogenization. Lower: Distribution of radioactivity in different cell fractions 3 d after feeding a male blue crab a diet containing ^{14}C -tetrachlorobenzene (2×10^5 counts per minute).

(Rice et al. 1989; Lee 1991, 1996). Distribution of TBT and its metabolites in tissues of blue crabs exposed to ^{14}C -TBT in the food is shown in Fig. 9. The data in this figure demonstrate the rapid metabolism and elimination of TBT, with the hepatopancreas being the primary tissue responsible for TBT metabolism. Tributyltin inhibits growth, as measured by protein and lipovitellin accumulation, of blue crab oocytes at concentrations of $2 \mu\text{g TBT L}^{-1}$, whereas hatching success of embryos is reduced by 50% at $0.047 \mu\text{g TBT L}^{-1}$ (Lee et al. 1996). Exposure of regenerating fiddler crabs to TBT ($0.5 \mu\text{g L}^{-1}$) results in retardation of regeneration, delayed ecdysis, and deformities of limbs (Weis et al. 1987, 1992).

Conclusions

There is much information available on the fate and metabolism of xenobiotics in blue crabs. The biochemical mechanisms involved in the toxicity and sublethal effects of some classes of xenobiotics are known, such as the action of organophosphorus pesticides to inhibit acetylcholinesterase and thus interfere with the nervous system (Fukuto 1990). Little, if anything, is known about the effects of xenobiotics on blue crabs at the population level. The increases in the use of juvenile hormone mimics such as methoprene, designed to disrupt chitin synthesis in insect larvae, mosquitoes, and gypsy moths, is a threat to juvenile blue crabs and other crustaceans, which inhabit the same habitat as the target insects. The increased development of regions adjacent to estuaries in the southeastern United States appears to have affected grass shrimp populations in affected estuarine creeks (Finely et al. 1999). Population decreases are correlated with increases in concentrations of polycyclic aromatic hydrocarbons, and in some areas where farms are adjacent to estuaries, population decreases are correlated with various pesticides. It would be predicted that blue crabs, which are important members of the food web in these creeks, would be similarly affected. In view of the complex life cycle of blue crabs, it is important to examine the effects of xenobiotics on growth, development, molting, and reproduction as they relate to population effects. Eventually such studies

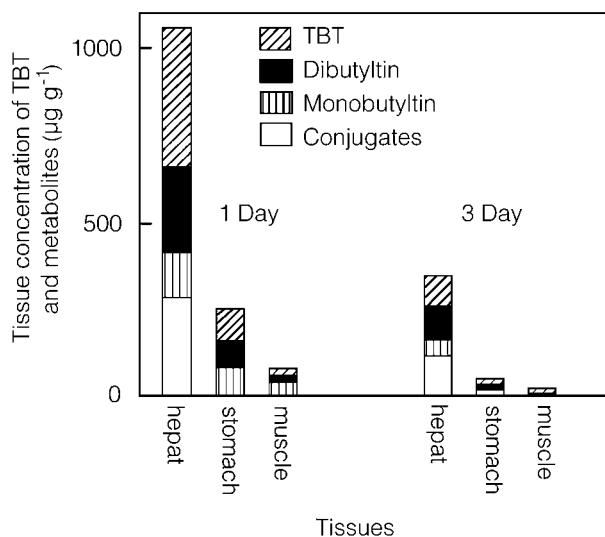


Figure 9. ^{14}C -Tributyltin (TBT) in the food ($12 \mu\text{g g}^{-1}$) of male blue crab. Tissues removed at the times indicated followed by tissue extraction and thin-layer chromatography of lipid extracts to separate metabolites. Separated metabolites were quantified with a scintillation counter. Conjugates in the cytosol were hydrolyzed by hydrolysis enzymes and quantified. (hepat= hepatopancreas.)

could be used to predict if exposure to contaminants can cause reproductive output to fall below critical levels required to maintain the population.

METALS

Some trace metals such as cobalt, copper, iron, manganese, molybdenum, nickel, selenium, and zinc are essential for the function of a wide variety of proteins, including enzymes, oxygen-binding proteins, proteins involved in electron transport, and DNA-binding proteins. When present in excess, trace metals can become toxic to the organisms that depend on them. In that case, an essential element has become an environmental pollutant. Other metals such as cadmium, mercury, lead, and silver do not have any known biological function and are toxic to organisms above certain threshold levels.

Marine pollution constitutes an ever-growing problem, especially in estuarine and coastal areas adjacent to densely-populated and industrialized

regions. Adverse environmental effects associated with redistribution of trace metals due to mining and fossil fuel combustion have long been recognized (Table 2, compiled from Leland and Kuwabara 1985 and Wren et al. 1995). The fact that metal pollutants are persistent in the environment makes these contaminants especially hazardous (Viarengo 1989). In this section we first examine the present knowledge about metal (essential and toxic) uptake and inter-organ transport in the blue crab. The emphasis is on copper and cadmium because these trace metals have been most extensively studied. We then discuss the molecular defense mechanisms used by the crab for protection against metal toxicity. Next we consider how metals, when the defense mechanisms are overwhelmed, exert their toxic effects at the cellular/molecular and organismal (growth/molting, development, and reproduction) levels. Finally, we relate the extensive studies carried out under controlled laboratory conditions to the limited number of studies carried out in the field.

Tissue Responses, Metal Accumulation and Transport

There are two possible routes of exposure to trace metals in the environment: food and water. Blue crabs accumulate cadmium from food in the hepatopancreas and cadmium from water in the gills. Only small amounts of the metal accumulate in muscle tissue (Engel 1983; Brouwer et al. 1984,

1992a). In contrast, copper from food and water appears to accumulate primarily in the hepatopancreas irrespective of the source (See Detoxification Mechanisms below for details). Very little, if anything, is known about the processes involved in uptake and export of trace metals from tissues in the blue crab or any other decapod crustacean. However, in view of the very conserved nature of the mechanisms and proteins involved in the uptake, intracellular distribution, and export of copper in the cells of organisms as diverse as yeast and humans (Valentine and Gralla 1997), it seems likely that similar mechanisms occur in crustacea as well, to help maintain copper homeostasis. There is indeed increasing evidence that decapod crustaceans are capable of regulating copper body burdens within narrow limits. The shore crab *Carcinus maenas* and the shrimp *Palaemon elegans*, for example, maintain constant body copper concentrations under varying external dissolved copper levels, until a threshold of dissolved metal concentration is reached ($170 \mu\text{g Cu L}^{-1}$ and $100 \mu\text{g Cu L}^{-1}$, respectively), beyond which net accumulation of copper begins (White and Rainbow 1982, 1985). The same appears to apply to copper accumulation by the crayfish *Procambarus clarkii* and by larvae of the mud crab *Rhithropanopeus harrisi* (Sanders et al. 1983; Anderson et al. 1997).

The oxygen-binding protein, hemocyanin, of the blue crab has a large number of high-affinity copper-binding sites, in addition to the two coppers that make up the active site (Brouwer et al. 1982,

Table 2. Concentrations of selected trace metals in the earth's crust and their ranges, reflecting localized anthropogenic inputs, in seawater and marine sediments.

Element	Earth's crust (mg kg ⁻¹)	Seawater ($\mu\text{g L}^{-1}$)	Sediments (mg kg ⁻¹)	Sources
Cadmium	0.16	0.03 - 0.3	1 - 225,000	Copper-nickel production; Electroplating; Ni-Cd battery plants
Copper	68	1 - 500	2 - 700	Copper mining; Steel production; Coal combustion
Lead	13	0.005 - 0.4	10 - 200	Lead smelting; Lead alkyl production; Battery production
Mercury	0.08	0.0001 - 0.7	0.01 - 800	Fossil fuel combustion; Chlor-alkali plants; Fungicides
Zinc	76	0.01 - 20	5 - 100,000	Mining; Municipal sewage discharge

1983). This *in vitro* observation appears to have biological implications. When *Carcinus maenas* is exposed to elevated levels of water-borne copper, the metal is taken up by the gills. Subsequent binding to circulating hemolymph proteins, mainly hemocyanin, ensures the transport of copper in a less toxic form to the tissues (Rtal et al. 1996; Truchot and Rtal 1998). When copper is injected into the hemocoel of the crab *Scylla serrata*, 63% of the metal becomes bound to hemocyanin and 20% to hemocyte proteins. The copper levels return to pre-injection levels within 4 h post injection with concomitant decrease of hemocyanin, suggesting a role of hemocyanin in copper transport (Balaji et al. 1989).

In addition to copper, blue crab hemocyanin can bind a large number of cadmium ions (Brouwer et al. 1983). Cadmium binds to sites that are normally occupied by calcium, which acts *in vivo* as a modulator of the oxygen-binding characteristics of hemocyanin. The *in vitro* substitution of cadmium for calcium increases the oxygen affinity of hemocyanin (Brouwer et al. 1982). When blue crabs are exposed to water-borne cadmium, the metal rapidly appears in the gill and hemolymph, where it is bound to hemocyanin. However the cadmium concentrations attained in the hemolymph are too low to affect oxygen transport by hemocyanin. During depuration, cadmium is transferred from the gill, via the hemolymph, to the hepatopancreas (Brouwer et al. 1984).

Cadmium in the gill and hepatopancreas is bound to the low-molecular weight metal-binding protein, metallothionein (MT). Two CdMT isoforms are present in the hepatopancreas, but only one in the gills (Brouwer et al. 1984; see Detoxification Mechanisms below for more details). Uptake of cadmium and zinc across the gill epithelia into the hemolymph where it is bound to hemocyanin and passed on to the tissues (hepatopancreas) is a common pathway in decapod crustacea (Wright 1977b; Jennings and Rainbow 1979; Wright and Brewer 1979; Bjerregaard 1990; Martin and Rainbow 1998). The mechanism by which cadmium is taken up from the hemolymph into the hepatopancreas is unknown. Studies with the blue crab suggest that the amino acid histidine chelates the cadmium and

that the histidine-cadmium complex competes with unchelated histidine for absorption by the mucosal epithelium (Pecon and Powell 1981).

The rate of cadmium uptake by the gills of the blue crab increases with decreasing salinity (Hutchinson 1974), similar to findings for cadmium uptake by *C. maenas* and *P. pugio* (Wright 1977a; Engel and Fowler 1979). These observations indicate that cadmium is taken up as Cd^{2+} and not as CdCl_2 . Indirect evidence suggests that Cd^{2+} is taken up through Ca^{2+} ion channels in the plasma membrane. The accumulation of cadmium by the shore crab *C. maenas* is inversely proportional to the calcium concentration in the external medium (Wright 1977c), whereas transport of cadmium across the apical surface of *C. maenas* gills is inhibited by calcium and by the non-specific Ca channel blocker, lanthanum (Lucu and Obersnel 1996). Similarly, cadmium uptake by the mollusc *Donax rugosus* is inhibited by the calcium channel blocker verapamil. The inhibition is not complete, indicating that other transport mechanisms may exist as well (Sidoumou et al. 1997). The toxicity of water-borne cadmium may therefore lie mainly in perturbation of calcium metabolism (Hogstrand et al. 1996).

Molecular and Cellular Responses

Detoxification Mechanisms

First Line of Defense: Metallothionein and Glutathione. Metal ions are both essential and toxic elements. To cope with potentially hazardous levels of heavy metal ions, organisms have developed an integrated metal-regulatory network that controls the concentration and availability of these elements. A major component of this network is a family of low-molecular weight (6500 Da; 57-62 amino acids), cysteine-rich (18-21 cysteines mole^{-1}), metal-binding proteins called metallothioneins (Kagi and Kojima 1987). These proteins are expressed in many different cell lines and tissues after exposure to heavy metals such as cadmium, copper, mercury, silver, and zinc; glucocorticoid hormones; interferon; interleukin-I; bacterial endotoxin; UV radiation; and oxidative stress (Cousins 1985; Hamer 1986; Engel and Brouwer 1989; Bauman et al. 1991; Roesijadi 1992;

Tamai et al. 1993). In mammals there are at least two major MT isoforms, designated as MT-I and MT-II, whose induction can be metal and hormone specific (Richards et al. 1984). Nuclear magnetic resonance (NMR) spectroscopy (Otvos and Armitage 1980; Messerle et al. 1992) and X-ray diffraction studies (Robbins et al. 1991) have shown that the cadmium/zinc form of mammalian MT is a dumbbell-shaped molecule, composed of an N-terminal, 9-cysteine/3-metal cluster and a C-terminal, 11 cysteine/4-metal cluster.

The presence of MT has been demonstrated in several decapod crustacea, and the proteins have been characterized with different degrees of detail (Overnell 1986; Engel and Brouwer 1989; Roesijadi 1992). The first crustacean MT amino acid sequences published were those of the two MT isoforms of *Scylla serrata* (Lerch et al. 1982). Whereas mammalian MTs have 20 cysteine residues and bind 7 g atoms of Cd mole⁻¹ of MT, the crab MTs have 18 cysteine residues and bind 6 g atoms Cd mole⁻¹ of MT. The amino acid sequences of MTs from *Homarus americanus* (Brouwer et al. 1989), *Carcinus maenas* (Pedersen et al. 1994), *Callinectes sapidus* (Brouwer et al. 1995), and the freshwater crayfish *Astacus astacus* (Pedersen et al. 1996) show a high degree of structural similarity to the MTs from *S. serrata*.

The structure and function of blue crab MTs have been studied in great detail. Anion-exchange chromatography shows one major constitutive ZnCuMT (ZnMT-I) in control crabs (Brouwer et al. 1992a), whereas five apparent CdMT isoforms are present in the hepatopancreas of crabs fed cadmium-enriched diets (CdMT-Ia-Ib-Ic and CdMT-IIa-IIb) (Brouwer et al. 1995). The amino acid sequences of CdMT-Ia and Ic are identical. CdMT-Ib differs from MT-Ia only in having an extra N-terminal methionine. Similarly, CdMT-IIb is identical with IIa

except for an extra Met at its N-terminal position. The same N-terminal heterogeneity has been observed for CdMTs from *C. maenas* and *A. astacus* (Pedersen et al. 1994, 1996). Thus, of the five observed CdMTs in the blue crab, only two are the products of distinct genes. The 3D structure of blue crab Cd6MT-I has been determined by 2D NMR spectroscopy (Narula et al. 1993, 1995). The protein is folded into two separate domains. Each domain contains a cluster of three metal ions, tetrahedrally co-ordinated to the sulfur atoms of the nine cysteine residues present in each domain.

When blue crabs are fed copper-enriched diets, two CuMT isoforms are found in the hepatopancreas: CuMT-Ia/Ib and CuMT-III (Schlenk and Brouwer 1991; Brouwer et al. 1992a; Schlenk et al. 1993; Brouwer 1996). Each MT isoform binds approximately 10 copper atoms per mole of protein (Brouwer and Brouwer-Hoexum 1991; Brouwer 1996). CuMT-III is induced to a much greater extent than CuMT-I. CuMT-I and CdMT-I are the same protein, with different metals bound to it. CuMT-III is a unique MT isoform that can be induced by copper but not by cadmium. The amino acid compositions of CdMT-II and CuMT-III are different (Brouwer et al. 1992a). The partial amino acid sequence of CuMT-III has been determined as described before (Brouwer et al. 1995), in collaboration with Dr. Jan Enghild of Duke University (Table 3).

We have cloned and sequenced the cDNA that encodes CuMT-III. The protein consists of 63 amino acids, 21 of which are cysteines (Syring et al. 1999). The blue crab CuMT-III amino acid sequence is very different from the CdMT-I and CdMT-II sequences. CuMT-III shows greatest homology with a Cu-specific MT from the snail *Helix pomatia* (Dallinger et al. 1997). The presence of

Table 3. Amino acid sequence alignment of *Callinectes sapidus* CdMT and CuMT isoforms^a.

CdMT - Ia	PGPC-CNDKVCQEGGCKAG---CQCTSCRCPCSQKCTSGCKCATKEECSKTCTKPCSCCPK
CdMT - IIa	PDPC-CNDKCECKEGETGTG---CKCKSCRCPPCDKCSSECKCTSKEECSKTCSKPCSCCP-
CuMT - III	---PCGCGTSCCKGSGKCCCGGSTCNCCTTPCKQ-----
	* * * * * * * * * * * * *

^aAlignment was done using CLUSTAL W (1.74). * Indicate identical amino acids.

closely-related copper-specific MTs in molluscs and crustaceans, both of which are dependent on hemocyanin for oxygen transport, suggests that CuMT is involved in regulation of copper associated with hemocyanin metabolism (Brouwer et al. 2002).

The biological function of the CuMT isoforms is dictated, in part, by their metal-binding properties. Examination of the CN-induced dissociation of the CuMT-I and CuMT-III complexes [$\text{Cu(I)-MT} + 3 \text{CN} \leftrightarrow \text{Cu(I)(CN)}_3 + \text{MT}$] by UV spectroscopy shows that CuMT-III has a higher affinity for copper ($K \sim 1.96 \times 10^{21} \text{ M}^{-1}$) than CuMT-I ($K \sim 5.35 \times 10^{19} \text{ M}^{-1}$) (Brouwer 1996). Studies of the binding of Cu(I) to apoMT-I and apoMT-III using fluorescence spectroscopy show that Cu(I) binding to apoMT-I is a strictly cooperative process, excluding intermediates in which metal-binding clusters are partially filled with copper. In contrast, the Cu(I)-thiolate cluster structure in CuMT-III is formed in a highly cooperative fashion only after more than four Cu(I) ions are bound (Brouwer and Hoexum Brouwer 1998). Analysis of the kinetics of cyanide-induced release of Cu(I) from blue crab CuMT isoforms by stopped-flow spectrophotometry shows that CuMT-I releases its copper at a faster rate than does CuMT-III. The greater thermodynamic stability of CuMT-III appears to find its origin, at least in part, in a slower rate of $\text{Cu(I)MT} \leftrightarrow \text{Cu(I)} + \text{MT}$ dissociation (see Brouwer 1996 for detailed analysis). The biological significance of the presence of two structurally and functionally distinct CuMT isoforms in *C. sapidus* is unknown.

As discussed above, long-term exposure of blue crabs to copper in food results in induction of the synthesis of two CuMT isoforms. What happens to copper before the synthesis of MT is turned on? Short-term copper exposure of hepatopancreas tissue explants shows that Cu taken up by the cells during the first 60 min combines with glutathione (GSH). Thereafter, Cu binds to newly-synthesized MT, with concomitant decrease in Cu(I)-GSH (Brouwer and Hoexum Brouwer 1998). Copper, when bound to GSH, does not participate in free radical chemistry (Hanna and Mason 1992), and formation of Cu-GSH complexes in the cell is a common mechanism for rapid detoxification of excess copper (Freedman

et al. 1989; Lin et al. 1993). The Cu(I)-GSH complex can transfer its metal to blue crab apoMT (Brouwer and Hoexum Brouwer 1998), and GSH-CuMT complexes appear to exist in the hepatopancreas of lobsters and blue crabs (Brouwer and Brouwer Hoexum 1991, 1992; Brouwer et al. 1993a; Brouwer 1996). A putative GSH-binding site in CdZn-MT has been identified by equilibrium binding and molecular-modeling studies (Brouwer et al. 1993b). Glutathione is able to "repair" oxidized MT and restore its metal-binding capacity (Brouwer et al. 1993a). Recent studies indicate that reduced glutathione (GSH) and glutathione disulfide (GSSG) are critical modulators of zinc transfer from ZnMT to zinc-dependent enzymes (Jacob et al. 1998; Jiang et al. 1998). It appears therefore that MT and GSH/GSSG play a critical role in detoxification and intracellular distribution of copper and zinc.

As reviewed above, copper and cadmium from food accumulate in the hepatopancreas. When blue crabs are exposed to cadmium in the water, most of the metal accumulates in the gill (Fig. 10). Most of the cadmium is bound to CdMT-II, while CdMT-I is virtually absent, indicating that the expression of the two cadmium-inducible MT genes is tissue specific (Brouwer et al. 1984, 1995). Only a small fraction is found in the hepatopancreas (Fig. 10). Surprisingly, when crabs are exposed to copper in the water, most of the copper accumulates in the hepatopancreas and only a small portion is found in the gills (Fig. 11). This indicates that copper is efficiently exported from the gill epithelial cells into the hemolymph with subsequent uptake by the hepatopancreas, a tissue that is heavily dependent on copper for hemocyanin synthesis (Rainer and Brouwer 1993).

In summary, the blue crab has three genes encoding three distinct MT isoforms. CdMT-I and CdMT-II are expressed in the hepatopancreas in response to cadmium in food. Cadmium in water activates the transcription of CdMT-II in gill tissues. The copper-specific CuMT-III is expressed in the hepatopancreas only, in response to copper in food and water.

Copper and MT, or MT degradation products, appear to accumulate in the hepatopancreas of the

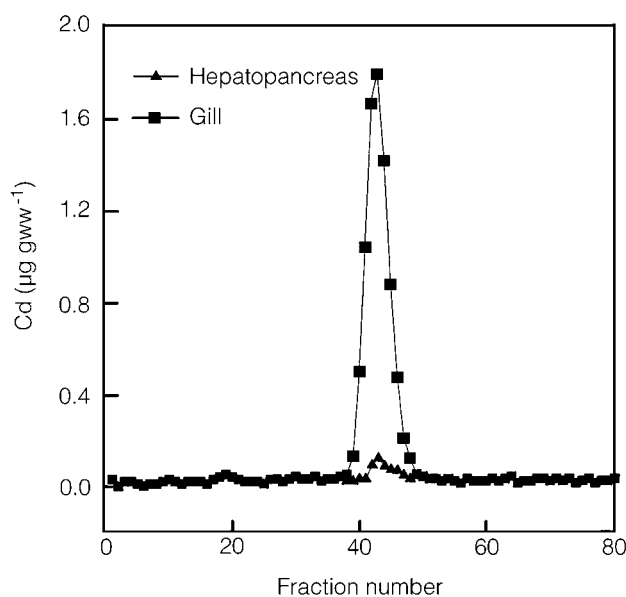


Figure 10. Blue crabs were exposed for 7 d to 1 ppm cadmium ($\sim 9 \mu\text{M CdCl}_2$) dissolved in seawater. After exposure, crabs were anaesthetized on ice and their hepatopancreas and gills were dissected and stored at -90°C . One gram of tissue was homogenized with a tissue tearor in degassed, ice-cold 3 ml 20 mM Tris pH 7.6, containing 0.5 mM β mercaptoethanol. Homogenates were centrifuged for 15 min at $21,000 \times g$. The supernatant was passed through glass wool and then centrifuged at $200,000 \times g$ for 70 min at 4°C . The supernatant was applied to a 1.5×70 cm column of Sephadex G-75. The column was eluted in the cold with nitrogen-saturated homogenization buffer at a flow rate of 12 ml h^{-1} . Fractions of 2 ml were collected and analyzed for cadmium using flame absorption atomic spectroscopy. Metal concentrations were normalized with respect to gram wet weight (gww) of tissue. Most of the cadmium accumulated in the gill ($16 \mu\text{g Cd MT}^{-1} \text{ gww}^{-1}$; determined by addition of cadmium in the fractions that make up the metallothionein [MT] peak). Only a small fraction was found in the hepatopancreas ($0.4 \mu\text{g Cd MT}^{-1} \text{ gww}^{-1}$).

shrimp *Penaeus semisulcatus* during the intermolt feeding stages, as evidenced by the presence of dense, lysosomal-derived, copper- and sulfur-containing granules in the R-cells. Copper is lost from the hepatopancreas during the non-feeding stages. It has been proposed that copper is stored in the hepato-

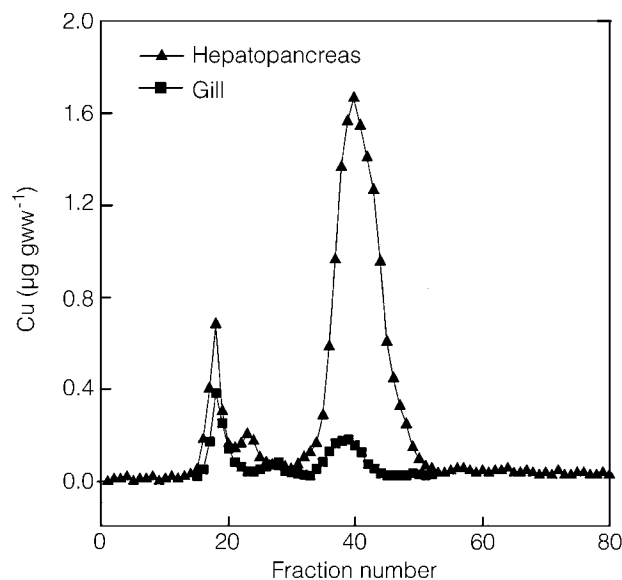


Figure 11. Blue crabs were exposed for 7 d to 1 ppm copper ($\sim 16 \mu\text{M CuCl}_2 \cdot 2\text{H}_2\text{O}$) dissolved in seawater. Preparation of tissue homogenates and conditions of Sephadex chromatography are described in the legend for Figure 10. Most of the copper accumulated in the hepatopancreas ($13 \mu\text{g Cu MT}^{-1} \text{ gww}^{-1}$). Only a small fraction was found in the gills ($1 \mu\text{g Cu MT}^{-1} \text{ gww}^{-1}$).

pancreas to provide copper for hemocyanin synthesis immediately after molting (Al-Mohanna and Nott 1989). Another type of granule appears during the pre-molt stages, in which the major elements are calcium and phosphorus. Such granules are also observed in the hepatopancreas of *C. sapidus* and *C. maenas* (Becker et al. 1974; Hopkin and Nott 1979). These granules have been implicated in the detoxification of lead (Hopkin and Nott 1979). There are no reports on the presence of copper- and sulfur-rich granules in crabs.

Second Line of Defense: Antioxidant Defense Systems. Copper can catalyze the production of highly toxic hydroxyl free radicals from intracellularly generated hydrogen peroxide: $\text{Cu(I)} + \text{H}_2\text{O}_2 \rightarrow \text{Cu(II)} + \text{HO}^- + \text{HO}\cdot$ (Halliwell and Gutteridge 1990). It is therefore essential, as we have seen above, that intracellular levels of free copper be kept at a minimum by sequestering copper in complexes, such as MT and GSH, that render the metal unable

to take part in redox reactions with activated oxygen species (superoxide and hydrogen peroxide). In addition to copper chelators, cells contain a variety of antioxidant defenses that limit oxidative damage that may result from metal-catalyzed formation of activated oxygen species. The enzyme superoxide dismutase (MnSOD in mitochondria and CuZnSOD in cytosol) catalyzes the conversion of superoxide into oxygen and hydrogen peroxide (Fridovich 1997). Catalase and GSH-peroxidase remove hydrogen peroxide and organic peroxides. Glutathione disulfide, formed in the reaction catalyzed by GSH-peroxidase, is recycled by the enzyme GSH-reductase. Cells contain nonenzymic antioxidants such as the lipid soluble α -tocopherol that can limit lipid peroxidation (Buettner 1993), and the water-soluble vitamin ascorbate and the intracellular tripeptide glutathione that remove free radicals from the cytosol (Reed 1990).

Long-term exposure of blue crabs to copper in food leads to increased activity of two antioxidant enzymes: GSH-peroxidase and MnSOD. No CuZnSOD is found. Activities of catalase and GSH-reductase, and the intracellular levels of GSH are unaffected by copper (Brouwer and Hoexum Brouwer 1998). In conclusion, blue crabs use the metal-chelators GSH and MT as a first line of defense against excess Cu. The antioxidant enzymes SOD and GSH-peroxidase constitute a second line of defense to help prevent or repair oxidative damage from Cu not sequestered by the chelators.

Superoxide dismutase is present in the cytosol (CuZnSOD) and mitochondria (MnSOD) of all oxygen-respiring eukaryotes examined until recently. However, we have recently found that blue crabs and all other crustaceans that use hemocyanin for oxygen transport do not have CuZnSOD. Blue crabs have replaced CuZnSOD with a MnSOD precursor that has an abnormal mitochondrial-targeting sequence (Brouwer et al. 1997; Grater and Brouwer 1998). N-terminal amino acid sequences of mitochondrial and cytosolic blue crab MnSOD differ in several positions. The MnSODs are thus encoded by two different genes. The paradigm that all aerobic eukaryotes contain intracellular CuZn-

SOD and that MnSOD occurs exclusively in the mitochondria appears not to apply to a large group of marine arthropods (Brouwer et al. 1997). We hypothesize that the replacement of a copper-dependent SOD by a manganese-dependent SOD may be associated with the high requirement of copper for hemocyanin synthesis. This hypothesis is corroborated by the observation that crustacea dependent on hemoglobin for oxygen transport (such as brine shrimp, [cerio]daphnia, barnacles, and copepods) have normal cytosolic CuZnSOD (Brouwer et al. 2003).

Mechanisms of Metal Toxicity

Metal-catalyzed Oxidative Damage. As outlined in the previous paragraphs, long-term copper exposure results in preferential induction of CuMT-III and leads to increased activity of two antioxidant enzymes: GSH-peroxidase and MnSOD. To examine if the defense mechanisms against copper toxicity are sufficient to protect against copper-mediated oxidative tissue damage, hepatopancreas tissues from control and copper-exposed blue crabs were analyzed for lipid peroxidation and protein oxidation (Brouwer and Hoexum Brouwer 1998). Levels of malondialdehyde, a common product of lipid peroxidation, were significantly greater in the copper-exposed crabs than in control animals, indicating that the defense mechanisms induced by copper exposure were not entirely sufficient to prevent oxidative damage to cellular membranes. However, protein oxidation was kept to a minimum. It seems therefore that copper chelation and increased antioxidant defenses are effective, in view of the large amounts of copper accumulated in the cells, in limiting oxidative tissue damage (Brouwer and Hoexum Brouwer 1998). Similar results have been reported for *Scylla serrata* (Reddy 1997). DNA is an important target of metal-catalyzed, oxygen-derived free radicals in vertebrates (Halliwell and Gutteridge 1990). Oxidative DNA damage associated with metal accumulation has been observed in the Sydney rock oyster *Saccostrea commercialis* (Avery et al. 1996), but no information exists regarding metal-catalyzed oxidative DNA damage in crustaceans.

Perturbation of Protein and Enzyme Function. Group IB and IIB transition metals bind with high affinity ($\text{Ag} \approx \text{Hg} > \text{Cu} > \text{Cd} > \text{Zn}$) to the cysteine sulfhydryl (SH) groups in proteins, and to a lesser extent to the side chains of histidine and aspartic and glutamic acid, which may lead to structural alterations and impairment of the protein's biological function. As discussed above, both copper and cadmium bind to blue crab hemocyanin *in vitro* and *in vivo*, which results in altered oxygen binding properties (Brouwer et al. 1982, 1983). However, the hemolymph concentrations of these metals resulting from uptake from water are too small to affect hemocyanin function to a noticeable extent. Metal-perturbed enzyme functions in blue crabs have not been studied.

Carinus maenas and rock crabs *Cancer irroratus* exposed to water-borne copper or cadmium exhibit loss of osmoregulatory function (Thurberg et al. 1973), and waterborne copper causes extensive cytological damages to the gill epithelium of *C. maenas* (Rtal et al. 1996). Specifically, copper and cadmium inhibit the activity of *C. maenas* gill Na,K-ATPase, an enzyme vital to the osmoregulatory processes in aquatic animals, resulting in reduced hemolymph electrolyte levels (Hansen et al. 1992a; Postel et al. 1998). Similarly, copper, cadmium and zinc in combination inhibit gill Na,K-ATPases in the male Norway lobster *Nephrops norvegicus* but there is no significant effect in treated females (Canli and Stagg 1996). Gill Na,K-ATPase appears to be a major target of the toxic action of waterborne trace metals. In addition, as discussed above, water-borne cadmium appears to exert its toxic action through perturbation of calcium metabolism in the gills.

Copper exposure from water results in reduction of the activities of glycolytic enzymes (hexokinase, phosphofructokinase, and pyruvate kinase) in gills and hepatopancreas (but not in chela muscle) of *C. maenas*, resulting in increased levels of lactate and glucose in hemolymph. However, the energy charge potential ($\text{ATP} + \text{ADP} + \text{AMP}$) is unaffected by copper exposure, indicating that the toxicity mechanism does not involve energy deprivation (Hansen et al. 1992b). Chronic exposure of the American lobster to cadmium causes an increase of the activity

of the glycolytic enzyme glucosephosphate isomerase in the heart of the animals. The metal also stimulates the activities of lactate dehydrogenase and malate dehydrogenase, two enzymes related to the cellular redox balance (Gould 1980). The data on the effects of heavy metals on intermediary metabolism in marine decapod crustacea are fragmentary (Viarango 1989), and care must be taken in the generalization of the available data. In fact, the metal effect may vary greatly in the different tissues of the same animal (Gould 1980).

At the physiological level, short-term (24 h) and chronic (4 weeks) copper exposure of *C. maenas* causes an increase in the heart rate of both resting crabs and crabs subjected to physical stress (Bamber and Depledge 1997a). Normal cardiac activity is restored after a 6-week recovery period in clean water (Lundebye and Depledge 1998). Changes in osmoregulatory ability of *C. maenas* collected from a range of sites are sufficiently sensitive to detect differences in environmental contamination (Bamber and Depledge 1997b). Physiological color changes of *Uca pugilator* are affected by cadmium. Cadmium appears to inhibit the synthesis of the black pigment-dispersing hormone in neuroendocrine cells, resulting in decreased ability of exposed crabs to disperse their black pigment (Reddy and Fingerma 1996). In contrast to molecular biomarkers, physiological biomarkers signal integrated responses to pollutant exposure. Furthermore, impairment of physiological processes reduces the survival potential of an organism. Physiological biomarkers may therefore have greater ecological relevance than their molecular counterparts (Depledge et al. 1995; Lundebye and Depledge 1998).

Organismal Responses: Embryo/Larval Development, Growth, and Molting

Growth of blue crab oocytes and hatching of embryos are inhibited by copper and cadmium (Lee et al. 1996). In contrast, cadmium causes a significant increase in hatching rate of king crab *Lithodes santolla* embryos, with concomitant increase in number of abnormal larvae (Amin et al. 1998). Under optimum

conditions of salinity and temperature, 95% of blue crab megalopae survive to third crab instar in the presence of 50 ppb cadmium. In 150 ppb of cadmium the survival is reduced to 20%. The effects of cadmium and mercury on survival of megalopae and juvenile crabs are much more pronounced at lower salinities (Rosenberg and Costlow 1976; Frank and Robertson 1979; McKenney and Costlow 1981). Similar observations were made for *Rhithropanopeus harrisi*, with zoeal larvae being more susceptible to cadmium than the megalopae (Rosenberg and Costlow 1976). However, survival and larval duration are not affected when newly hatched larvae of *R. harrisi* are exposed to copper using free cupric ion activities corresponding to those found in seawater. Under those conditions, most cytosolic copper is associated with MT, which is related to free cupric ion activity. At higher copper activities the metal is also associated with very low molecular weight (VLMW) ligands. Inhibition of larval growth is correlated with copper accumulation in the MT and VLMW pools (Sanders et al. 1983; Sanders and Jenkins 1984).

Most studies on MTs have been directed toward the examination of induced MTs in organisms exposed to elevated concentrations of metals. Few have studied MTs in order to understand their biological function in the absence of such exposure. Investigations on the involvement of MT in copper and zinc partitioning during the molt cycle of the blue crab have shown that three significant changes occur in the metals bound to MT. The first is at the beginning of premolt when the metals bound to MT change from predominantly copper to zinc. These changes are thought to be correlated with reduced hemocyanin biosynthesis and an increased rate of Zn-carbonic anhydrase synthesis in preparation for molting. The second change occurs within 90 min after ecdysis, when there is a transient pulse of CuMT, probably correlated with the catabolism of hemocyanin. The CuMT complex may then be sequestered in lysosomes and eliminated into the gut and out in the feces. The third change occurs during the papershell stages, when MT once again becomes primarily a copper protein, preceding the biosynthesis of hemocyanin.

These investigations give further support to the hypothesis that the function of MT in organisms that are not metal-stressed is in the regulation of nutritional metals, associated with the degradation and synthesis of metalloproteins (Engel 1987; Engel and Brouwer 1987, 1989, 1991, 1993). They also show that the use of MT as a biomarker of metal exposure is fraught with danger (Engel and Roesjaji 1987). Because molting is critical to survival of crustaceans and because trace metals undergo dramatic changes during the molt cycle, it seems likely that perturbation of metal metabolism through metal exposure may affect the molting process and survival. Few studies have addressed this question. Copper exposure of *C. maenas* during ecdysis and postmolt may have an effect on postmolt calcium content of papershell crabs, possibly through a reduced uptake of calcium from the environment, but no apparent effect on molting success or survival is observed (Scott-Fordsman and Depledge 1997).

The processes of limb regeneration and molting in decapod crustaceans are generally coupled with one another under the control of the neuroendocrine system (Weis et al. 1992). Inorganic mercury, methylmercury, and cadmium reduce the rate of limb regeneration in the fiddler crab *Uca pugilator*. Retardation of regeneration is generally accompanied by a delay in ecdysis, so that limbs are usually fully formed by the time of molting (Weis 1976; Weis et al. 1991, 1992). The effects of cadmium are greatly enhanced at reduced salinities (Weis 1978, 1985). The potential effects of trace metals on reproduction by blue crabs or decapod crustaceans have, to our knowledge, not been studied.

Field Data

Blue crabs are important members of the estuarine food web. Because of their omnivorous feeding characteristics and association with sediments, blue crabs may potentially accumulate significant amounts of metals. Foundry Cove, located on the Hudson River, was heavily contaminated with cadmium by effluent discharged from a Ni-Cd battery plant between 1953 and 1979 (Kneip and Hazen 1979). The area has now been remediated in an effort to

remove the metal-contaminated sediments. High levels of cadmium ($7\text{--}11\mu\text{g g}^{-1}$ tissue) are found in the hepatopancreas of blue crabs collected in the cove, and the metal appears to be bound to MT (Wiedow et al. 1982). The hepatopancreas and gills of the field-exposed crabs also have elevated concentrations of copper. The gill has an MT that contains mostly cadmium and no copper, whereas the MTs in the hepatopancreas contain cadmium, copper, and zinc (Engel and Brouwer 1984a), identical to what is observed when crabs are exposed to waterborne cadmium and copper under controlled laboratory conditions. Blue crabs collected from Baltimore Harbor have high copper concentrations in the digestive gland. Most of the copper is bound to two MT isoforms (Engel and Brouwer 1984b; Engel et al. 1985) similar to results obtained after dietary copper exposure in the laboratory. Interestingly, CdMT also has been found in crabs *Cancer pagurus* collected from Orkney and Shetland, north of Scotland, far removed from known sources of industrial pollution (Overnell and Trehwella 1979). Underwater outcrops of cadmium-rich minerals are the likely source of the metal.

Hepatopancreas, gill, and muscle tissues of blue crabs with shell disease from the Pamlico River, North Carolina, and of non-diseased crabs from the Albemarle-Pamlico estuarine system in North Carolina have been analyzed for 13 different trace metals. Sediments from the Pamlico River show enrichment of arsenic, cadmium, manganese, titanium, and vanadium which is reflected in higher concentrations of these metals in the crabs. However, no trend is evident with regard to diseased versus non-diseased crabs. The higher metal concentrations in the edible portions of the crabs do not pose a significant health risk to humans (Gemperline et al. 1992; Weinstein et al. 1992). Similarly, analysis of metals (Al, As, Cd, Cu, Pb, Hg, Ni, Se, Ag, Ti, Zn) in tissues from crabs collected from two estuaries in Connecticut indicated no risk to human health (Jop 1997). At present there are two fish consumption advisories for mercury in blue crabs: Point Comfort, Lavaca Bay, Texas, and Brunswick, Georgia. Both locations are Superfund sites where the sources of the mer-

cury were chlor-alkali plants. The concern is for human health and not for populations of blue crabs. From the available data, there does not seem to be any measurable effect of mercury on blue crab recruitment, survival, and reproduction within the affected area of Lavaca Bay (Engel and Thayer 1998).

Concluding Remarks

A variety of organic and inorganic contaminants, including polycyclic aromatic hydrocarbons, organohalogens, pesticides, organometallics, and heavy metals can be present in the blue crab's estuarine environment. Our knowledge of the effects of these contaminants on blue crabs decreases from the molecular, cellular, and organismal levels to the population level. For example, much has been learned about the molecular and cellular mechanisms used by the blue crab to detoxify organic contaminants and heavy metals. In addition, the mechanisms underlying the toxicity and sublethal effects of some classes of contaminants are known. Recent studies have shown that polycyclic aromatic hydrocarbons (PAHs) enhance ecdysone (molt hormone)-dependent gene transcription and cell proliferation. At the organismal level PAHs inhibit growth and molting of juvenile blue crabs, suggesting that PAHs act as endocrine disrupters in the crab. Tributyltin inhibits growth of blue crab oocytes and reduces hatching success of embryos. Heavy metals such as mercury, copper, and cadmium inhibit hatching of blue crab embryos and reduce survival of megalopae and juvenile crabs. However, most studies on the effects of contaminants on crabs have been carried out in the laboratory under conditions that may not be environmentally relevant. To date there is little compelling evidence that contaminants have had direct effects on blue crab populations. A greater threat to blue crab populations may be posed by increased nutrient loading, alterations of freshwater inflow, and physical destruction of estuarine and coastal habitats that accompany increasing human population densities and development near the coast (Engel and Thayer 1998).

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