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Why are Early Life Stages of Aquatic Organisms more Sensitive to Toxicants than Adults?

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1. Introduction

1.1. The selection of a suitable test species

Toxicity tests are designed to determine the specific concentrations of chemicals that induce a measured effect on a target organism. However, the potency of any toxicant can be influenced by the characteristics of the chemical, as well as environmental (temperature, pH, water chemistry, salinity) and species (life stage, sensitivity, pre-exposure) specific factors. Environmental factors can either modify the toxicant itself or the immediate environment of an organism, increasing or decreasing the effects of the toxicants. Species specific factors can alter the organism/toxicant interaction by modifying the rate of uptake, distribution, elimination and detoxification pathways. Therefore when conducting toxicity tests, it is important to have controlled environmental conditions and select suitable test species to ensure reliable, relevant, reproducible, defensible and ecologically significant results. The selection of a suitable test species can be based on several criteria:

- the species should be widely available
- they should be easily maintained under laboratory conditions and provide sufficient numbers of an appropriate size and age
- the genetics, genetic composition and history of the organisms should be known
- they should be the most sensitive species in the environment.
- should be recreationally, ecologically and commercially important
- organisms should be in good physiological condition
- it should be indigenous or representative of the eco-region being studied

Since the first toxicity tests were performed over 60 years ago, fish and invertebrates continue to be the most popular test species because there exist a significant knowledge base on their physiology, biochemistry, behavior, reproduction, life cycle and ecological importance. However there continues to be an ongoing debate as to whether certain life stages are more sensitive to toxicants than others. The general assumption in toxicology has been to utilize the 'most sensitive' life stage which toxicologists assume to be the earliest life stages for a given species. Selecting the most sensitive life stage provides a quick, relatively easy and sensitive toxicity test with the added advantage of having a low cost and test duration. In fact, many standardized test protocols often specify the preferred life stage to be used for testing. For example, US Environmental Protection Agency (US EPA) [EPA-600/4-80/001, EPA-812-R-02-012], and American Society for Testing and Materials (ASTM) [ASTM E1192-97(2008), ASTM E1267-03(2008) and ASTM E724-98(2004)] test protocols recommend the use of early life stages; first instar of daphnia, juvenile mysids, juvenile fish or embryos of mollusks as the most suitable for toxicity tests. The choice of early life stages has been based on the premise that they are the most susceptible to toxicants and that toxicity data using the most vulnerable life stage would offer protection to all life stages in the natural environment.

2. Early life stages in toxicity tests

Numerous studies have reported that the early life stages of fish and invertebrates were more sensitive to toxicants than the adult organisms (Herkovits et al. 1997, Schmieder et al. 2000, Hutchinson et al. 1998, Mohammed et al. 2009). Schmieder et al. (2000) reported that for medake, embryo-larval stages showed 50% mortality when 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) residues in the eggs were 1396 pg/g, however, for adults 50% mortality occurred when the whole body concentration of 2,3,7,8-TCDD residue was 2400 pg/g. Using data from the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Acute Toxicity (EAT) data base, Hutchinson et al. (1998) analysed the EC50 data from partial and full life cycle studies and reported that the sensitivity of aquatic invertebrate larvae were greater than or equal to juveniles for 66% of the substances tested, while the sensitivity of juveniles was greater than or equal to the adults' for 54% of the substances tested. Hutchinson et al. (1998) also reported that for fish, NOEC data indicated that larvae were more sensitive than embryos for 68% of substances, while the sensitivity of fish larvae was greater than or equal to that of juveniles for 83% of the substances tested. Based on fish EC50 data, juveniles were more sensitive than adults to 92% of the substances tested (Hutchinson et al. 1998). For fishes, it has been reported that the larvae are more sensitivity than embryos, and embryos are more sensitive than adults (larvae > embryos > adults). However, for invertebrates such as molluscs, embryos may sometimes appear to be more sensitive than larvae. Therefore, in scales of relative sensitivity, the position of specific life stages may vary depending on species.

3. Early life stages as the most sensitive stage

Variations in sensitivity between life stages have been reported for organisms such as *Callosobruchus maculatas*, *Metamysidopsis insularis* and *Danio rerio*. Macova et al. (2008) reported that juvenile *D. rerio* exposed to 2-phenoxyethanol had a significantly lower LC50 (338.22 +/- 15.22 mg/L) than embryonic stages (486.35 +/- 25.53 mg/L) indicating a higher larval sensitivity. Fisher et al. (1994) showed that veliger larva had similar sensitivity to Bayer 73 and 3-trifluoromethyl-4-nitrophenol (TFM), whereas plantigrades and adults were less sensitive. Hardersen and Wratten (2000) found that the most susceptible life stage of *Xanthocnemis zealandica* following exposure to azinphos-methyl and carbaryl, was instar 7, while the least susceptible were instars 2 and 13. Lotufo and Fleeger (1997) also reported significant differences in sensitivity of nauplii, copepodites, and adults of *Schizopera knabeni* following exposure to phenanthrene. However, Borlongan et al. (1998) showed that for *Cerithidea cingulata* (a brackish water pond snail) sensitivity typically increased as the snail grew and matured.

Other studies have reported that early life stages such as first instar of daphnia, juvenile mysids, juvenile fish and embryos are more susceptible than adults following exposure to toxicants such as heavy metal (Bodar et al. 1989; Gopalakrishnan 2008; Hoang and Klaine 2007; Green et al. 1986; Verriopoulos and Morai'tou-Apostolopoulou 1982). For example, George et al. (1996) reported that the larva (yolk sac stage) of *Scophthalmus maximus* was more sensitive than other larval stages (larval and posthatch larval stages) following exposure to cadmium. Green et al. (1986) also reported that for the crustacean *Asellus aquaticus* exposed to cadmium, the juveniles (96 hr LC50 = 80 µg Cd/L) were more sensitive than the embryos (96 hr LC50 = >2,000 µg Cd/L). Ringwood (1990) reported that older larval of the bivalve *Isognomon californicum* were approximately 10 times more sensitive than adults while embryos and early larval stages were more than 50 times more sensitive than adults. Kennedy et al. (2006) also reported that adult *Dreissena polymorpha* had a 48 hr LC50 of 1,214 µg Cu/L which was several orders of magnitude higher than the 24hr LC50 (13 µg Cu/L) for earlier life stages (72-h old trochophores). Verriopoulos and Morai'tou-apostolopoulou (1982) also reported that the most sensitive life stage of *Tisbe holothuriae* to both copper and cadmium was the one-day-old nauplius which had a 48h LC50 of 0.3142 mg Cu/L and 0.5384 mg Cd/L, while the five-days-old nauplii had a 48h LC50 of 0.3415 mg Cu/L and 0.645 mg Cd/L, and the ten-days-old nauplii showed a 48h LC50 of 0.5289 mg Cu/L and 0.9061 mg Cd/L. The most resistance stage was the ten-days-old copepodids but generally, the resistance of *Tisbe holothuriae* to copper and cadmium progressively increased with larval age.

4. Probable causes for variations in sensitivity between life stages

The apparent variability in sensitivity between early life stages and adults may be due to several factors; surface area/volume ratio (particularly with young fish); the greater likelihood that juveniles may have accumulated less fat than adults thus having less capacity to store lipophilic substances; greater uptake of toxicant from the environment; under developed

homeostatic mechanism to deal with the toxicants; immature immune systems and under developed organs (liver and kidney) which has an important role in detoxification and elimination of toxicants.

There are various ideas that seek to explain why early life stages are more sensitive than adults. These often take into consideration specific behavioral, morphological, physiological and biochemical characteristics which may be different between life stages. Some of the main ideas include:

1. Organ systems may become sensitive to the effects of toxicant at certain periods during early development but once developed, they may no longer be vulnerable (Ozoh 1979, Bentivena and Piatkowski 1998).
2. The time taken for toxicants to reach target sites may be shorter in early life stages, because of their smaller size when compared to later stages and adults.
3. Most embryo and larval forms may have poorly developed organs such as gills, liver and kidneys. They also have permeable skin which, in early life stages, is the primary means of ionic regulation. The skin presents a larger surface area for the uptake of toxicants, resulting in increased susceptibility of the larva when compared to the adults.
4. In crustaceans research has shown that metals can concentrate in the body covering thus reducing their entry into the body. The completion of body covering diminishes the entry of metal into the body, thereby increasing the resistance of older forms.
5. Some toxicants may be sequestered in fat tissue or specific proteins preventing them from reaching target organs.

The increased susceptibility of early life stages may also be related to other factors such as; differential rates of absorption/uptake distribution or detoxification. Organisms have also evolved intricate regulatory (physiological, immunological and biochemical) mechanisms which allow them to survive, grow and reproduce. Some of these mechanisms are also important in the elimination, detoxification or reduction of the effects of toxicant. For example, kidneys and liver may be involved in the elimination of toxicants, while various proteins and enzymes such as Metallothioneins and Mixed Function Oxygenases are induced following exposure to metals and hydrocarbons. It is often suggested that the difference in the development of these mechanism and immature detoxification pathways in early life stages can also be a basis for the apparent increased sensitivity of juveniles exposed to toxicants. A few specific factors for increased sensitivity will be discussed below.

4.1. Avoidance strategies

The higher sensitivity of early life stages may be explained by behavioral, morphological, physiological or biochemical changes. Free swimming species are able to avoid toxicants, while some sessile species such as bivalves may close their valves to avoid contact with the toxicants. Disruption in behavioral responses may include:

1. impaired feeding ability resulting in poor diet, which can cause reduced growth and longevity;
2. altered predator - avoidance behavior;
3. impaired schooling leading to increased mortality and/or altered reproductive function,
4. movement away from the source of the toxicant, or
5. as in the case of bivalves, closure of the valves for varying periods of time in order to reduce exposure (Weis, 2005).

Behavioral responses can have greater impacts on earlier life stages, which show significantly less physiological and morphological development than adults. Kennedy et al. (2006) showed that adult *Dreissena polymorpha* (unlike the free swimming larva or early life stages) closed their valves when exposed to copper at $777 \pm 40 \mu\text{g Cu/L}$, while exposure to $99 \pm 9 \mu\text{g Cu/L}$ caused partial valve closure and/or retraction of siphons. It has been suggested that they may possess chemoreceptors which can detect elevated levels of Copper which triggers closure of the valves. Scott et al. (2003) also reported that exposure of *Oncorhynchus mykiss* to $2 \mu\text{g Cd/L}$ for 7 days eliminated normal antipredator behaviors whereas exposures of shorter duration or lower concentration had no effect on normal behavior.

4.2. Morphological characteristics

Avoidance strategies undoubtedly result in decreased exposure, but it is unlikely that chemical avoidance alone can account for the difference in sensitivity between adults and early life stages. In some species such as *I. californicum*, *A. aquaticus* and *D. rerio*, embryos have been shown to be less sensitive than other life stages. In this instance, embryonic membranes can act as a physical barrier which reduces exposure and consequently reduces toxicity. Eggs of *I. californicum* contain a single membrane about $2 \mu\text{m}$ thick which is shed about 8hr post fertilization. Similarly in fish the chorionic membranes in embryos may act as an effective barrier to toxicants, lowering the sensitivity of the embryo when compared to larval forms. In some fish and isopods species, the embryos may be encased in several layers of membranes. The membrane not only forms a physical barrier, but may also bind metals, effectively reducing their passage to the embryos, as does the chorion in fish eggs (Beattie and Pascoe 1978). Plhalová et al. (2010) reported that exposure of the embryonic stage of *D. rerio* to terbutryn gave a 144hr LC₅₀ of $8.04 \pm 1.05 \text{ mg/L}$ while for the juvenile stage the 96hr LC₅₀ was $5.71 \pm 0.46 \text{ mg/L}$ which suggested that juvenile stages were more sensitive to terbutryn than the embryonic stages. Green et al (1986) also showed that the embryos of *Asellus aquaticus* (L) were more resistant to the effects of cadmium than the early juveniles. The eggs of *A. aquaticus* possess four membranes which are successively shed as the embryo passes through the various stages of development (Holditch and Tolba 1981). When the last membrane is shed, they reported that the sensitivity may increase by as much as 20 times (Beattie and Pascoe 1978). However, the membrane barrier may only be effective at certain concentrations above which the capacity to bind or adsorb metals is exceeded and reducing its effectiveness to lower toxicity. Green et al. (1986) showed that when embryos of *Asellus aquaticus* were exposed to $1,750 \mu\text{g Cd/L}$, the last embryonic membranes offered little or no protection and embryos responded in a similar manner as the smallest juveniles. At 5 and $17.5 \mu\text{g Cd/L}$, however, the last membrane affords considerable protection to the embryo, significantly prolonging its survival time in comparison early

juveniles. Generally, the extent to which it can modify the sensitivity of the embryo relative to other life stages may be related to the type and permeability of membrane and whether they remain intact during the toxicity test

In many fish species, larval forms are generally more sensitive than juveniles and adult forms. Newly hatched larvae constitute a particularly critical and sensitive life stage, because at hatching the embryos lose their protective membrane and are fully exposed to potential toxicants (Arufe et al. 2004). A significant characteristic of most larval stages is the fast changing morphology giving rise to the adult forms. Organ systems may become sensitive to the effects of toxicant at certain periods during early development but once developed, they may no longer be vulnerable (Ozoh 1979, Bentivena and Piatkowski 1998). Middaugh and Dean (1977) reported that cadmium was more toxic to the 7-day-old larvae ($LC_{50} = 12 \text{ mg Cd/L}$) of *Fundulus heteroclitus* than the adults ($LC_{50} = 43 \text{ mg Cd/L}$). Similarly, Hilmy et al. (1985) reported that for the larval forms of *Mugil cephalus* the LC_{50} was 8 mg Cd/L compared with 34 mg Cd/L for juveniles. George et al. (1996) also reported that newly hatched larvae of *Scophthalmus maximus* (yolk sac stage) had a 48-hr LC_{50} value of $0.18\text{--}0.23 \text{ mg Cd/L}$, however, day 4 posthatch larvae showed a 48-hr LC_{50} of 2 mg Cd/L while the 10-day posthatch larvae showed a 48-hr LC_{50} of 5 mg Cd/L which indicated decreasing sensitivity with increasing age. Kazlauskienė and Stasiūnaitė (1999) also reported that for rainbow trout, partially and fully hatched larvae were the most sensitive life stage, while eggs, early eye stage and those immediately after fertilization were least sensitive. Williams et al. (1986) reported that the larvae of *Chironomus riparius* showed increased tolerance with increasing age when exposed to cadmium. The most resistant stage (fourth instar) had a 24 h LC_{50} of $2,400 \text{ mg Cd/L}$, approximately 950 times greater than the corresponding value of 2.1 mg Cd/L recorded for the most sensitive (first instar) stage. The apparent higher sensitivity of larval forms may be related to various factors such as: higher levels of uptake, poorly or underdeveloped organ systems, incompletely formed liver and kidneys, poorly developed immunological systems, and low levels of detoxification proteins. Fish larvae typically do not possess gills, have a large surface-to-volume ratio and possess permeable skin which enables respiration and ionic transport (Tytler and Bell, 1989) as well as the free uptake of metals such as Cd^{2+} (Carpene and George, 1981; Jenkins and Sanders, 1986). As the skin develops it begins to differentiate, becoming multilayered and its permeability to both gases and solutes decreases. Since differentiation occurs gradual during larval development there would appear to be a decreasing sensitivity with increasing age. The skin also secretes a mucus layer which can sequester toxicants such as divalent metal ions (Coombs et al. 1972) further reducing cutaneous absorption. After hatching, marine fish larvae also drink water to maintain their osmotic balance (Brown and Tytler, 1993; Tytler and Blaxter, 1988; Tytler and Ireland, 1994) and actively feed, further increasing the likelihood of uptake from diet and water. However, in some species such as *Scophthalmus maximus*, organogenesis occurs rapidly, usually within 3 to 4 days after hatching. This results in the formation of vital organ systems such as the circulatory system, liver, gills, kidneys and thickening of the skin. However, gills which is one of the major organ for elimination, do not become fully formed until 12–14 days after hatching when the larvae are about 5 mm long (Al-Maghazachi and Gibson, 1984; Segner et al. 1994). In some species such as the Senegalese sole (*Solea senegalensis*) haemopoietic cells in the kidney and were first observed on 6 days after hatching, whereas the thymus was first observed 9 days after hatching (Cunha et al. 2003, Padro et al. 2011). As organs become functional they begin to eliminate or detoxify toxicants, thereby decreasing the sensitivity of the adult forms relative to the early life stages.

4.3. Detoxification mechanisms

Toxicants may induce synthesis of specific proteins specific proteins such as Mix Function Oxygenases and Metallothioneins which may detoxify or sequester toxicants, thus reducing their toxic effects. However, early life stages may lack fully-expressed enzyme systems for efficient detoxification and elimination of toxicants because of slow organ development. In most adult organism detoxification and elimination processes follow one of two pathways depending on whether the toxicant is a metal or organic compound (Figure 1). As previously stated, difference in the development of these mechanism and immature detoxification pathways in early life stages can also be a basis for the apparent increased sensitivity of juveniles exposed to toxicants.

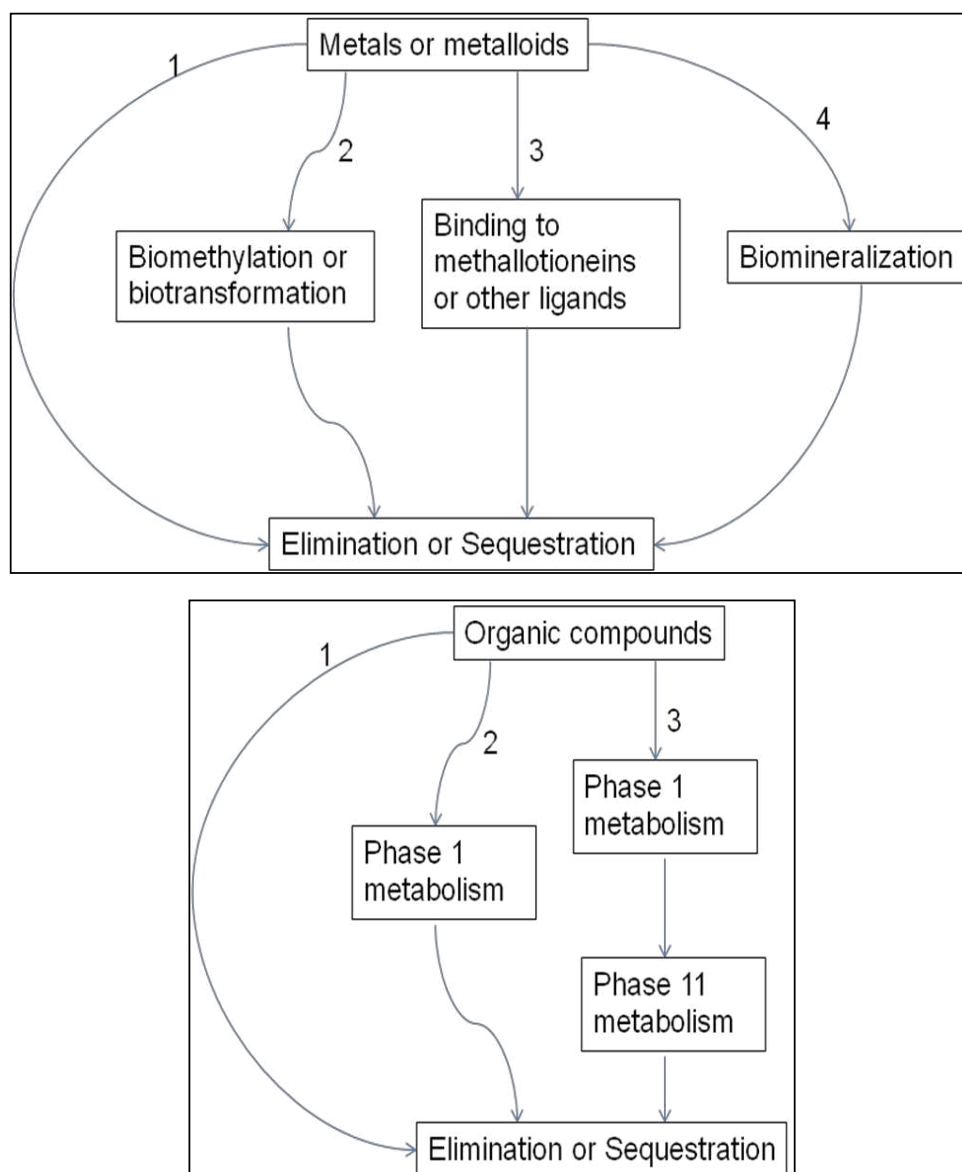


Figure 1. Detoxification pathways for metals and organic compounds

Metallothioneins are non-enzymatic cysteine rich, low molecular weight proteins of about 7 kDa and apparent molecular weight of 13 kDa. The metallothioneins pool is made up of various isoforms each having different physiological roles and different induction pathways and are important in homeostasis of metals such as copper and zinc, and detoxification of heavy metal (Butler and Roesijadi, 2001). Mason and Jenkins (1995) proposed two roles for metallothioneins in the regulation of metals in organisms.

1. They may comprise a non-toxic zinc and copper reservoir available for the synthesis of metalloenzymes, allowing the homeostasis of many cellular processes (Brouwer et al. 1989; Viarengo and Nott 1993; Roesijadi 1996).
2. Metallothioneins can reduce the nonspecific binding of non-essential metals within cells, and so restrict their toxic potential (Roesijadi 1992, 1996; Zaroogian and Jackim 2000).

The induction of metallothioneins confers metal tolerance to organisms (Klaasseen et al. 1999) due to their ability to bind and sequester some heavy metals. However, the ability of metallothioneins to reduce metal toxicity can vary with the age of organism.

The sequestration of metal ions by metallothionein is considered to be one of the most common detoxification pathways for metals in adult organisms. Its presence in organisms can therefore also be used to help explain the variable susceptibility of different life stage to metals. Synthesis of metallothionein is strongly induced by transcriptional activation of metallothionein gene expression following exposure to metals (George et al. 1992, 1996; George and Olsson, 1994; Zafarullah et al. 1989). Laville (1988) showed that in mice, metallothionein mRNA in liver depended on the age at which exposure to cadmium occurred. Exposure to 2mg Cd/kg resulted in a small increase (two- to threefold) in levels of metallothionein mRNA in livers of 7- and 14-day-old mice. However, cadmium treatment of 28- and 56-day-old mice resulted in 12- to 19-fold increases in levels of metallothionein mRNA in liver. George et al. (1996) used metallothionein gene expression (mRNA) to map changes in protein expression during development of *Scophthalmus maximus* in response to cadmium exposure. They reported that metallothionein mRNA expression in newly hatched larvae was lower in the liver at metamorphosis and immediately prior to and during hatching compared to embryos (24 hr postfertilisation). Elevate levels of metallothionein in embryos may be related to its role in the control of homeostasis of essential metals such as zinc and copper. Following hatching (4.5 and 5 days postfertilization) metallothionein mRNA expression dropped to about 50% of the level detected in early-stage embryos (24 and 72 hr after fertilization). Newly hatched larvae feeding on endogenous yolk reserves were reported to be very sensitive to Cd exposure and metallothionein mRNA levels were not induced by exposure to 0.1 ppm Cd for 48 h (George et al. 1996). Following hatching (2 and 4 posthatch) larvae switch from endogenous to exogenous feeding, this resulted in a decreased sensitivity to Cd and 48 h exposure to 0.1 or 0.5 ppm Cd resulted in a threefold - to fivefold increase of metallothionein mRNA levels. These studies therefore indicate a direct relationship between metallothionein induction and decreasing sensitivity. However, Sassi et al. (2012) showed that for gilthead sea bream larvae relative transcript levels of mt were increased at 5 and 10 mg/L of Cd(2+) which they suggested was probably to detoxify excess metals. Zhang et al. 2012 was also able to show that for juvenile

grunt (*Terapon jarbua*) inorganic As(III) and As(V) in the diet and waterborne phases were rapidly biotransformed to the less toxic arsenobetaine (AsB, 89-97%). After exposure to inorganic As, *T. jarbua* developed detoxified strategies, such as the reduction of As(V) to As(III) followed by methylation to less toxic organic forms, as well as the synthesis of metal-binding proteins such as metallothionein-like proteins.

All organism have at least some ability to metabolize organic compounds. These often involve some enzyme mediate detoxification pathway requiring one or more enzymes such as cytochrome P450 monooxygenase, epoxide hydrolase and other conjugating enzymes (Figure 1) associated with the liver or kidney. In most adult organisms, these pathways are well developed (Shailaja and D'Silva 2003; Tuvikene 1995; Eisler, 1987). In juveniles, induction of these may also occur, once organ systems are fully functional. Oikari et al. (2002) was able to show that in juvenile rainbow trout, exposure to contaminated sediments significantly induced trout liver CYP1A activity. However Sassi et al. (2012) reported that for gilthead sea bream larvae were unable to show transcription of Gpx in following exposure to cadmium. Gpx is responsible for the break down hydrogen peroxide as in adult organisms.

5. Conclusion

The greater sensitivity of early life stages when compared to adults can therefore be explained by a number of physiological, morphological, behavioral and biochemical characteristics. It may appear that in early life stages these responses are either underdeveloped or have not yet developed fully thus contributing to the increased sensitivity of these early life stages when compared to the adults.

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