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虱目魚對砷、鋅、銅混合毒物之吸收、排除及生物濃縮研究

Uptake, depuration and bioconcentration of arsenic, zinc and copper mixtures in milkfish (*Chanos chanos*)

Abstract

The aim of this study is to investigate the time-integrated uptake and depuration of individual trace elements by milkfish, *Chanos chanos*, following exposure to a mixture containing arsenic (As), zinc (Zn) and copper (Cu). The trace elements used during the experiments will be chosen to represent the pollutants found in the culture ponds of milkfish in the blackfoot disease (BFD) area, southwest Taiwan. A 14-day exposure experiment under laboratory conditions was conducted to assess the uptake rate constants (k_1) and depuration rate constants (k_2) as well as the bioconcentration factor (BCF) of milkfish, based on a simple 1st-order one-compartmental model, to access the bioaccumulation effects. The interactions among the elements and the subsequent uptake and depuration rates associated with the individual elements were analyzed. The effects of the individual elements in the mixture will also be analyzed. The results demonstrate that Zn can reduce the accumulation of As and Cu because the high Zn accumulation ability of milkfish. The competition of Zn toward As and Cu may cause the reduction of accumulation in milkfish of the later toxins. Since Cu will enhance the metabolism of the fish , it might be the reason that milkfish will accumulate more As and Zn, when Cu was added into the stock.

Introduction

Trace elements, such as arsenic (As), zinc (Zn) and copper (Cu), are introduced into the environment by a wide spectrum of natural and anthropogenic sources (Turner et al., 1986). These elements are non-biodegradable, and once they enter organisms from the environment, bioconcentration may occur in tissue by means of metabolic and biosorption processes (Hodson, 1988; Carpené et al., 1990; Wicklund-Glynn, 1991). Many studies have shown that trace elements can be accumulated in fish (Villegas-Navarro and Villarreal-Treviño, 1989; Mohan and Choudhary, 1991; Peres and Pihan, 1991; Pelgrom et al., 1995). Uptake of sublethal concentrations of these elements may lead to altered physiological processes, which reduce the normal functioning of the organism (Grobler et al., 1989; Liao et al., 2003). From an environmental point of view, a study on bioconcentration is important because elements usually occur in low concentrations and subtle physiological effects go unnoticed until gross chronic reactions (e.g. changes in populations structure, altered reproduction, etc.) become apparent (Kumar and Mathur, 1991).

Among trace elements, As is well known as a toxin (Thomas, 1994). Arsenic has been classified as a carcinogen, based on human epidemiological data; it is known to increase the risk of producing or inciting cancer (IARC, 1987; Abernathy *et al.*, 2003). Since As is mainly transported by water, it can easily be accumulated by aquatic organisms (Spehar *et al.*, 1980; Phillips, 1990; Liu *et al.*, 2003; Ng *et al.*, 2003). Several studies have been conducted and demonstrated that an overexposure of As could result in accumulation in fish and lead to adverse health effects (Donohue and Abernathy, 1999; Lin *et al.,* 2001, 2004; Liao and Ling, 2003). Consumption of As-polluted fish might cause an overexposure of As and pose a cancer risk to human health via the food chain (Huang et al., 2003; Lin and Lin, 2004). It has also been well documented that As is the major risk factor for blackfoot disease (BFD), which is a peripheral vascular disease that ends with dry gangrene and spontaneous amputation of affected extremities (Chiou *et al.*, 1995). The increase in internal organ and skin cancers as well as BFD disease was significantly associated with the use of high-As groundwater (Chen *et al.*, 1980, 1999).

Although Zn and Cu are essential nutrients for normal metabolic functioning (Mertz, 1981; Watanabe et al., 1997; Hogstrand and Wood, 1996), they may become toxic to aquatic organisms, particularly fish, when ambient concentrations exceed physiological thresholds (Vallee and Falchuk, 1993; Wilson and Taylor, 1993; Taylor et al., 2000). These elements can be incorporated into the food chain and concentrated by aquatic organisms to a level that affects their physiological state (Laurén and McDonald, 1987a, 1987b; Liao et al., 2002; Liao and Ling, 2004). Ultimately they pose a health hazard to humans.

Many studies have demonstrated a modifying influence of water quality on Zn and Cu toxicity to fish. For example, pH affects Zn and Cu speciation, which in turn affects bioavailability (Cusimano et al., 1985); calcium (Ca^{2+}) associated with water hardness tends to reduce toxicity by competitively inhibiting Cu binding to fish gills (Pagenkopf, 1983; Laurén and McDonald, 1986; Playle et al., 1992; Erickson et al., 1996); and increasing concentrations of dissolved organic matter sequester waterborne Cu from biological uptake (Playle et al., 1993; Hollis et al., 1997).

As effluent from many sources enters natural waters, the negative impacts on the aquatic ecosystem are due to a mixture of elements, rather than individual component elements. When mixtures of trace elements are accumulated in organisms, they may show a number of synergistic, antagonistic or additive effects (Lewis, 1978; Mukhopadhyay and Konar, 1985). Most of those researches have been concerned with the physiological effects and bioconcentration patterns of individual elements. Researches on the interaction of mixtures of trace elements are limited mainly to mammalian toxicological studies (Wepener et al., 2001). A limited study investigating the uptake of Cu and Zn in fish exposed to a mixture containing Cu and Zn has been undertaken by Dethloff et al. (1999). Bioaccumulation in fish following exposure to a mixture containing As, Zn and Cu still remains unstudied.

Milkfish (*Chanos chanos*) is common seafood in Taiwan. Most of the milkfish culture ponds are located in the coastal region of southwest Taiwan, which is subjected to groundwater polluted with As and well known as the blackfoot disease (BFD) area (Lin *et al.*, 2004). A high amount (38,000-49,000 ton ha^{-1}) of freshwater is needed for milkfish culture. Several studies have been conducted to demonstrate that to use the groundwater for aquaculture may cause an overexposure of As (Lin *et al.*, 2001, 2004; Liao and Ling, 2003). Milkfish from the aquacultural ponds in the BFD area can be also contaminated by Zn and Cu from the ambient water.

The purpose of this study is to evaluate the bioaccumulation of individual elements from mixtures containing As, Zn and Cu in milkfish. The uptake and depuration rate constants, as well as the bioconcentration factor of milkfish will be estimated. The interactions between the elements and the subsequent uptake and depuration rates associated with the particular chemical properties of individual elements will be also analyzed. This information could contribute to understand the uptake, depuration and bioconcentration effects, following exposure to mixtures containing As, Zn and Cu.

Materials and Methods

Acclimation

Milkfish (*Chanos chanos*), averaging 8 cm in length and 50 g in mass, will be obtained from a commercial hatchery in Donan, Taiwan. Prior to experimental use, the fish will be kept in 8 glass aquarium tanks (30 fish per tank), each containing 50 L aerated and circulated distilled water at 25 ± 0.5°C. The fish will be fed daily with commercial milkfish bran.

Following acclimation to laboratory conditions for a period of two weeks, 24 healthy fish from each tank will be selected and allowed to acclimatize in the experimental tanks for at least one week prior to chemical exposure. Distilled water will be used during the entire acclimation and experimental period.

The water temperature of the experimental tanks will be kept at 25 ± 0.5°C and pH values will be measured. The fish will be subjected to an interval of 14-h light and 10-h dark; which is simulated to the condition prevailed in southwest Taiwan during the summer months.

Chemical Exposure

Concentrations of As, Zn and Cu will be selected to represent ecologically relevant concentrations, based on actual measured values, obtained from the chemical monitoring programs in the BFD area (Lin et al., 2001, 2004; Lin and Lin, 2005). The As^{3+}, Zn^{2+} and Cu^{2+} are the most common forms we found in milkfish ponds in the BFD area, and they are considered the most toxic ones from As, Zn and Cu species.

The concentration of 1 mg L^{-1} of As, Zn and Cu chosen for this experiment is lower than the fetal concentration for milkfish. Toxins will be added to 100 L reservoir tanks as $HAsO_2$, $CuCl_2$, and $ZnCl_2$ solutions. The solutions with toxins (As, Zn, Cu) and toxicant mixtures (As-Zn, As-Cu, Zn-Cu and As-Zn-Cu) will be added into the aquarium tanks, to replace the original water. Control fish will be maintained under similar conditions, but without the addition of the toxin to the water.

A 14-day uptake/depuration experiment will be conducted. First, milkfish will be exposed to the toxins for 7 d; and then, the residual fish will be transferred to non-toxicant tanks with only distilled water for another 7 d. Three fish will be sampled at 1, 2, 4, 7, 8, 11, 14 d. During the experimental period, fish will be fed every day. Good care will be taken to ensure that no excess food is left in the tanks after feeding. During sampling the fish will be anesthetized with tricaine methanesulfonate (MS222), weighed (0.01 g), washed in distilled water, and then stored at -20°C for further determination of total As, Zn and Cu contents in their flesh.

Water samples (500 ml for each) will be taken following the sampling schedule mentioned above. After acidification by adding 5 ml 1N HNO_3, the water samples will be kept at 15°C. The fish and water samples will then sent to the Super Micro Mass Research and Technology Center, Cheng Shiu Institute of Technology for analysis of total As, Zn and Cu.

Chemical Analysis

The frozen flesh of milkfish will be dehydrated in a dryer (40℃) for 96 h, and then grounded into powder. Aliquots of dry flesh powder weighing 0.5 g will be placed into a 250 ml beaker. Nitric acid (65%, 10 ml) will be added for an overnight digestion. The beaker with flesh solution, after the digestion, will be heated with a water bath at 70-80℃ for 2-4 h until the total volume is reduced to 1-2 ml. The solution will be transferred to a volumetric flask (50 ml), and then filled with 0.01N of HNO3 to make a 50 ml of final solution. After filtration, this 50 ml solution will be transferred to test tubes for As, Zn and Cu analysis using ICP-MS (Agilent 7500a). Analytical quality control will be achieved by digesting and analyzing identical amounts of rehydrated (90% H_2O) standard reference materials (DORM-2, Dogfish Liver-2-organic matrix, NRC-CNRC, Canada). Recovery rates will be ranged from 95% to 97%.

Calculation of BCF and rate constants

When steady-state chemical concentrations of tissue are attained, the equilibrium bioconcentration factor (BCF) of the milkfish can be calculated from the ratio of the chemical concentration in fish to that in water. The BCF can also be calculated from the ratio of the uptake rate constant to the depuration rate constant as,

$$\mathrm{BCF} = \frac{C_b}{C_w} = \frac{k_1}{k_2} \tag{1}$$

where C_b (μg g^{-1}) is the chemical concentration in biota; C_w (μg ml^{-1}) is the chemical concentration in water; k_1 is the uptake rate constant (ml g^{-1} d^{-1} or g g^{-1} d^{-1}); and k_2 is the depuration rate constant (d^{-1}).

Eqs. 1 is based on a well-established model that is first-order one-compartment and that is used to estimate accumulated chemicals resulting from exposures to water-borne contaminants,

(a) at uptake phase,

$$\frac{dC_b}{dt} = k_1 C_w - k_2 C_b \tag{2}$$

(b) at depuration phase,

$$\frac{dC_b}{dt} = -k_2 C_b \tag{3}$$

The solution of Eq. 2 at the constant C_w is,

$$C_b(t) = C_b(t=0) + C_w \frac{k_1}{k_2}(1 - e^{-k_2 t}) \quad (4)$$

As the first-order one-compartment model assumes that k_2 is not a function of tissue concentration, k_2 is often determined by depurating contaminated organisms in uncontaminated water and determining k_2 directly in that test organism. Therefore, after the fish are transferred to clean water tanks, the depuration rate constants (k_2) can be calculated by the linear regression of log-transformed tissue toxin concentrations on depuration time (d) as,

$$\ln C_b(t) = \ln C_b(t=T) - k_2 t \quad (5)$$

where T is the time when depuration begins. The k_1 and k_2 can also be estimated by fitting Eq. 4 to measured tissue toxin concentration data from the uptake experiments using an iterative, nonlinear, least-squares curve-fitting technique (SAS, Version 6.11). Variances in k_2 values derived from two methods were tested for homogeneity using an *F*-test. Values were then compared using *t*-test. The BCFs will be calculated from Eqs. 1.

The significance of the data will be determined using the analysis of variance (ANOVA) and the Tukey test with a 95% confidence limit (Zar, 1996).

Results and Discussion

The resulting data shows that the As, Cu, Zn concentrations in pond water were 63.92 ± 57.71 μg L^{-1}, 69.36 ± 27.81 μg L^{-1} and 11.09 ± 15.22 μg L^{-1}, respectively (Table 1), while in fish were 0.94 ± 1.34 μg g^{-1}, 2.01 ± 0.96 μg g^{-1} and 40.31 ± 17.25 μg g^{-1}, respectively (Table 2) (Lin, unpublished data). The high BCF values of As, Cu and Zn accumulated in fish (12.60 ± 4.68, 32.31 ± 12.53 and 4029.04 ± 1623.96, respectively) show that those cultured milkfish from the BFD area are contaminated by the ambient water and have a high tolerance against the pollutants. Ingestion of As, Zn and/or Cu contaminated milkfish could result in overexposure in inhabitants and lead to adverse health effects (Lin *et al.* 2004).

Table 1. Arsenic (As), Zinc (Zn) and Copper (Cu) concentrations in water of milkfish

culture ponds（μg L-1）in the BFD area.

Location	Concentration in Water（Mean ± SE）		
	As	Zn	Cu
Putai	88.10 ± 65.63*	21.65 ± 11.06	85.70 ± 8.34*
Yichu	102.57 ± 63.62*	5.97 ± 1.91	60.01 ± 2.44*
Hsuehchia	30.44 ± 6.82	7.45 ± 0.23	40.78 ± 15.92*
Peimen	34.58 ± 3.76	8.49 ± 0.23	121.88 ± 7.18*
Average	63.92 ± 57.71	11.90 ± 15.22	69.36 ± 27.81

*：As > 50 μg L^{-1}; Zn > 500 μg L^{-1}; Cu > 30 μg L^{-1}

Table 2. Arsenic (As), Zinc (Zn) and Copper (Cu) levels in milkfish (μg L-1) from culture ponds in the BFD area.

Location	Level in Fish（Mean ± SE）		
	As	Zn	Cu
Putai	1.89 ± 1.66	46.33 ± 13.75	2.11 ± 0.03
Yichu	1.29 ± 0.93	33.10 ± 4.61	1.84 ± 0.11
Hsuehchia	0.32 ± 0.00	39.92 ± 2.63	1.76 ± 0.31
Peimen	0.40 ± 0.11	32.57 ± 2.65	2.63 ± 0.45
Average	0.94 ± 1.34	40.31 ± 17.25	2.01 ± 0.96

The increase of uptake rate factor (k_1), depuration rate factor (k_2) and bioconcentration factor (BCF) values of As accumulation in milkfish was observed when Cu was added into the As stock. Those values were decreased when the As stock was in combination with Zn additive. Accumulation of Zn in milkfish was enhanced when the fish was exposed to As-Zn mixture, while it is reduced when exposed to Zn-Cu mixture. When milkfish were exposed to As-Cu mixture and Zn-Cu mixture, the Cu accumulation in milkfish was decreased. It shows that Zn will reduce the accumulation of As and Cu in milkfish, while Cu enhanced the accumulation of As and Zn. When As was added into Zn stock, the accumulation of Zn was decreased. In the As-Cu mixture stock, milkfish accumulate more Cu than that in Cu stock. It indicates that As will enhance the accumulation of Cu in milkfish.

Table 3. The uptake rate factor (k1), depuration rate factor (k2) and bioconcentration factor (BCF) of Arsenic (As), Zinc (Zn) and Copper (Cu) accumulated in milkfish.

Stock	Additive	k_1	k_2	BCF	R	R^2
As	/	981.73	5.17	189.74	0.83	0.68
	Zn	812.83	3.08	264.20	0.90	0.81
	Cu	1313.76	8.49	154.67	0.87	0.76
Zn	/	657.71	2.17	302.80	0.83	0.69
	As	1005.75	3.01	333.80	0.80	0.64
	Cu	882.74	3.22	273.89	0.80	0.65
Cu	/	13.07	1.24	10.52	0.96	0.91
	As	6.90	0.66	10.50	0.83	0.68
	Zn	7.04	1.28	5.50	0.82	0.67

The results demonstrate that Zn can reduce the accumulation of As and Cu because the high Zn accumulation ability of milkfish (Lin *et al.*, 2005). The competition of Zn toward As and Cu may cause the reduction of accumulation in milkfish of the later toxins. Since Cu will enhance the metabolism of the fish (Wepener *et al*., 2001), it might be the reason that milkfish will accumulate more As and Zn, when Cu was added into the stock.

Table 4. The effects of the individual elements, Arsenic (As), Zinc (Zn) and Copper (Cu) in the mixture on the individual toxin accumulation in milkfish.

Stock	Additive		
	As	Zn	Cu
As	/	Decreased	Increased
Zn	Increased	/	Increased
Cu	Decreased	Decreased	/

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