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RESEARCH ARTICLE

Mixture risk assessment due to ingestion of arsenic, copper, and zinc from milkfish farmed in contaminated coastal areas

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Abstract Human health risks associated with the consumption of metal-contaminated fish over extended periods have become a concern particularly in Taiwan, where fish is consumed on a large scale. This study applied the interaction-based hazard index (HI) to assess the mixture health risks for fishers and non-fishers who consume the arsenic (As), copper (Cu), and zinc (Zn) contaminated milkfish from As-contaminated coastal areas in Taiwan, taking into account joint toxic actions and potential toxic interactions. We showed that the interactions of As–Zn and Cu–Zn were antagonistic, whereas As–Cu interaction was additive. We found that HI estimates without interactions considered were 1.3–1.6 times higher than interactive HIs. Probability distributions of HI estimates for non-fishers were less than 1, whereas all 97.5%-tile HI estimates for fishers were >1. Analytical results revealed that the level of inorganic As in milkfish was the main contributor to HIs, indicating a health risk posed to consumers of fish farmed in As-contaminated areas. However, we found that Zn supplementation could significantly decrease As-induced risk of hematological effect by activating a Zn-dependent enzyme. In order to improve the accuracy of health risk due to exposure to multiple metals, further toxicological data, regular environmental monitoring, dietary survey, and refinement approaches for interactive risk assessment are warranted.

Keywords Mixture risk assessment · Human health · Metal mixture interactions · Milkfish · Arsenic · Copper · Zinc · Interaction-based hazard index

Introduction

In Taiwan, the high level of seafood consumption is a major route of exposure to heavy metals (Han et al. 1998; Chien et al. 2002). Milkfish is a commonly consumed and the main cultured fish in

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Taiwan. Its cultivation is a promising business in Taiwan because of its high market value, with US$168 million dollars in 2014 (FACOA 2014). Most of the milkfish aquaculture ponds are located in the southwest coastal area of Taiwan, which is also a highly developed industrial area. Because of the limited water resource and highly polluted surface water, a large amount of groundwater is used to supply fishponds in this area. Unfortunately, groundwater in southwestern Taiwan had high arsenic (As) concentrations during the 1960s, ranging from 10 to 1820 μg L^{-1} (Kuo 1968; Tseng et al. 1968). A long-term field investigation of many aquaculture ponds in the southwest coastal area of Taiwan during 1998–2003 reported average As concentrations in pond water ranging from 13.0 to 350 μg L^{-1} (Lin and Liao 2008; Lin et al. 2001).

Copper (Cu) and zinc (Zn) have also been commonly detected in aquaculture waters due to inherent in nature and from intensive human activities. Cu is the cheapest and most extensively used agent for eradicating filamentous algae and blue-green algae in aquaculture ponds in Taiwan (Tsai et al. 2013). In Taiwan, reported Cu concentrations in water from fish farms are 63–120 (Tsai et al. 2013) and 32.1 ± 14.3 μg L^{-1} (Ling et al. 2013), and average Zn concentrations in the water in aquaria are 38–160 μg L^{-1} (Lin and Liao 1999).

It has long been known that As can cause various cancers (Chen et al. 1992), hyperpigmentation, and keratosis (Tseng et al. 2000), and is strongly associated with blackfoot disease, a peripheral vascular disease endemic in southwest coastal areas in Taiwan (Chen et al. 1985). The trace elements Cu and Zn are essential to maintain physiological processes and functions in humans. They play an important role in defense mechanisms against free radical damage, particularly through Zn/Cu superoxide dismutase (Chan et al. 1998; ATSDR 2004a). However, intakes of Cu and Zn beyond normal dietary requirements can lead to adverse health effects. A high dose of Cu can induce hepatic and renal lesions, and overexposure to Zn is associated with hematotoxic, pancreatic, adrenal abnormalities, and impaired immune function (ATSDR 2004a).

Previous works have widely studied the assessment of the risk posed by metals in seafood, although such investigations exclusively examined single metals (Lin and Liao 2008; Liu et al. 2005; Chou et al. 2006; Jang et al. 2006; Liang et al. 2010, 2011, 2013; Ling et al. 2014; Raknuzzaman et al. 2016). Fewer studies have explored the risks due to co-exposure to As, Cu, and Zn in seafood (Chien et al. 2002; Lin 2009; Yi et al. 2011; Giri and Singh 2014). These studies assessed the risk by using the hazard index (HI), a common component-based formula for assessing non-cancer risk. The HI is based on the key assumption of dose additivity (i.e., non-interaction) among chemical components.

The traditional non-interaction HI method may underestimate or overestimate human health risks in cases involving interaction effects among components. To circumvent this problem, the United States Environmental Protection Agency (USEPA) (2000) proposed a refined formula, termed the interaction-based HI. In this formula, a weight-of-evidence (WOE) analysis is applied to judge the magnitude of toxic interaction for binary mixtures based on the quality of scientific data.

Default calculation of the HI has traditionally used the reference dose (RfD) derived from the critical effect, defined as the toxic effect occurring at the lowest dose (USEPA 2000). Thus, RfD-based HI method generally cannot be used to explore the risk of toxic effects of interest. It may also overestimate the risk of effects less sensitive than those that produce a critical effect (USEPA 2000; ATSDR 2004b). A recommended alternative is the target-organ toxicity dose (TTD), a RfD-like safety guidance valve that is unlikely to affect a specific target organ (Mumtaz et al. 1997; ATSDR 2004b). The Agency for Toxic Substances and Disease Registry (ATSDR) (2004b) therefore adopted the concept of TTD and combined it with the WOE analysis to provide a guideline for the assessment of joint toxic action and endpoint-specific HI of chemical mixtures, taking into account target-organ-specific effects and toxic interactions.

The mixture risk approaches proposed by the USEPA (2000) and ATSDR (2004b) have been adopted to assess human health risks posed by a mixture of metals in drinking water (Ryker and Small 2008) and food crops (Cao et al. 2011). These approaches, however, have not yet been applied to the mixture risk assessment for consumption of fish contaminated with multiple metals. The objectives of this study were threefold: (1) to identify joint toxic actions and potential toxic interactions for mixtures of As, Cu, and Zn on the basis of available empirical data; (2) to apply the USEPA and ATSDR's approaches for assessing the mixture health risk due to As, Cu, and Zn in milkfish farmed in As-contaminated areas; and (3) to explore the impact of toxic interactions on the mixture health risk.

Materials and methods

Problem formulation

Putai, Yichu, Peimen, and Hsuehchia located in southwestern Taiwan were selected as study sites. These four sites are As-contaminated coastal towns and are also referred to as the arseniasis-endemic areas in the past few decades (Chen et al. 1985). In these areas, milkfish is the most important seafood, having the highest cultured area (Liang et al. 2010). However, the pond water is polluted with As, Cu, and Zn due to the extraction of As-contaminated groundwater, industrial activities, and regular use of algaecide. Several studies have shown that milkfish can accumulate As, Cu, and Zn to high levels that are positively correlated with ambient concentrations

(Chen et al. 2000; Chou et al. 2006; Lin and Liao 2008; Lin 2009).

Dietary intake of fish contaminated with metals is a potential health concern to local residents, especially those in fishing communities, who generally consume more milkfish than the nationwide average. Although it is recognized that multiple metal exposure and toxic interactions (i.e., additive, synergism, and antagonism) could affect health risk, most studies have not yet considered them (Chien et al. 2002; Lin and Liao 2008; Liu et al. 2005; Chou et al. 2006; Jang et al. 2006; Lin 2009; Liang et al. 2010, 2011, 2013; Ling et al. 2014).

Exposure assessment

Data on the concentrations of As, Cu, and Zn in milkfish were adopted from two previous field surveys in As-contaminated areas: (1) inorganic As levels in milkfish from 12 groundwater-cultured ponds were measured by Lin and Liao (2008) and (2) levels of Cu and Zn in milkfish from 8 groundwater-cultured ponds were collected by Lin (2009). Data on milkfish consumption for fishers and non-fishers in As-contaminated areas were derived from Lin and Liao (2008) and Chou et al. (2006), respectively. Lin and Liao (2008) used a brief questionnaire to interview 141 subsistence fishers to determine the rate at which they consume milkfish. Chou et al. (2006) used results from an unpublished investigation to obtain data for levels of milkfish consumption of non-fishers in As-contaminated areas.

Hazard characterization

We characterized the toxic interactions for binary mixtures of As, Cu, and Zn mainly according to ATSDR's interaction profiles (ATSDR 2004a) and published literature (Krishnan and Brodeur 1994; Modi et al. 2005, 2006; Chou et al. 2007; Antonio Garcia et al. 2013). Based on the results of toxicological and epidemiological literature, ATSDR's interaction profiles considered empirical observations and mechanisms of toxicity to evaluate joint toxic actions of chemical mixtures and to infer what type of interaction could occur among chemicals in the mixture (Pohl et al. 2003, 2004). The brief conclusions on the toxicity of the mixture and the relevance to public health then were drawn. A peer review process is further conducted to ensure the accuracy of data presented and the validity of conclusions (Pohl et al. 2003). An interaction profile (ATSDR 2004a) proposed an antagonistic relationship between Cu and Zn with respect to hepatic and hematological effects on the basis of several oral studies in rats.

Krishnan and Brodeur (1994) incorporated laboratory observations with human experience to verify the occurrence of supra- and infra-additive interactions among environmental pollutants and observed antagonistic interactions in As–Zn and Cu–Zn mixtures. Chou et al. (2007) proposed an additive interaction between As and Cu on the basis of a similar mode of action with regard to oxidative stress. Using δ-aminolevulinic acid dehydratase (ALAD), a heme biosynthesis enzyme, as a biomarker, Modi et al. (2005, 2006) conducted a series of experiments on male mice and rats to examine the hematologically protective effects of Zn against As toxicity. They showed that Zn supplementation results in significant recovery from As-induced inhibition of blood ALAD activity. By exposing rat pups to As, Antonio Garcia et al. (2013) demonstrated that administering Zn effectively restored the hematological parameters (red blood cell count, hemoglobin levels, and hematocrit) to the levels of the control group. On the other hand, Zn also causes hematological toxicity, mainly by interfering with the homeostasis of Cu, which is essential for heme synthesis (ATSDR 2004a). In rat and mouse studies, co-exposure to As did not affect blood Zn level (Modi et al. 2005, 2006). Although As also affects heme synthesis (ATSDR 2004c), mechanistic understanding and toxicological data are not adequate to determine the interaction for the effect of As on the hematological toxicity of Zn.

The USEPA and ATSDR developed the information-rich database on chemical-specific toxicity values that can be used to perform non-cancer risk assessment. RfD values were adopted from USEPA's Integrated Risk Information System (IRIS) database or from USEPA's Regional Screening Level (RSL) Summary Table, while TTD values were obtained from ATSDR's interaction profiles. The derivation of TTDs is analogous to the derivation of ATSDR's target-organ-specific minimal risk levels (MRLs) (ATSDR 2004b). Thus, when TTD values were not available, MRLs were used (ATSDR 2004c; Ryker and Small 2008). The joint toxic actions of As, Cu, and Zn then could be determined according to TTDs and MRLs.

Risk characterization

The traditional non-interactive HI method was first used to estimate the non-cancer health risks posed by consumption of a mixture of As, Cu, and Zn in milkfish. Non-interactive HI ($HI_{non\text{-}INT}$) can be calculated as the sum of component-specific hazard quotients (HQ_i),

$$\mathrm{HI}_{\text{non-INT}} = \sum_{i=1}^{n} \mathrm{HQ}_i = \sum_{i=1}^{n} \frac{\mathrm{CDI}_i}{\mathrm{RfD}_i} \tag{1}$$

$$\mathrm{CDI}_i = \frac{C_i \times IR \times EF \times ED \times 10^{-3}}{BW \times AT_{\mathrm{nc}}} \tag{2}$$

where CDI_i is the chronic daily intake (mg kg^{-1} day^{-1}) for chemical i, RfD_i is the reference dose (mg kg^{-1} day^{-1}), C_i is the chemical concentration in milkfish (μg g^{-1}), IR is the milkfish consumption rate (g day^{-1}), EF is the exposure frequency (day year^{-1}), ED is the exposure duration (year), BW is the body weight of Taiwanese adult (kg), AT_{nc} is the averaging time for non-carcinogens (day), and 10^{-3} is the unit conversion factor.

We then adopted the USEPA's interaction-based HI method (USEPA 2000) to examine the impact of interaction on the mixture risk assessment. The interactive HI (HI_{INT}) was formulated by having an adjustment factor for each HQ_i as follows:

$$HI_{INT} = \sum_{i=1}^{n} \left(HQ_i \times \sum_{i \neq j}^{n} f_{ij} \times M_{ij}^{B_{ij}\theta_{ij}} \right) \quad (3)$$

$$f_{ij} = \frac{HQ_j}{HI_{non\text{-}INT} - HQ_i} \quad (4)$$

$$\theta_{ij} = \frac{(HQ_i \times HQ_j)^{0.5}}{(HQ_i + HQ_j) \times 0.5} \quad (5)$$

where f_{ij} is the toxic hazard of j chemical relative to the total HI from all chemicals potentially interacting with chemical i, M_{ij} is the magnitude of interaction representing the influence of chemical j on the toxicity of chemical i, B_{ij} is the binary WOE score reflecting the strength of evidence that chemical j influences the toxicity of chemical i, and θ_{ij} is the degree to which chemicals i and j are present in equitoxic amounts.

Furthermore, we employed the ATSDR's TTD modification method (ATSDR 2004b) to assess the target-organ-specific HI_{INT} of the selected metals through Eqs. (3, 4, and 5), using TTD instead of RfD for each HQ_i. HI >1 indicates a potential health hazard associated with multiple metals.

The USEPA (2000) sets a default value of five for M_{ij} in Eqs. (3, 4, and 5). We adopted a more detailed and regimented WOE methodology used by the ATSDR (2004b) to determine B_{ij}. In the WOE methodology, a qualitative classification scheme was constructed to create B_{ij} scores for characterizing the effect of each chemical on the toxicity of every other chemical. The qualitative classification scheme consists of the expected direction of interaction (additive, greater than additive, less than additive, or indeterminate) and the numerical weighting scores for evaluating the quality of data by taking into account mechanistic understanding, toxicological significance, and modifying factors (ATSDR 2004b, Supplementary Table S1). The B_{ij} scores can then be determined by multiplying the direction of interaction and the data quality weighting scores ranging from −1 (the highest possible confidence in less-than-additive interactions) through 0 to +1 (the highest possible confidence in greater-than-additive interactions) (ATSDR 2004b).

Uncertainty and sensitivity analysis

A Monte Carlo (MC) technique was applied to generate 2.5 and 97.5 percentile as 95% confidence interval (CI) for quantifying the uncertainty of model parameters. The Kolmogorov–Smirnov statistics was used to determine the goodness of fit of distributions for parameters. We also performed the MC simulation to quantify the uncertainty and its impact on the estimations of expected non-cancer risks ($HI_{non\text{-}INT}$ and HI_{INT}). The MC simulation was implemented with 10,000 iterations to ensure the stability of probability distributions. A sensitivity analysis was used to examine the contribution of each critical variable on the non-cancer risks. Contribution to variance was calculated by squaring the rank correlation coefficients and normalizing them to 100%. The Oracle® Crystal Ball software (version 11.1, Oracle Corporation, Redwood Shores, CA, USA) was used to implement the MC simulation and sensitivity analysis.

Results and discussion

Chronic daily intakes of As, Cu, and Zn from milkfish

Figure 1a–d and Supplementary Table S2 show that the concentrations of inorganic As, Cu, and Zn in milkfish in As-contaminated areas were 0.121 ± 0.075 (mean ± standard deviation) to 0.639 ± 0.795, 1.759 ± 0.305 to 2.634 ± 0.448, and 32.570 ± 2.651 to 46.330 ± 13.752 μg g^{-1}, respectively. We found that the milkfish ingestion rates of fishers ranged from 138.47 ± 45.32 to 274.58 ± 106.06 g day^{-1} and were much higher than those of non-fishers at N(6.71, 1.95 g day^{-1}) (Table 1).

We estimated the chronic daily intakes (CDIs) of inorganic As, Cu, and Zn (Fig. 2a–d) in the four study sites for fishers and non-fishers through Eq. (2), using component-specific concentrations in milkfish (Fig. 1a–d) and essential parameter values (likelihood and point estimates) listed in Table 1. Average CDIs of fishers were 2.71×10^{-4}–1.27×10^{-3}, 3.75×10^{-3}–7.77×10^{-3}, and 7.31×10^{-2}–1.40×10^{-1} mg kg^{-1} day^{-1} for inorganic As, Cu, and Zn, respectively (Fig. 2a–d). The highest CDI of inorganic As was found in Hsuehchia (median: 8.04×10^{-4}, 95% CI: 1.03×10^{-4}–5.29×10^{-3} mg kg^{-1} day^{-1}). Residents in Yichu had the highest CDIs for Cu (7.24×10^{-3}, 3.29×10^{-3}–1.53×10^{-2} mg kg^{-1} day^{-1}) and Zn (1.30×10^{-1}, 5.66×10^{-2}–2.85×10^{-1} mg kg^{-1} day^{-1}). Results also reveal that the CDIs of fishers were approximately 19–46 times higher than that of non-fishers.

The Joint FAO/WHO Expert Committee for Food Additives (JECFA) recommended a provisional tolerable weekly intake (PTWI) for inorganic As of 15 μg kg^{-1} $week^{-1}$ (i.e., 0.0021 mg kg^{-1} day^{-1}) and provisional maximum tolerable daily intakes (PMTDIs) for Cu and Zn of 0.5 and 0.3–1 mg kg^{-1} day^{-1}, respectively (JECFA 2014). In this study, CDIs of Cu and Zn fall within the

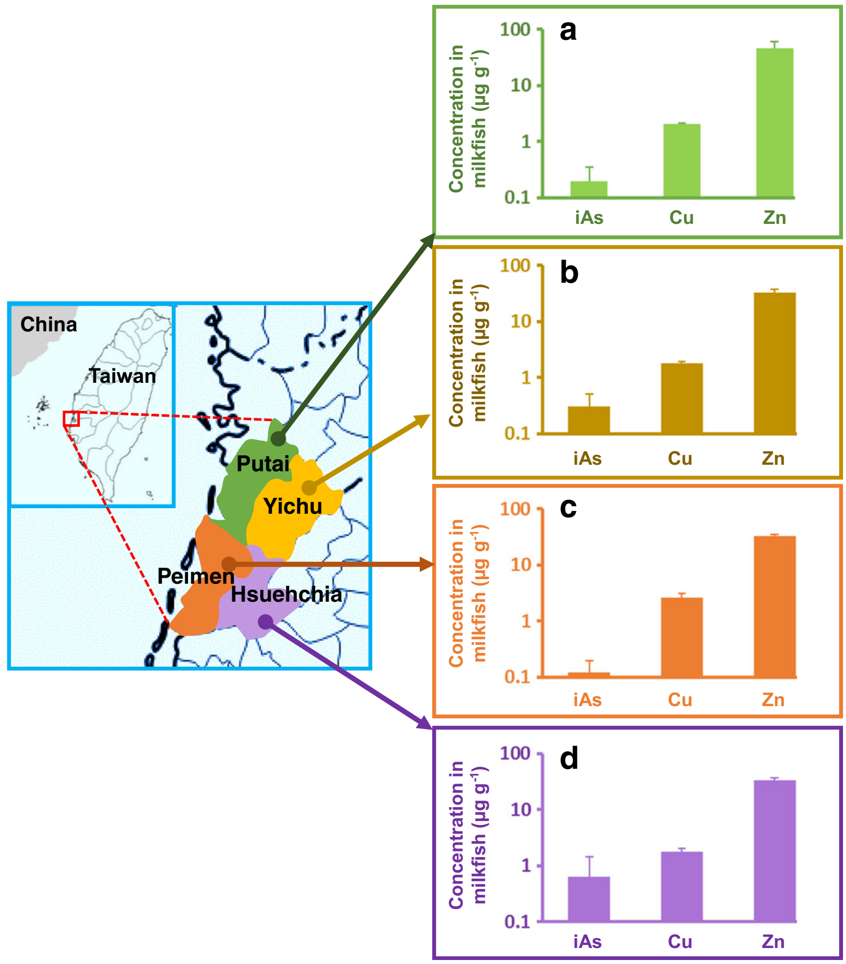

Fig. 1 Site-specific study data for inorganic arsenic (iAs), Cu, and Zn concentrations in milkfish farmed located at (**a**) Putai, (**b**) Yichu, (**c**) Peimen, and (**d**) Hsuehchia in As-contaminated areas

PMTDIs, whereas those of inorganic As could exceed nearly 2.5 times the recommended safe level.

In light of the epidemiological evidence of adverse effects (lung and urinary tract cancers) of inorganic As at

Table 1 Probability distributions and point values of parameters used to estimate non-interactive and interactive hazard index ($N(a, b)$ denotes the normal distribution with mean a and SD b)

Parameters	Symbol	Estimated value
Milkfish ingestion rate of fishers[a]	IR (g day^{-1})	
Putai		N(179.81, 74.79)
Yichu		N(274.58, 106.06)
Peimen		N(145.12, 50.74)
Hsuehchia		N(138.47, 45.32)
Milkfish ingestion rate of non-fishers[b]	IR (g day^{-1})	N(6.71, 1.95)
Exposure frequency[c]	EF (day year^{-1})	350
Exposure duration[c]	ED (year)	30
Body weight of Taiwanese adult[d]	BW (kg)	N(63.07, 7.15)
Averaging time for non-carcinogens[e]	AT_{nc} (day)	10,950

[a] Estimated based on Lin and Liao (2008)

[b] Estimated based on Chou et al. (2006)

[c] Adopted from USEPA (1991)

[d] Adopted from the National Health Interview Survey, National Health Research Institutes, Taiwan (NHRI 2005)

[e] Exposure duration 30 years × 365 days (USEPA 1991)

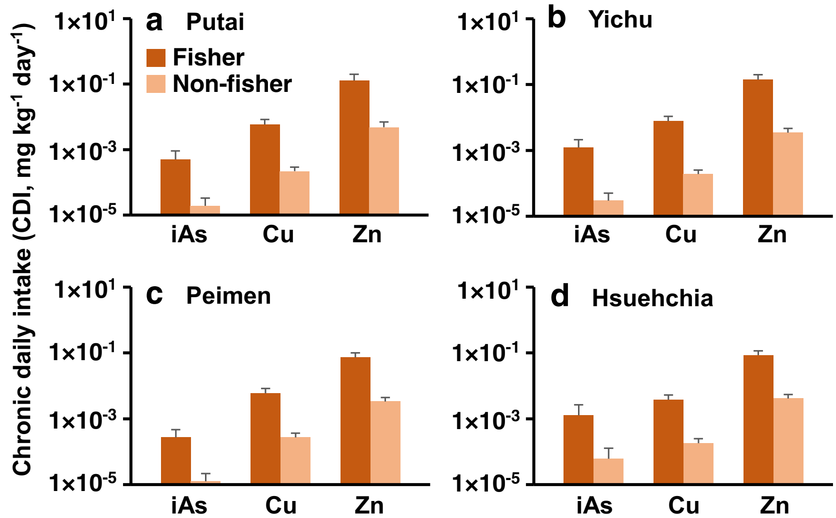

Fig. 2 Component-specific chronic daily intake (CDI) for milkfish-consuming fishers and non-fishers residing in (**a**) Putai, (**b**) Yichu, (**c**) Peimen, and (**d**) Hsuehchia in As-contaminated coastal towns of Taiwan. *iAs* inorganic arsenic

concentrations in drinking water that below the PTWI, the European Food Safety Authority (EFSA) Panel concluded that the PTWI of inorganic As is no longer appropriate and should be lowered (EFSA 2009). The PTWI was withdrawn by the JECFA in 2011. Therefore, the data for cancers of lung, skin, and bladder as well as dermal lesions, which are the main adverse effects reported to be associated with long-term ingestion of inorganic As in humans, were considered by the EFSA Panel as possibly providing an appropriate reference point (EFSA 2009). A benchmark response of 1% extra risk was then selected, and a range of benchmark dose lower confidence limit ($BMDL_{01}$) values were also identified. Consequently, the EFSA Panel suggests that the overall range of $BMDL_{01}$ values between 0.3 and 8 μg kg^{-1} day^{-1} should be used instead of a single reference point in the risk assessment for inorganic As (EFSA 2009). By comparison, our CDIs of inorganic As could be 17.7 times higher than the $BMDL_{01}$ values.

Joint toxic effects and toxic interactions among As, Cu, and Zn

Table 2 summarizes the values of RfD, TTD, and MRL for inorganic As, Cu, and Zn. According to TTDs and MRLs, the joint toxic actions for As–Cu and As–Zn were gastrointestinal and hematological effects, respectively. The potential interactions of binary mixtures and the strength of evidence of the interaction among As, Cu, and Zn represented as B_{ij} scores are shown in Table 3. For instance, the B_{ij} scores for As–Cu interactions obtained by multiplying the direction of interaction (=), the ratings of III for mechanistic understanding, and C for toxicological significance were zero, reflecting an additive effect between As and Cu at low confidence (Table 3). In contrast, an antagonistic interaction for the effect of Zn on hematological toxicity of As with moderate confidence (<IIA = −0.71) was found. On the other hand, the interaction for the effect of As on Zn was indeterminate because of a lack of toxicological and mechanistic data. Thus, the potential effect of As–Zn mixture on hematological toxicity might be antagonistic interaction. For the Cu–Zn mixture, the effect of Cu on the hematological toxicity of Zn (<IIA = −0.71) and the effect of Zn on the hepatic toxicity of Cu (<IB = −0.71) were antagonistic, showing moderate to high confidence.

Mixture health risk from milkfish consumption

Given the component-specific HQ_i (Fig. 3a) and the B_{ij} scores for each pair in the combinations (Table 3), the site-specific $HI_{non\text{-}INT}$ and HI_{INT} for fishers and non-fishers (Fig. 3b) could be calculated through Eqs. (1, 3, 4, and 5), respectively. Figure 3a shows that HQ estimates of As were apparently higher than those of Cu and Zn in the four study sites. Figure 3b reveals that HI_{INT} estimates were lower than $HI_{non\text{-}INT}$, indicating risk assessment without considering toxic interactions among chemicals in the mixture might be overestimated if the interactions were less than additive. For non-fishers in As-contaminated areas, distributions of $HI_{non\text{-}INT}$ and HI_{INT} were less than the standard of one, with a mean consumption rate of 6.71 g day^{-1} (Fig. 3b). However, for fishers with a mean consumption rate of 138.47–274.58 g day^{-1}, all 97.5%-tile HI estimates were >1, revealing significant contributions from milkfish consumption (Fig. 3b). Especially, fishers residing in Hsuehchia had the highest non-cancer risks, that is, 3.01 (95% CI: 0.66–18.42) for $HI_{non\text{-}INT}$ and 2.04 (0.42–13.90) for HI_{INT}.

Figure 4 presents the target-organ-specific HI_{INT} for As–Cu and As–Zn mixtures. Results show that fishers had markedly higher risks of gastrointestinal and hematological effects compared with non-fishers. Most of 97.5%-tile HI_{INT} estimates for fishers were >1 for gastrointestinal effect (range:

Table 2 Reference dose and organ/system toxicity values for inorganic As, Cu, and Zn

Chemical	RfD (mg kg^{-1} day^{-1})	Organ or system toxicity values	
		TTD (mg kg^{-1} day^{-1})	MRLs (mg kg^{-1} day^{-1})
Inorganic As	0.0003[a]	0.0003 (neurological)[c] 0.09 (renal)[c] 0.0003 (cardiovascular)[c] 0.0006 (hematological)[c]	0.0005 (gastrointestinal)[d, e]
Cu	0.04[b]	0.14 (hepatic)[f]	0.01 (gastrointestinal)[d]
Zn	0.3[a]		0.3 (hematological)[d]

RfD reference dose, *TTD* target-organ toxicity dose, *MRLs* minimal risk levels

[a] Adopted from USEPA's IRIS database (http://www.epa.gov/iris)

[b] Adopted from Regional Screening Level (RSL) Summary Table (USEPA 2016)

[c] Adopted from ADSTR (2004c)

[d] Adopted from Minimal Risk Levels (MRLs) (ATSDR 2016)

[e] 0.0005 mg kg^{-1} day^{-1} was obtained through the acute MRLs of inorganic As (0.005 mg kg^{-1} day^{-1}) divided by acute-to-chronic extrapolation uncertainty factor (10)

[f] Adopted from ADSTR (2004a)

2.54–10.57; Fig. 4a) and hematological effect (range: 0.90–5.85; Fig. 4b). For fishers in Hsuehchia with highest exposure risk, HI_{INT} estimates of gastrointestinal and hematological effects were 1.19 (95% CI: 0.47–10.57) and 0.86 (0.25–5.85), respectively (Fig. 4). Particularly, we found that the hematological effect presented a lower risk. In As–Zn mixture, HI_{INT} estimates for hematological effect were obviously lower than HQs of As (Fig. 4b).

Results of sensitivity analysis of HI_{INT} estimates for fishers in Hsuehchia reveal that the level of inorganic As in milkfish was the most sensitive to HI_{INT}, followed by the milkfish ingestion rate (Fig. 5). Our results indicate that milkfish As content was the most important determinant of HI_{INT}, which was consistent with most previous studies conducted by Lin (2009), Yi et al. (2011), and Giri and Singh (2014) comparing the non-cancer risks due to As, Cu, and Zn in consumed fish. Liang et al. (2013) pointed out that milkfish had a greater probability of exceeding the PTWI than do tilapia and shellfish, indicating that regulation of As levels in milkfish farms

Table 3 Matrix of binary weight-of-evidence score (B_{ij}) for the mixtures of As, Cu, and Zn

Effect of	On toxicity of		
	As	Cu	Zn
As		=IIIC (0)[a]	? (0) h[d]
Cu	=IIIC (0) nr[a]		<IIA (−0.71) h[c]
Zn	<IIA (−0.71) h[b]	<IB (−0.71) p[c]	

Qualitative classification scheme in WOE methodology (Supplementary Table S1): "=, >, <, ?" stand for additive, greater than additive, less than additive, or indeterminate; "I, II, III" express the extent of mechanistic understanding with the corresponding rating scores of 1.0, 0.71, and 0.32, respectively; "A, B, C" express the extent of toxicological significance with the corresponding rating scores of 1.0, 0.71, and 0.32, respectively

nr other toxicities except renal toxicity, *h* hematological, *p* hepatic

[a] Adopted from Chou et al. (2007)

[b] Determined based on Modi et al. (2005, 2006), Chou et al. (2007), and Antonio Garcia et al. (2013)

[c] Adopted from ATSDR (2004a)

[d] Determined based on ATSDR (2004a, c)

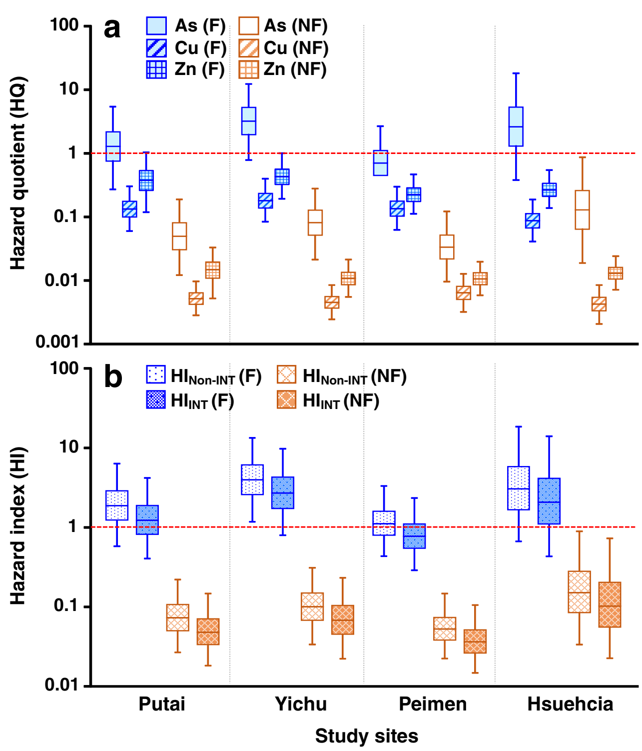

Fig. 3 Box-and-whisker plot representing (**a**) hazard quotients (HQ) as well as (**b**) non-interactive ($HI_{non-INT}$) and interactive (HI_{INT}) hazard indexes for fishers (F) and non-fishers (NF) exposed to a mixture of As, Cu, and Zn due to consuming milkfish farmed in As-contaminated areas

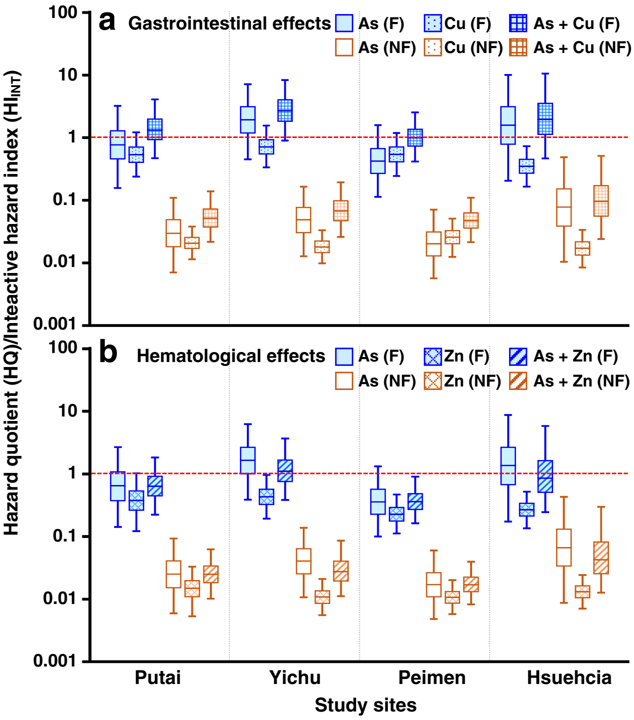

Fig. 4 Box-and-whisker plot of target-organ-specific risk represented as hazard quotients (HQ) and interactive hazard indexes (HI_{INT}) for milkfish-consuming fishers (F) and non-fishers in As-contaminated areas. (**a**) Gastrointestinal and (**b**) hematological effects for As–Cu and As–Zn mixtures, respectively

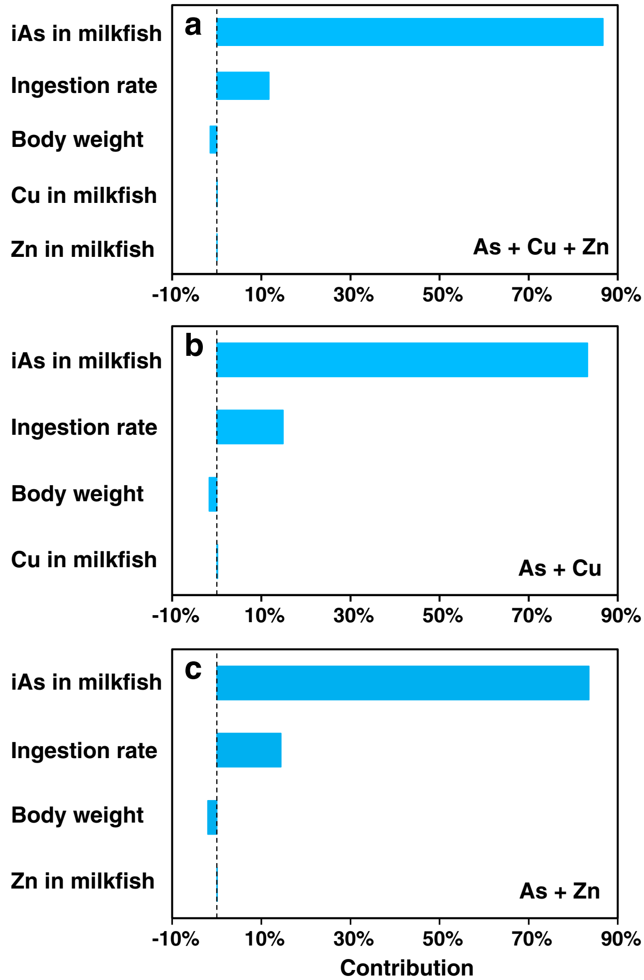

Fig. 5 Results of sensitivity analysis of each parameter contribution to the interactive hazard indexes (HI_{INT}) for fishers in Hsuehchia, the town with highest exposure risk, by different risk calculation scenarios. **a** As–Cu–Zn. **b** As–Cu. **c** As–Zn mixtures. *iAs* inorganic arsenic

should take precedence. Our results also suggest that levels of inorganic As in milkfish increase the HI_{INT} by approximately 80% (Fig. 5), thus implying that As accumulation in milkfish could be reduced by improving the water quality of milkfish farms and consequently, significantly reduce health risks to consumers.

The quality of mixture risk assessment depends strongly on the toxic interaction scores (B_{ij}) among multiple pairs of metals. To our knowledge, current information on mixture toxicity is insufficient for determining the joint toxic actions and the corresponding toxic interactions for mixtures of two or more chemicals. Although the As–Cu and As–Zn interactions could not be easily characterized in this study because of unavailable mixture data from ATSDR, some published evidence could be used to support our evaluations that potential As–Cu interaction for gastrointestinal effect was additive and that As–Zn interaction for hematological effect was antagonistic.

Numerous experiments on rats (Ademuyiwa et al. 1996; Uthus 2001; Cui and Okayasu 2008) and guinea pigs (Hunder et al. 1999) have demonstrated that As–Cu interactions occur exclusively in the kidneys. Furthermore, As did not influence the Cu content of intestine, liver, and other organs (Elsenhans et al. 1987; Uthus 2001; Cui and Okayasu 2008). As is well known to be toxic by inhibiting ALAD activity, thus impairing heme synthesis and ultimately causing anemia (Flora et al. 2008; Chakrabarty 2015). Zn exhibits protective behavior against As-induced hematological toxicities, in particular, anemia, by reversing the inhibition of ALAD (Modi et al. 2005, 2006; Chakrabarty 2015) and by restoring the red blood cell count, hemoglobin levels, and hematocrit (Antonio Garcia et al. 2013). The recovery of hematological toxicities can be partially due to the fact that ALAD is a Zn-dependent enzyme, thus promoting heme synthesis (Modi et al. 2005, 2006; Antonio Garcia et al. 2013; Chakrabarty 2015).

Taking binary interactions between metals into consideration, we found that $HI_{non\text{-}INT}$ estimates were 1.3–1.6 times higher than HI_{INT}. We also found that the hematological effect, which seems to be due to blood ALAD activation by Zn, presented a lower risk (Modi et al. 2005, 2006; Antonio Garcia et al. 2013; Chakrabarty 2015). Additionally, red blood cells are vulnerable to oxidative damage (Gürer et al. 1998). As such, reducing oxidative stress by increasing the activity of antioxidant enzymes and metallothionein expression (Chakrabarty 2015; Ganger et al. 2016) may also be a possible protective mechanism of Zn against As-induced hematotoxicity. The World Health Organization recommends supplementation with trace elements such as Zn for reversing arsenicosis (Howard 2003).

In addition to toxic interaction, HI_{INT} can be affected by the magnitude of interaction (M_{ij}). Ryker and Small (2008) found that with strong interaction in a mixture (i.e., B_{ij} is close to 1), M_{ij} may strongly influence HI_{INT}. They also indicated that M_{ij} may reach the maximum value of 10. Furthermore, consumption variability among subpopulations, such as that due to age, gender, and susceptibles, has considerable influence on the health risk associated with fish consumption (Liang et al. 2013). Although the TTD method avoids conservatism of the critical effect and may use fewer uncertainty factors than does RfD, this approach is not yet widely used and is not the only means of calculating the interaction risk (USEPA 2000; Ryker and Small 2008; EFSA 2013). Alternative methods such as benchmark dose, relative potency factor, and toxic equivalence factor methods could be applied when possible and when suitable data are available (USEPA 2000; Ryker and Small 2008; EFSA 2013).

This study is intended to seek out the currently available methodology to assess mixture health risk and to explore the impact of toxic interaction on health risk. The mixture risk approaches developed by the USEPA (2000) and ATSDR (2004b) allow a prediction of mixture risks different from dose additivity by considering information on binary mixtures between chemicals. The application of these approaches to mixture risk assessment was not only for metal mixture (Ryker and Small 2008; Cao et al. 2011) but also for mixtures of organic compounds, such as persistent organic pollutants (Pohl et al. 2004), air pollutants, and pesticides (Ragas et al. 2011).

Conclusion

This work presents a risk assessment approach for mixtures by integrating the USEPA and ATSDR's interactive mixture methods, taking into account joint toxic actions and toxicity interactions. We used this approach to assess the mixture health risk due to consumption of a mixture of As, Cu, and Zn in milkfish farmed in As-contaminated areas. We found markedly different HI estimates between fishers and non-fishers due to the individual variability of milkfish consumption. Sensitivity analysis showed that the level of inorganic As in milkfish is the main contributor to health risk, thus indicating a health concern for consumers of milkfish farmed in As-contaminated areas. Our results demonstrated that the health risk may be overestimated by calculations that do not consider antagonistic As–Zn and Cu–Zn interactions. Furthermore, we found a particularly lower risk of hematological effects due to As–Zn interactions probably because Zn supplementation reduces As-induced hazards. Our risk assessment was heavily based on studies of mixture toxicity in the literature. Scientific evidence of toxic interactions among metals in concerned target organs or systems is still limited. In addition to further toxicological data, regular environmental monitoring, dietary survey, and refinement approaches for interactive risk assessment are warranted to ensure the accuracy of assessments of health risks due to consuming fish contaminated with multiple metals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Research involving human participants and animal rights The article does not contain any studies with human participants or animals performed by any of the authors.

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