Evaluation of artificially contaminated fish with formaldehyde under laboratory conditions and exposure assessment in freshwater fish in Southern Bangladesh

Submitted to the Journal of

Chemosphere

Md. Sazedul Hoque[a*], Liesbeth Jacxsens[b], Md. Boktheir Hossain[a], A.K.M. Nowsad Alam[c], S.M.Oasiqul Azad[a], Bruno De Meulenaer[b], Carl Lachat[b] and Matiur Rahman[d]

[a]Department of Fisheries Technology, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh.

[b]NutriFOODchem Unit, Department of Food Safety and Food Quality (Partner in Food2Know), Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

[c]Department of Fisheries Technology, Bangladesh Agricultural University, Mymensingh-2200, Bangladesh.

[d]National Food Safety Laboratory, Institute of Public Health, Mohakhali, Dhaka 1212, Bangladesh

*Corresponding author

Address: Department of Fisheries Technology, Patuakhali Science and Technology University, Dumki, Patuakhali-8602, Bangladesh. E-mail address: sazedul.fst@pstu.ac.bd (M. S. Hoque) Phone : + 8801716244719; Fax: +880427566009.

Abstract

Formalin can be added as preservative to fresh foods to prevent spoilage and extend shelf life. Formalin contains 37-40% formaldehyde, which is classified as carcinogenic to humans. To assess the public health risk associated with formaldehyde exposure in freshwater fish in Southern Bangladesh, formaldehyde concentrations (mg/kg) were determined in tilapia, Indian major carp rui, Chinese carp and a minor carp from local market and in laboratory simulations (0.5, 1.0, 2.0 and 4.0% formaldehyde solution for 5, 15, 30 and 60 min) with spectrophotometric and high performance liquid chromatography (HPLC) methods. A food frequency questionnaire was used to collect fish consumption (kg/kg BW.d) data from 400 respondents. A probabilistic exposure assessment was conducted using @Risk®7.0 software. Fish treated with formalin at increasing concentrations and exposure time showed increased trends of formaldehyde acquisition irrespective of fish species and analytical methods used ($p<0.05$). Compared to spectrophotometry, the HPLC method was shown to be more sensitive and is therefore the preferred method for formalin quantification. Maximum exposure to formaldehyde (0.28 mg/kg BW.d) was calculated for tilapia using HPLC analysis. Margin of exposure (MoE) provides high priority (<10,000) for tilapia and Indian major carp rui at P99 under spectrophotometric analysis whereas as determined using HPLC, tilapia had MoE values much lower than 10,000 at P99, P95 and P90 (both total population and consumers). Exposure to formaldehyde associated with freshwater fish consumption is a public health concern in Southern Bangladesh and needs further assessment and risk management strategies.

Key words: Fish, formalin, formaldehyde, exposure assessment, risk characterization.

1. Introduction

In Bangladesh, fish and fishery products are key for food security as they supply 60% of animal protein, provide employment opportunities (approx. 16 million people) and contribute 3.69% of the gross domestic product (DoF, 2015). Available reports suggest that formaldehyde is added in the form of formalin (by dipping or spraying) to marketed fish in Bangladesh to prevent spoilage and increase shelf life (Hoque *et al.* 2016; Jaman *et al.* 2015; Yeasmin *et al.* 2010). Formalin typically contains a 37-40% concentration of formaldehyde. Volatile toxic aldehydes like formaldehyde are considered food contaminants and a safety and public health concern (Bianchi *et al*. 2007; Claeys *et al*. 2009). A higher concentration of formaldehyde was reported for imported fish due to the additional time it takes through various steps along the supply chain before the fish reaches domestic retail markets in Bangladesh (Rahman *et al*. 2016). Consumer concerns regarding the formaldehyde content in fish and other food are growing and determination of the presence of formaldehyde is therefore needed.

Rapid, accurate, easy-to-use and affordable methods are available to screen for food contaminants and toxicants (Chiou *et al*. 2015). The Bangladesh Council of Scientific and Industrial Research (BCSIR) has developed a simple kit to detect presence of formaldehyde in fish (Rahman *et al*. 2016; Yeasmin *et al.* 2010). However, this kit can only be used for qualitative testing of formaldehyde, i.e. whether formaldehyde is present or absent. Qualitative tests and detection kits have been used previously in Bangladesh to determine formaldehyde in freshwater and marine water fish i.e. by Islam *et al.* (2015) for Indian major carp (rui and catla) fish in Dhaka region; Indian major carp (rui, catla), hilsa shad, minor carp fish in Jessore district (Paul *et al.* 2014) and Sylhet city (Rahman *et al*. 2012). Spectrophotometry and high performance liquid chromatography (HPLC) are quantitative methods for formaldehyde determination. The spectrophotometric

method using Nash's reagent and trichloroacetic acid (TCA) based extraction is considered a reliable, convenient, fast and safe procedure for quantitative estimation of formaldehyde in fish (Benjakul *et al*. 2006; Zhang *et al.* 2015). Jaman *et al.* (2015) conducted quantitative tests of formaldehyde presence in Indian major carp rui, tilapia, Thai climbing perch, Ganges river sprat, bombay duck and ribbon fish in Mymensingh using spectrophotometry. Compared to spectrophotometry, the HPLC method is more selective and more sensitive. HPLC requires however more expensive equipment and more know-how to operate, which is not widely available in a developing country such as Bangladesh. Wahed *et al.* (2016) used HPLC method for determination of formaldehyde in marketed fish and other food in Bangladesh and concluded that the method has good analytical performance in terms of specificity, linearity, precision, recovery and robustness, i.e. potential as a reference standard method. The different formaldehyde content found in the different studies was therefore due to differences in performance between methods used for determination of formaldehyde content.

Fish and fishery products can contain high levels of formaldehyde from artificially added and endogenous sources where the foremost source is endogenous (Zhang *et al.* 2015). Formaldehyde can be produced naturally in fish by the degradation of trimethylamine oxide (TMAO) in presence of enzyme trimethylamine oxide demethylase (TMAOase), which catalyzes the conversion of TMAO into dimethylamine and methanal (also known as formalin) (Bianchi *et al*. 2007; Stanley and Hultin, 1984; Yeh *et al*. 2013; Zhang *et al.* 2015). The naturally high levels of formaldehyde in fish complicate the accurate detection of illegally added formaldehyde (Wahed *et al.* 2015). It should be noted that in view of the high reactivity of formaldehyde towards proteins, a substantial part of the formaldehyde will be bound to proteins (Vandemoortele and Meulenaer, 2015). Naturally available formaldehyde in the fish muscle is covalently bound to functional groups of

proteins and forms a cross linkage among the peptide chains (Sikorski *et al.* 1982). Formaldehyde binds with lysine and arginine residues in peptides. The reaction of formaldehyde with a peptide or protein starts with N-formylated products (Liu *et al*. 2016) and formation of unstable methylol adducts on amino and thiol groups, and also Schiff base on a lysine residue can form stable cross-links with several amino acid residues (Metz *et al*. 2006). It is very likely that the formation of such formaldehyde-protein adducts would be stimulated by a cooking treatment, thus restricting further its evaporation, while increasing the amount of formaldehyde potentially liberated from these complexes during the digestion process, which is relevant from a food safety perspective (Vandemoortele and Meulenaer, 2015; Vandemoortele *et al*. 2017).

Formaldehyde has acute or chronic toxicity with carcinogenic and mutagenic properties. The International Agency for Research on Cancer hence categorized formaldehyde in the group 1,'as carcinogenic to humans' (IARC, 2004). Formaldehyde has potential carcinogenic modes of action due to its mutagenicity (formaldehyde induced DNA–protein and protein–protein cross-links) (Claeys *et al*. 2009; Monakhova *et al*. 2012). Humans could be exposed to this hazardous chemical from eating of formaldehyde contaminated fish, or via inhalation, skin or eye contact (Claeys *et al*. 2009). Epidemiological studies reported that oral exposure to formaldehyde at 0.17 ppm and greater can induce ulceration of the gastrointestinal tract. Those working with chemicals, furniture and in the funeral service industry have greater prevalence of irritation of the eyes or respiratory tract including loss of cilia, goblet cell hyperplasia, and cuboidal and squamous cell metaplasia in the nasal cavity (Naya and Nakanishi, 2005).

Apart from cultivated mushrooms in Belgium (Claeys *et al*. 2009), marketed fish in Malaysia (Aminah *et al*. 2013) and different food products in China (Tang *et al*. 2009), research regarding the exposure associated with formaldehyde is scarce. To date, no assessment of exposure and risks

of formaldehyde through fish consumption in Bangladesh has been conducted. The aim of the present study is to (i) estimate intake data and to determine formaldehyde concentration in commonly consumed fish from different steps along the fish supply chain in Patuakhali district, Southern Bangladesh, and, (ii) to assess the effect of treatment conditions using two different analytical methods under laboratory conditions. Using these data, a further probabilistic exposure assessment and risk characterization is carried out. This study assesses the risks of formaldehyde exposure in fish and provides guidance for risk managers and the national food safety authorities in Bangladesh as well as in South East Asia.

2. Materials and methods

2.1 Study area and fish sample collection

Fresh fish samples were collected from different sites covering major steps along the fish distribution channel, *viz.* harvest site (fish farm in Patuakhali Science and Technology University; PSTU), landing center/auction center, wholesale market and retail market in Patuakhali district of Bangladesh. Four of the most commonly available freshwater fish i.e., tilapia (*Oreochromis niloticus*), Indian major carp (*Labeo rohita*), Chinese carp (*Hypophthalmichthys molitrix*) and a minor carp (*Amblypharyngodon mola*) were selected. Collected fish samples were covered in polythene pouch (zip-lock), preserved in an ice box and brought to the laboratory. In addition, tilapia from the PSTU fish farm were treated with formaldehyde in the laboratory at different concentrations and contact times (dipping into 0.5, 1.0, 2.0 and 4.0% formaldehyde solution for 5, 15, 30 and 60 min). Formaldehyde concentration of all fish samples from different steps along the fish supply chain in Patuakhali district of Bangladesh and laboratory treated fish were determined

using the spectrophotometric method. The difference in performance between the spectrophotometric and HPLC method was also compared using tilapia as representative sample.

2.2 Determination of formaldehyde concentration

2.2.1 Chemicals

Ammonium acetate, acetyl acetone, acetic acid, trichloroacetic acid (TCA), potassium hydroxide (KOH) and hydrochloric acid (HCl) were purchased from Merck, India. Formaldehyde (37% formaldehyde) in water certified reference material (CRM) (4815 mg/L) and solvents were of analytical grade (SIGMA–Aldrich, Buchs SG, Switzerland). 2,4 dinitrophenylhydrazine (2,4 DNPH) was purchased from Merck (Darmstadt, Germany).

2.2.2 Determination of formaldehyde concentration using spectrophotometry

The spectrophotometric method with some modification (Benjakul *et al.* 2003) using Nash's reagent was applied to determine the formaldehyde content (mg/kg) in fish. Nash's Reagent was used as an indicator which helps to detect the absorbance of formaldehyde (Nash, 1953). To prepare Nash's reagent, 15 g ammonium acetate was diluted in a 100 ml Erlenmeyer flask with an addition of 0.3 ml of acetyl acetone and 0.2 ml of acetic acid. About 30 g fish flesh was blended for 10 minutes, using a Philips HR-2106 blender. Sixty (60) mL of 6% w/w TCA was added for extraction of formaldehyde from the fish flesh. The extracted solution was then filtered by a Whatman No.1 filter paper. The pH of the solution was determined by a pH meter (pH 211, Hanna Instruments, Italy). The addition of TCA resulted in a reduced pH value of the sample which was then adjusted between 6.00-6.50 by using potassium hydroxide (KOH) and hydrochloric acid (HCl). Five (5) mL of sample solution was taken in a 50 mL volumetric/conical flask and kept in a freezer (Walton W2D-1H5, Bangladesh) at -20 $^{\circ}$C for 1 h. The sample was taken out of the

freezer and 2 mL of previously prepared Nash's reagent was added as indicator. The fish sample was then heated in the water bath (Fisherbrand FB60301, China) at 60 ˚C for 30 minutes. The absorbance of the sample in a cuvette was measured at 415 nm immediately by UV/Vis spectrophotometer (T60 UV/Visible Spectrophotometer, PG Instruments, U.K). The sample reading (triplicate) was placed in the standard curve for the calculation of formaldehyde concentration in the fish sample.

2.2.3 Determination of formaldehyde concentration using HPLC

The modified method of Wahed *et al.* (2015) for the determination of formaldehyde concentration (mg/kg) using HPLC was applied. A 2,4 dinitrophenylhydrazine (2,4 DNPH) working solution was recrystallized prior to use by dissolving 10 g of 2,4 DNPH in 100 mL in hot analytical grade acetonitrile to form a saturated solution. After complete dissolution, the solution was cooled to room temperature, capped in a brown bottle and stored overnight at 4 °C for crystallization. The crystals were collected by vacuum filtration. A 150 mg of 2,4 DNPH crystals were accurately weighed, dissolved in 49.5 mL of acetonitrile and mixed with 0.5 mL of phosphoric acid (85%). Derivatization kinetics followed the procedure described by Claeys *et al*. (2009) with slight modification. Muscle parts of the fish were used for the analysis. To sample aliquots of 5 g, 5 mL of acetonitrile were added, the sample was vortexed and then sonicated for 30 min. The samples were then centrifuged at 5000 rpm for 5 min and the supernatant was passed through a 90 mm diameter Whatman®541 (Hardened Ashless) filter paper (SIGMA–Aldrich, Buchs SG, Switzerland). Two and half milliliter of 2,4 DNPH was added to the extract and mixed well. Samples were incubated at 40 °C for 60 min in a shaking water bath (model BS-11, Oxon, UK). Formaldehyde was quantitatively converted to its Schiff base in 60 min. During analysis,

derivatization time was set to 60 min. After incubation, the acetonitrile layer was collected, membrane filtered (0.45 µm) and injected into the HPLC.

Analyses were performed on a C_{18} Luna column (25 cm × 4.6 mm id., 5 µm particle size), (Phenomenex, Utrecht, The Netherlands) using a HPLC (model SPD-M20A) coupled to a photodiode array detector (both manufactured by Shimadzu, Kyoto, Japan). The wavelength was set to 355 nm and the oven temperature at 30 °C. Separation was achieved using isocratic elution with a mixture of water/methanol (35:65, v/v). The flow rate was 1.0 mL/min and the injection volume 20 µL. The total run time was 12 min. The regression square coefficient (R^2), LODs and LOQs were 0.99, 0.39 (mg/L), 1.30 (mg/L) for matrix free and 0.99, 1.75 (mg/L), 5.83 (mg/L) for matrix-matched calibrations, respectively.

2.3 Collection of fish consumption data

Consumption data were collected via convenience sampling i.e. 400 adult respondents in Patuakhali district of Bangladesh from June 2015 to February 2016 were invited to participate in the survey. Socio-demographic (age, sex, weight, family member, income source etc.) data of the respondents were also collected. Fish consumption data were collected by Master students from PSTU who received training as interviewers beforehand, using face-to-face interviews. The interviewers explained the procedures and objective of the survey to the respondents, after which they administered a structured food frequency questionnaire. The respondents were asked to estimate the portion size of each fish consumed by the family. This estimate was divided by the total household size to obtain the quantity of fish consumed by the respondents, under the assumption that all consumers in the respondent's family consume a similar amount of fish. Next, the respondents were asked 'how often do you eat a particular fish, categorized as daily or 7 times a week, 5 times a week, 3 times a week, once a week, once per two weeks, once per four weeks or

never. The responses were first converted to a daily consumption using a conversion factor (i.e. 7 times a week corresponds to 1/day; 5 times a week corresponds to 5/7, 3 times a week corresponds to 3/7, 1 time a week corresponds to 1/7, 1 time per two weeks corresponds to 1/15, 1 time per four weeks corresponds to 1/30 and never corresponds to non-consumer who do not like or eat that specific fish), followed by a multiplication of the amount of fish consumed by the respondent. The estimate was divided by the body weight (kg) of the respective respondent as representing the body weight of the whole family (adult members). The body weight of the respondent was measured using a digital weighing scale (Sagas weighing scale, India). Using these estimates, the fish consumption dataset kg/kg BW.d was obtained for both consumer and non-consumer in respect to each fish species.

2.4 Probabilistic exposure assessment

To evaluate the population risk associated with consumption of formaldehyde contaminated fish, a probabilistic exposure assessment was conducted. It was assumed that the food processing factor (washing, freezing or cooking) of fish as a traditional consumer practice of Bangladesh does not affect the formaldehyde concentration in fish (worst case scenario). As noted earlier in this paper, in view of the high reactivity of formaldehyde towards proteins, part of the formaldehyde is likely to be bound to proteins, and formation of such formaldehyde-protein adducts could be stimulated by cooking, restricting its evaporation. Calculations were done for the actual fish consumer (consumer of specific fish) and for the total population (consumers and non-consumers). The total population (consumer and non-consumer) refers to the total number of respondents (e.g. 400) whereas consumer refers to number of respondents who actually consume specific fish (e.g. out of 400, 350 respondents consume rui fish); in this case for rui, 350 respondents are consumers and

227 the remaining 50 respondents are non-consumers. The inclusion of non-consumers was used to
228 assess the chronic exposure.

229 @Risk®7.0 for Microsoft Excel 2010 (Palisade Corporation, USA), was used with different fish
230 consumption data (kg/kg BW.d) and formaldehyde concentration (mg/kg) distributions from both
231 spectrophotometric and HPLC method were combined into an exposure distribution (mg/kg
232 BW.d). Best fit distributions were determined for consumption and formaldehyde concentration
233 (both spectrophotometric and HPLC method) using the Chi-square statistics,
234 probability/probability (P/P) and quantile/quantile (Q/Q) plot. For the exposure calculations of the
235 whole population (including consumers and non-consumers), a logical "if" function was applied
236 combining the zero consumption of the fraction of non-consumers and the distribution of the
237 consumption for the fraction of the consumers. First-order Monte-Carlo simulations were
238 undertaken considering 50,000 iterations. The simulations were repeated three times to ensure that
239 stable estimates. Formaldehyde intake (mean, standard deviation, maximum, minimum and
240 percentiles) was determined from the output of the simulation model.

241 ***2.5 Risk characterization***

242 Risk characterization of the carcinogenic and genotoxic formaldehyde was carried out compared
243 with the results from the probabilistic exposure assessments with the corresponding margin of
244 exposure (MoE) approach using the benchmark dose lower confidence level ($BMDL_{10}$) of 23 mg
245 kg^{-1} day^{-1} for formaldehyde (Monakhova *et al*. 2012). To calculate the MoE, formaldehyde
246 exposure estimated for both total population and consumers were used from both
247 spectrophotometric (all fish species) and HPLC (Tilapia only) methods. MoE were calculated from
248 a chosen point of departure (PoD) on the dose–response curve (lower limit of the benchmark dose

estimate at 95% confidence where 10% of responses achieved $BMDL_{10}$) divided by the human dietary exposure estimated, using following formula:

$$\text{MoE} = \frac{\text{BMDL10}}{\text{Human exposure}}$$

2.6 Statistical analysis

For the both spectrophotometric and HPLC method, each sample was analyzed in triplicate (n=3). Data were subjected to analysis of variance (ANOVA) and comparison of means was carried out by Duncan's multiple range test (Steel and Torrie, 1980). Statistical significance was accepted at a P value of <0.05. The statistical analysis was performed using the SPSS package (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Prevalence and concentration of formaldehyde in different fish from different steps along the supply chain in Southern Bangladesh

The formaldehyde concentration of four different fish collected in different steps along the supply chain is shown in Table 1. In spectrophotometric analysis, a significant variation in formaldehyde concentration was observed when the same species of fish was collected from different steps along the supply chain. Indian major carp rui had the highest (1.68 mg/kg) formaldehyde concentration when collected from the retail market while lowest concentrations were found (0.77 mg/kg) in samples collected from the fish farm ($p<0.05$). In case of Chinese carp, the highest and lowest formaldehyde concentrations were 0.82 and 1.50 mg/kg for samples collected from fish landing center and fish farm, respectively. Among the four different fish, the highest (2.08 mg/kg) formaldehyde concentration was found in tilapia samples from the landing site ($p<0.05$). Minor

carp contained a very low amount of formaldehyde compared to other fish and along any steps of the supply chain considered (range 0.43 to 0.93 mg/kg). Using the same analytical method, variations in formaldehyde concentration in tilapia from different locations were reported previously. Jaman *et al.* (2015) found formaldehyde concentrations of 1.85, 2.53 and 2.50 mg/kg in tilapia fish from different fish markets in Mymensingh district in Bangladesh, which were higher than the present findings. With the same spectrophotometric method, Jaman *et al.* (2015) reported formaldehyde concentrations of 1.45 mg/kg for rui and 7.00 to 7.35 mg/kg for small indigenous species kachki. Aminah *et al.* (2013), Bianchi *et al.* (2007) and Noordiana *et al.* (2011) also observed differences in formaldehyde content in different fish species.

A significant higher formaldehyde concentration was observed in HPLC analyzed fish irrespective of sample source/origin ($p<0.05$) (Table 1). Differences in formaldehyde concentration determined with the two different methods might be due to differences in sample preparation, extractions, recovery and detection limit of the respective methods. Chiou *et al*. (2015) reported that the accuracy of conventional testing methods was generally not as good as that of sophisticated instrumental methods such as HPLC, high performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) of which the limit of detection (LOD) is much lower and could be competent for quantitative analysis. Chiou *et al*. (2015) also reported that chromatography could easily separate and detect corresponding hydrazine derivates from reaction between formaldehyde and 2,4-dinitrophenylydrazine (2,4-DNPH) chemical derivatives. In the present study 2,4-DNPH was also used to determine the formaldehyde concentration in tilapia fish by using HPLC, this was not used in Nash's reagent basis spectrophotometric method, and might explain the different results obtained from these two studies.

Furthermore, a non-significant difference in formaldehyde concentration between tilapia fish samples from the university fish farm and other steps along the supply chain was found ($p<0.05$, Table 1), indicating a natural occurrence of formaldehyde in fish. Several studies reported a natural occurrence of formaldehyde in fish, aquatic products and seafood (Bianchi *et al*. 2007; Chiou *et al*. 2015; Claeys *et al*. 2009). During post-mortem changes, TMAO is formed in fish from the post-mortem enzymatic reduction of TMAO to equi-molar amounts of formaldehyde and di-methylamine. The differences in formaldehyde content in different fish could be due to differences in the amount of TMAO formed upon death and during storage of fish (Bianchi *et al.* 2007; Nielsen and Jørgensen, 2004). Wahed *et al*. (2016) also reported that formaldehyde occurs naturally in free and bound forms. Therefore, the findings from present and previous studies indicate that formaldehyde content in fish could vary with species, habitat of fish, compositional differences, processing method used, storage time, storage temperature and differences in response to reaction between fish protein and formaldehyde.

3.2 Formaldehyde concentration in laboratory treated fish measured by spectrophotometric method

Figure 1 indicates that at the same concentration of formaldehyde dipping solution, formaldehyde concentration in fish muscle gradually increases with increasing dipping time regardless of fish species. On the other hand, at the same dipping time with increasing concentration of formaldehyde dipping solution, increased trends in formaldehyde concentration were found for each fish. At 0.5% concentration formaldehyde dipping solution for 5 min, Indian major carp fish muscle had a formaldehyde concentration of 5.65 mg/kg. For the same species, at the same concentration with the increasing dipping time at 15, 30 and 60 min, the formaldehyde concentration significantly ($p<0.05$) increased to 6.60, 7.77 and 8.32 mg/kg, respectively. The result also shows that for the same species and same dipping time (5 min), the formaldehyde

concentration gradually increased from 5.65 to 95.15 mg/kg when fish was treated with 0.5% to 4% formaldehyde solution (Figure 1). Similar trends in the increase of formaldehyde concentrations were observed for higher dipping time (15, 30 and 60 min) when other higher concentration (1, 2 and 4%) formaldehyde treatments were used. A significant increase ($p<0.05$) in formaldehyde concentration with increased concentrations of formaldehyde dipping solution and dipping time was also noticed, regardless of the fish. For all fish species treated under laboratory conditions, the concentration of formaldehyde dipping solution more markedly influenced the formaldehyde concentrations in the samples than dipping time. Yeasmin *et al*. (2013) also studied Indian major carp rui fish dipped in different concentrations (5 – 35%) of formalin solution for 5 min each and reported that at increased concentrations of dipping solution a correspondingly longer duration was required for removal of formalin in water. Removal of formalin from the surface of fish body also depends on the concentration of formalin with the time needed for removal being directly proportionate to the concentration of formalin used to treat the fish.

Figure 1 compares the formaldehyde concentration in different fish with the same concentration of formaldehyde dipping solution and dipping time used. There were differences ($p<0.05$) in formaldehyde concentration between the fish given a particular exposure concentration and dipping time. At 0.5% formaldehyde concentration dipping solution and 5 min dipping time, Indian major carp rui contained higher formaldehyde concentration (5.65 mg/kg) than any other species where Chinese carp contained the lowest value (2.92 mg/kg) ($p<0.05$). However, of the four species, the Chinese carp had a higher formaldehyde concentration when 1, 2 and 4% solutions were used for 5 and 15 min respectively ($p<0.05$). At the highest concentration dipping solution (4%) and dipping time (60 min) treatment, minor carp showed the highest formaldehyde

concentration value (186.82 mg/kg) ($p<0.05$). Under the same laboratory treatment condition, the variations in formaldehyde concentration between different fish species might be due to differences in biochemical composition of fish muscle. Analysis of proximate biochemical compositions of the studied fish (data not shown) confirm differences in major protein composition between the studied fish species. Chemical reactions between fish muscle of different biochemical composition and formaldehyde might have resulted in differences in formaldehyde concentrations between the species. Naturally produced or available formaldehyde in the fish muscle is covalently bound to functional groups of proteins and forms a cross linkage among the peptide chains (Sikorski *et al.* 1982). Yeh *et al*. (2013) distinguished formaldehyde bonding types in fishery products as free, bound and total formaldehyde. In another study Metz *et al*. (2006) and Yeh *et al*. (2013) reported several unstable and stable reactions of formaldehyde with several amino acid residues.

3.3 Formaldehyde concentration in laboratory treated tilapia fish measured by spectrophotometric and HPLC method

Formaldehyde concentrations in tilapia collected from different steps along the supply chain were determined using both spectrophotometric and HPLC methods. A considerable increase in detection of formaldehyde concentration by HPLC over spectrophotometric method ($p<0.05$) was noted (Table 1). Therefore, the HPLC method was also applied to measure formaldehyde concentrations in tilapia under laboratory conditions. Figure 2 compares formaldehyde concentrations of tilapia fish treated with different concentrations of formaldehyde dipping solution and dipping times under laboratory conditions determined with spectrophotometric and HPLC methods. At any concentration of formaldehyde dipping solution and dipping time used, significantly higher formaldehyde concentration readings were obtained by HPLC method in

365 comparison to the spectrophotometric method ($p<0.05$). At a condition of dipping into 0.5% for
366 5 min, the formaldehyde concentration determined by HPLC was approximately three times (from
367 3.43 to 10.87 mg/kg) higher than that by the spectrophotometric method. Similar to the
368 spectrophotometric results for different laboratory treated fish (Figure 1), the HPLC method also
369 showed increasing formaldehyde concentrations with formaldehyde dipping solutions at
370 increased concentrations and dipping time (Figure 2). At 0.5% dipping solution, formaldehyde
371 concentration detection at increasing dipping time from 5 to 60 min was about doubled for both
372 spectrophotometric (3.43 to 6.08 mg/kg) and HPLC (10.87 to 21.05 mg/kg) method. On the other
373 hand, with increasing concentrations of dipping solution from 0.5 to 4%, formaldehyde
374 concentration for the same time period (5 min), detection was around thirty times higher for both
375 spectrophotometric and HPLC methods. Nevertheless, the formaldehyde concentrations detected
376 by HPLC were much higher than those detected by the spectrophotometric method, which
377 indicates that the HPLC method is more sensitive and accurate than the spectrophotometric
378 method to determine formaldehyde concentrations in fish samples. In this last method,
379 colorimetric reaction in acid extracted sample distillates produces a purple color in presence of
380 formaldehyde. The intensity of the color is proportional to the formaldehyde concentration and
381 can be measured by UV spectrophotometer. Drawbacks of colorimetric methods are their poor
382 specificity, selectivity, prolonged analysis times and highly acidic conditions, which together lead
383 to over-reporting and/or false positives. Several studies reported that formaldehyde occurs
384 naturally in free and bound forms and produces different amino acid residues of protein with
385 different covalent cross-links, reversible, acid liable, acid resistant and schiff base reactions (Liu
386 *et al*. 2016; Metz *et al*. 2004; Yeh *et al*. 2013). Stanley and Hultin (1984) reported that
387 spontaneous reaction of formaldehyde with protein could make formaldehyde unavailable for

388 colorimetric reaction. This is the likely reason that in this study spectrophotometric analysis
389 showed lower formaldehyde concentrations than HPLC, under the same treatment.
390 Formaldehyde reported by spectrophotometric procedure should hence be considered free
391 formaldehyde as opposed to total formaldehyde. Yeh *et al*. (2013) found that the sum of the
392 concentrations of free and reversibly bound formaldehyde was higher by 19.3 mg/kg than the free
393 formaldehyde concentration in the HPLC method. Free formaldehyde constituted an average of
394 39% of total free and reversibly bound formaldehyde in the HPLC method. Mason *et al*. (2004)
395 stated that under harsh conditions of steam distillation with acid, formaldehyde released was the
396 sum of free and reversibly bound formaldehyde. Therefore, the measured formaldehyde
397 concentrations were relatively high. From this study, it can be concluded that performance of the
398 widely used spectrophotometric method is low compared to formaldehyde detection by HPLC.

399 ***3.4 Formaldehyde exposure assessment***

400 Using the probabilistic exposure assessment in this study, one data set for formaldehyde
401 concentration of all samples collected from different steps along the supply chain was constructed
402 for each fish. No difference in formaldehyde concentration was observed in tilapia fish collected
403 along different steps of supply chain using the HPLC method (Table 1).

404 ***3.4.1 Fish consumption data***

405 Cumulative consumption (kg/kg BW.d) of different fish by all respondents (non-consumer and
406 consumer) are presented in Figure 3. Fish consumption survey data were collected from 400 adults
407 in the Patuakhali district of Bangladesh. Amongst the total sample (n=400), non-consumers were
408 identified for the respective fish. Among the fish, the highest percentage (54.5%) of non-
409 consumers was identified for Chinese carp, followed by minor carp (38.0%). Highest fish
410 consumption was observed for Indian major carp rui and tilapia, where only 11.3 and 26.8% were

411 non-consumers, respectively. Mean fish consumption for Indian major carp rui, minor carp,
412 Chinese carp and tilapia was 0.00042, 0.00015, 0.00027 and 0.00044 kg/kg BW.d, for consumers;
413 and 0.00037, 0.00011, 0.00012 and 0.00031 kg/kg BW.d, for total population, respectively (Table
414 2).

415 Wahed *et al*. (2016) reported average consumption of fish at 50.3 g/person/d in Bangladesh.
416 National data estimate average fish consumption in Bangladesh at 56 g/person/d (DoF, 2015).
417 However, in our study, the estimated fish consumption was lower compared to the national average
418 fish consumption. Only four species were considered in the present study whereas in the national
419 data all fish consumption is considered. Differences in the consumption between species can be
420 clarified due to differences in characteristics and sensory properties. Chinese carp is a bony fish
421 with huge intramuscular bone and minor carp is a small indigenous species having less flesh, which
422 is consumed as whole fish with its skeletal bones. As an Indian major carp rui is preferred by
423 consumers for its high meat flesh, less bone and unique taste. However, non-consumption of Indian
424 major carp rui fish might be a result of low purchasing power of consumers as Indian major carp
425 rui is comparatively more expensive than the other three fish sampled. In Bangladesh, tilapia is
426 cheaper and known to be fish for the poor people. Consumers from higher socio-economic classes
427 generally do not consume tilapia due to its muddy flavor (Mikael *et al*. (2014). Belton *et al*., (2011)
428 reported a preference for Indian major carp rui, tilapia and Chinese carp by 26, 23 and 8%
429 respectively, of the consumers surveyed.

430 ***3.4.2. Probabilistic exposure assessment***

431 To identify the best fit distribution in the probabilistic exposure analysis, fish consumption and
432 natural occurrence of formaldehyde concentration in different fish measured with
433 spectrophotometric and HPLC methods were fitted in the @Risk software. From the result, the P–

P plot provided roughly a straight line joining the diagonals for both consumption (consumers) of different fish and formaldehyde concentration in the respective fish. When fitting the distribution for consumption of different fish, the consumer and total population (including consumers and non-consumers) were separately considered due to the presence of zero consumption patterns of the respective fish. The best fit distributions for both the formaldehyde concentration and consumption (consumers and total population, including consumers and non-consumers) of fish are shown in Table 2. The calculated dietary exposures due to formaldehyde concentration in fish measured with two different methods are shown in Table 3. Based on spectrophotometric concentration data, the mean formaldehyde intake due to Indian major carp fish consumption was 2.41E-04 and 4.87E-04 mg/kg BW.d for total population and consumers, respectively. Mean formaldehyde intake was much lower for minor carp (4.61E-05 and 9.24E-05 mg/kg BW.d) and Chinese carp (1.64E-04 and 3.25E-04 mg/kg BW.d) than Indian major carp and tilapia, in case of both total population and consumers. Formaldehyde intake for tilapia had the highest mean value (3.64E-04 and 7.30E-04 mg/kg BW.d). Moreover, a further increase in mean value of formaldehyde exposure 1.45E-03 and 2.91E-03 mg/kg BW.d was observed for tilapia when exposure was calculated based on formaldehyde concentration data from HPLC method.

Based on HPLC determined formaldehyde concentration data, tilapia consumers were exposed to a maximum of 0.28 mg/kg BW.d. Exposure above zero level is considered harmful for formaldehyde, as genotoxic carcinogen (JECFA, 1998) and should be maintained as low as reasonable amount (ALARA). Limitations in the detection method (poor sensitivity, specificity explained in section 3.3) might result in an underestimate of concentrations of formaldehyde ingested (Wahed *et al*. 2016). The real exposure of formaldehyde could hence be higher than that suggested by the method of analysis. Based on a proper formaldehyde detection method,

formaldehyde exposure due to consumption of tilapia fish is a health concern for the population studied in Southern Bangladesh. However, from deterministic exposure analysis Wahed *et al*. (2016) reported low or no risk of human exposure to formaldehyde in fish in Bangladesh. Average human exposure to formaldehyde from alcoholic beverages was estimated at $8.0x10^{-5}$ mg/kg/d and the resulting MoE was above 20,000 which may be considered a negligible risk of cancer from formaldehyde with alcohol consumption, but a priority for risk management (Monakhova *et al*. 2012). The formaldehyde exposure through the consumption of cultivated mushrooms was 0.19 μgkg^{-1} body weight day^{-1} for consumers only and 99 μgkg^{-1} body weight day^{-1} for total population and it was concluded that the dietary exposure to formaldehyde was not a cause for concern (Claeys *et al*. 2009). The present study indicates that, estimation of human exposure to formaldehyde in fish consumption is dependent on the method, with the HPLC method providing a more accurate way to determine free formaldehyde. Yeh *et al.* (2013) report a detailed analytical method applied for formaldehyde determination in fish and also suggested to measure the free formaldehyde as it is of toxicological interest.

3.5. Risk characterization due to formaldehyde exposure in fish based on toxicological value

To characterize the formaldehyde exposure risk, the margin of exposure (MoE) was calculated using the $BMDL_{10}$ of rodent data (Monakhova *et. al*. 2012) for the different fish under two different analytical methods (Table 4). According to EFSA (2005), a MoE greater than 10,000 could be considered as low health concern. Based on spectrophotometric data, the MoE for minor carp and Chinese carp calculated based on the exposures to formaldehyde concentrations at different percentiles (P50, P75, P95, P99) for both total population and consumers only were considerably higher than 10,000. However, calculated MoE at P99 was lower than 10,000 both for total population and consumers only, at 8,240 and 6,270 respectively for Indian major carp rui, and

5,560 and 3,940 respectively for tilapia. In addition, at P95 tilapia also showed MoE exposure (9,030) below 10,000 for consumers only.

Based on HPLC determined formaldehyde concentration data, MoE for tilapia was lower than 10,000 for formaldehyde exposure at P90, P95 and P99 in case of both total population and consumers only. For the same fish, MoE at the same percentile (P99) was much lower for the HPLC determined sample than the spectrophotometric one for both total population and consumer only. On the other hand, for the same analytical method (HPLC) and at higher percentile, MoE values were lower than that of lower percentile (MoE, P99<P95<P90) for both total population and consumer only, indicating potential health risk from consuming the respective fish in the studied area of Bangladesh.

3.6. Uncertainty evaluation of the exposure assessment

Uncertainties associated with exposure assessments need to be considered when interpreting the results. Factors intrinsic to fish consumption surveys such as under/over reporting of consumption data, misreporting of consumed fish and the erroneous estimation of consumed quantities (based on respondent's perception) could contribute to both under and over estimation of fish consumption as well as concentration of contaminants which affects the exposure assessment. Moreover, freezing, proper washing and cooking of fish can alter the concentration of formaldehyde compared to that in fresh marketed fish. The analytically determined formaldehyde concentration in the food can underestimate the actual amount of formaldehyde liberated during the digestion process, which is relevant from a food safety perspective. Further research could elaborate the various aspects that affect the evaporation of formaldehyde. Variations in LOD, LOQ and recovery values for different formaldehyde detection methods might lead to over/under estimation of formaldehyde concentrations which is also supported by the present study results in

Table 1 and Figure 2. Due to the natural occurrence of formaldehyde in fish and high reactivity of added chemicals, reaction with food protein and formation of new adducts could contribute to underestimation of formaldehyde in fish.

4. Conclusion

Under both marketed and laboratory conditions, the HPLC method was more effective in determining formaldehyde concentration than the spectrophotometric method. Differences in formaldehyde concentration between different steps along the fish supply chain were observed by the spectrophotometric method. However the HPLC method had higher detection levels and yielded similar formaldehyde concentration estimates in tilapia from different steps along the fish supply chain. Increased trends in formaldehyde concentration were observed in fish treated under laboratory conditions with increasing concentrations of dipping solution and dipping time, irrespective of species. Natural occurrence of formaldehyde in fish and different reactions between highly reactive formaldehyde and fish protein amino acid residues might result in different magnitudes in determined formaldehyde concentration in fish varying by species, site, concentration of dipping solution and dipping time.

Formaldehyde exposure from the consumption of Indian major carp rui, minor carp, Chinese carp and tilapia fish was assessed by probabilistic exposure assessment using fish consumption and formaldehyde concentrations respectively analyzed under two different methods. Food frequency questionnaire results indicate higher consumption of Indian major carp rui and tilapia than Chinese carp and minor carp. Estimates of exposure to formaldehyde from consumption of four different fish were lower with spectrophotometric analysis than with HPLC analysis for both total population and consumer only. Maximum exposure of formaldehyde (0.28 mg/kg BW.d) was estimated for tilapia (consumers only) under HPLC method which might cause health concerns.

MoE provides high priority (<10,000) of risk from exposure for tilapia and Indian major carp rui at P99 under spectrophotometric analysis for both total population and consumer only. Under HPLC analysis, tilapia had much lower MoE values for both total population and consumers only at higher to lower percentiles (P99, P95 and P90) indicating risk priorities. Exposure to formaldehyde with tilapia fish consumption is a possible health concern for the population in the Southern district of Bangladesh. Therefore, priority should be given to formulating a proper risk management strategy on the basis of knowledge of endogenous formaldehyde present in fish. The MoE results from this study could be used to compare the risk of formaldehyde intake from consumption of different species of fish, between the methods used for formaldehyde determination, and to prioritize risk management strategies for the fish consumer in Bangladesh.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

The authors are very grateful to the International Foundation for Science (IFS), Sweden for the research grant (research grant agreement number E/5670-1). The authors also would like to acknowledge ITP Food Safety program, Department of Food Safety and Food Quality, Faculty of Bioscience, Ghent University, Belgium and the Flemish Interuniversity Council (VLIR-UOS), Belgium for scientific and research stay support. The laboratory analytical support from National Food Safety Laboratory (NFSL), Dhaka, Bangladesh is also highly acknowledged. We acknowledge Dr. Martin L. van Brakel, Scientist, ECOFISH-Bangladesh project, WorldFish South Asia, Dhaka, Bangladesh for his additional comments on an earlier version of our manuscript.

References

Aminah, S. A., Zailina, H. and Fatimah, A. B. (2013). Health risk assessment of adults consuming commercial fish contaminated with formaldehyde. Food Pub. Heal. 3(1), 52-58.

Belton, B., Karim, M., Thilsted, S., Murshed-E-Jahan, K., Collis, W and Phillips, M. (2011). Review of aquaculture and fish consumption in Bangladesh. Studies and Reviews 2011. The WorldFish Center (Dhaka), Bangladesh. pp 53.

Benjakul, S., Visessanguan, W. and Tueksuban, J. (2003). Changes in physico-chemical properties and gel-forming ability of lizard fish (*Saurida tumbil*) during post-mortem storage in ice. Food Chem. 80, 535–544.

Bianchi, F., Careri, M., Musci, M. and Mangia, A. (2007). Fish and food safety, Determination of formaldehyde in 12 fish species by SPME extraction and GC-MS analysis. Food Chem. 100, 1049-1053.

Chiou, J., Leung, A. H. H., Lee, H. W. and Wong, W. (2015). Food Safety Special Issue: Rapid testing methods for food contaminants and toxicants. J. Integr. Agri. Doi：10.1016/S2095-3119(15)61119-4.

Claeys, W., Vleminckx, C., Dubois, A., Huyghebaert, A., Höfte, M., Daenens, P. and Schiffers, B. (2009). Formaldehyde in cultivated mushrooms: a negligible risk for the consumer. Food Addit. Contam. 26 (9), 1265–1272.

DoF, (2015). National fish week compendium. Department of Fisheries, Ministry of Fisheries and Livestock, Government of the People's Republic of Bangladesh, pp. 1-144.

EFSA, (2005). European Food Safety Authority opinion from the scientific committee on request from EFSA related to a harmonized approach of risk assessment of a substances which are both genotoxic and carcinogenic. EFSA J. 282, pp 1-31.

572 Hoque, M. S., Jacxsens, L., Meulenaer, B. D. and Nowsad, A. A. K.M. (2016). Quantitative risk
573 assessment for formalin treatment in fish preservation: food safety concern in local market
574 of Bangladesh. Procedia Food Sci. 6, 151-158.
575 IARC (International Agency for Research on Cancer) (2004). Formaldehyde 2-butoxyethanol and
576 1-tert-butoxypropan-2ol. Monographs on the evaluation of carcinogenic risk to humans.
577 Year book 88, IARC, Lyon, France.
578 Islam, R., Mahmud, S., Aziz, A., Sarker, A. and Nasreen, M. (2015). A comparative study of
579 present status of marketing of formalin treated fishes in six districts of Bangladesh. Food
580 Nutri. Sci. 6, 124-134.
581 Jaman, N., Hoque, M. S., Chakraborty, S. C., Hoq, M. E. and Seal, H. P. (2015). Determination of
582 formaldehyde content by spectrophotometric method in some fresh water and marine fishes
583 of Bangladesh. Intl. J. Fish. Aqua. Stud. 2(6), 94-98.
584 JECFA, (1998). Safety evaluation of certain food additives and contaminants, WHO Food
585 Additives Series 40, Aflatoxins. In: The Forty-Ninth Meeting of the Joint FAO/WHO
586 Expert Committee on Food Additives. World Health Organization, Geneva, pp. 359–469.
587 Liu, J., Chan, K. K. J. and Chan, W. (2016). Identification of protein thiazolidination as a novel
588 molecular signature for oxidative stress and formaldehyde exposure. Chem. Res. Toxicol.
589 29, 1865−1871.
590 Mason, D. J., Sykes, M. D., Panton, S. W. and Rippon, E. H. (2004). Determination of naturally-
591 occurring formaldehyde in raw and cooked Shiitake mushrooms by spectrophotometry and
592 liquid chromatographyemass spectrometry. Food Addit. Contam. 21, 1071-82.
593 Metz, B., Kersten, G. F.A., Baart, G. J. E., Jong, A.D., Meiring, H., Hove, J. T., Steenbergen, M.
594 J. V., Hennink, W.E., Crommelin, D. J. A. andJiskoot, W. (2006). Identification of

595 formaldehyde-induced modifications in proteins: reactions with insulin. Bioconjugate
596 Chem. 17, 815-822.
597 Mikael, A., Petersen, M. A., Alam, M. A., Rahman, M. M., Ali, M. L., Mahmud, S., Schlüter, L.
598 and Jørgensen, N.O. G. (2014). Geosmin off-flavour in pond-raised fish in southern
599 Bangladesh and occurrence of potential off-flavour producing organisms. Aqua. Env.
600 Interac. 5, 107–116.
601 Monakhova, Y. B., Jendral, J. A. and Lachenmeier, D. W. (2012). The margin of exposure to
602 formaldehyde in alcoholic beverages. Arh. Hig. Rada. Toksikol. 63, 227-237.
603 Nash, T. (1953). The chlorometric estimation of formaldehyde by means of the Hantzschreaction.
604 Biochem. J. 55, 416-421.
605 Naya, M. and Nakanishi, J. (2005). Risk assessment of formaldehyde for the general population in
606 Japan. Regul. Toxicol. Pharmacol. 43, 232–248.
607 Nielsen, M. K. and JØrgensen, B. M. (2004). Quantitative relationship between trimethylamine
608 oxide aldolase activity and formaldehyde accumulation in white muscle from Gadiform
609 fish during frozen Storage. J. Agric. Food Chem.52, 3814-3822.
610 Noordiana, N., Fatimah A. B. and Farhana, Y. C. B. (2011). Formaldehyde content and quality
611 characteristics of selected fish and seafood from wet markets. Int'l Food Res. J. 18, 125-
612 136.
613 Paul, L., Mondal, D. K., Paul, M., Riar, M. G. S. and Ali, A. (2014). Intensity of formalin misuse
614 for fish preservation in five markets of Jessore district, Bangladesh. Int'l J. Nat. Soci.
615 Sci. 1, 77-81.

Rahman, M. M., Ahmed, S., Hosen, M. and Talukder, A. K. (2012). Detection of formalin and quality characteristics of selected fish from wet markets at Sylhet city in Bangladesh. Bangla. Res. Publi. J. 7 (2), 161-169.

Rahman, S., Majumder, M. A. A., Ahasan, R., Ahmed, S. M. M., Das, P. and Rahman, N. (2016). The extent and magnitude of formalin adulteration in fish sold in domestic markets of Bangladesh: a literature review. Int'l J. Cons. Stud. 40, 152–157.

Sikorski, Z. (1982). Protein changes in frozen fish. CRC Crit' Rev' in Food Nutr. 8(1), 97.

Stanley, D.W. and Hultin, H.O. (1984). Amine and formaldehyde production in North American squid and their relation to quality Can. Inst. Food Sci. Technol. J. 17(3), 157-162.

Steel, R. G. D. and Torrie, J. H. (1980). Principles and procedures of statistics: A biometrical approach. New York: McGraw-Hill.

Tang, X., Bai, Y., Duong, A., Smith, M. T., Li, L. and Zhang, L. (2009). Formaldehyde in China: Production, consumption, exposure levels, and health effects. Env. Intl. 35, 1210-1224.

Vandemoortele, A. and Meulenaer, B. D. (2015). Behavior of malondialdehyde in oil-in-water emulsions. J. Agric. Food Chem. DOI: 10.1021/acs.jafc.5b01780.

Vandemoortele, A., Babat, P., Yakubu, M. and Meulenaer, B. D. (2017). Reactivity of free malondialdehyde during *In-Vitro* simulated gastrointestinal digestion. J. Agric. Food Chem.; 65, 2198−2204. DOI:10.1021/acs.jafc.7b00053.

Wahed, P., Razzaq, M. A., Dharmapuri, S. and Corrales, M. (2016). Determination of formaldehyde in food and feed by an in-house validated HPLC method. Food Chem. 202, 476–483.

637 Yeasmin, T., Reza, M. S., Khan, M. N. A., Shikha, F. H. and Kamal, M. (2010). Present status
638 of marketing of formalin treated fishes in domestic markets at Mymensingh district
639 in Bangladesh. Intl. J. Biol. Res. 1(4), 21-24.

640 Yeasmin, T., Reza, M. S., Shikha, F. H., Khan, M. N. A. and Kamal, M. (2013). Effect of washing
641 and chilled storage on the retention of formalin and quality of rohu fish (*Labeo rohita,*
642 Hamilton). Discourse J. Agric. Food Sci. 1(6), 118-122.

643 Yeh, T. S., Lin, T. C., Chen, C. C. and Wen, H. M. (2013). Analysis of free and bound
644 formaldehyde in squid and squid products by gas chromatography mass spectrometry. J.
645 Food Drug Anal. 21, 190-197.

646 Zhang, X., Hui, Y., Cai, Y., Huang, D. (2015). The research progress of endogenous formaldehyde
647 in aquatic products. World J. Eng. Tech. 3, 272-276.
648
649
650
651
652
653
654
655
656
657
658
659

Table 1: Formaldehyde concentration of different fish obtained from different steps of fish supply chain measured by using spectrophotometric and HPLC method

Method	Fish species	Formaldehyde concentration (mg/kg)			
		Fish farm	Fish landing center	Wholesale market	Retail Market
Spectropho-tometric	Indian major carp rui	0.77 ± 0.03^{cC}	1.03 ± 0.03^{bB}	1.03 ± 0.03^{bC}	1.68 ± 0.06^{aA}
	Minor carp	0.43 ± 0.03^{dD}	0.55 ± 0.00^{cD}	0.67 ± 0.03^{bD}	0.93 ± 0.03^{aD}
	Chinese carp	1.50 ± 0.00^{aA}	0.82 ± 0.03^{dC}	1.35 ± 0.00^{bB}	1.20 ± 0.05^{cC}
	*Tilapia	$^{B}1.08\pm0.03^{dB}$	$^{B}2.08\pm0.05^{aA}$	$^{B}1.83\pm0.03^{bA}$	$^{B}1.43\pm0.06^{cB}$
HPLC	*Tilapia	$^{A}6.62\pm0.84$a	$^{A}6.92\pm0.66$a	$^{A}6.44\pm0.65$a	$^{A}5.60\pm0.53$a

Means ± standard deviation (n=3); Different small alphabet in the same row represent significant difference ($p<0.05$) in formaldehyde contents in same species from different steps of supply chain; and different capital alphabet in the same column represent significant difference ($p<0.05$) in formaldehyde contents in different fish species from same steps of supply chain.

(*) indicates the comparison between the methods and different capital alphabet in the right side of value indicates significant difference ($p<0.05$).

682 Table 2: Best fit distributions and descriptive statistics (min, mean, median and max) for different
683 fish of formaldehyde concentrations (mg/kg) and consumption of fish (kg/kg BW.d) under
684 both analytical methods applied for the probabilistic exposure assessment.

Inputs of exposure assessment	Best fit distributionfunction	Min.	Mean	Median	Max.
Concentration of formaldehyde (mg/kg)					
Indian major carp rui	=RiskBetaGeneral(0.18516,0.26237,0.75000,1.7500,RiskName("FA Concentration in Indian major carp rui fish (mg/kg)"))	0.75	1.16	1.01	1.75
Minor carp	=RiskLogistic(0.61200,0.20802,RiskName("FA Concentration in Minor carp (mg/kg)"))	- ∞	0.61	0.62	+ ∞
Chinesecarp	=RiskExtValueMin(1.3351,0.19389,RiskName("FA Concentration in Chinese carp fish (mg/kg)"))	- ∞	1.22	1.26	+∞
Tilapia	=RiskExtValueMin(1.7954,0.32482,RiskName("FA Concentration in Tilapia fish (mg/kg)_Spectro"))	- ∞	1.61	1.68	+ ∞
Tilapia_HPLC	=RiskLogistic(6.38689,0.44836,RiskName("FA concentration in Tilapia fish (mg/kg)_HPLC"))	- ∞	6.39	6.39	+ ∞
Consumption of different fish species (kg/kg BW.d) by consumer					
Indian major carp rui	=RiskInvGauss(0.00043263,0.00023271,RiskShift(-1.53920e-005),RiskName("Consumption of Indian major carp rui (kg/kg BW.d)_Consumer"))	-1.54 E-005	0.00042	0.00022	+∞
Minor carp	=RiskLognorm(0.00015215,0.00019558,RiskShift(0.000000163637),RiskName("Consumption of Minor carp (kg/kg BW.d)_Consumer"))	1.64 E-006	0.00015	0.00010	+∞
Chinesecarp	=RiskGamma(0.95289,0.00026658,RiskShift(1.15741e-005),RiskName("Consumption of Chinese carp (kg/kg BW.d) _Consumer"))	1.16 E-005	0.00027	0.00018	+∞
Tilapia	=RiskLognorm(0.00044963,0.00077965,RiskShift(5.26553e-006),RiskName("Consumption of Tilapia (kg/kg BW.d)_Consumer"))	5.27 E-006	0.00044	0.00023	+∞
Consumption of different fish species (kg/kg BW.d) by total population					
Indian major carp rui	=IF(RAND()>fraction of non-consumer,RiskInvgauss(0.00043263,0.00023271,RiskShift(-0.000015392),RiskName("Consumption of Indian major carp rui (kg/kg BW.d)_Consumer")),0)	0.00	0.00037	0.00017	+ ∞
Minor carp	=IF(RAND()>fraction of non-consumer,RiskInvgauss(0.00043263,0.00023271,RiskShift(-0.000015392),RiskName("Consumption of Minor carp (kg/kg BW.d)_Consumer")),0)	0.00	0.00011	0.00005	- ∞
Chinesecarp	=IF(RAND()>fraction of non-consumer,RiskGamma(0.95289,0.00026658,RiskShift(0.0000115741),RiskName("Consumption of Chinese carp (kg/kg BW.d) _Consumer")),0)	0.00	0.00012	0.00	+ ∞
Tilapia	=IF(RAND()>fraction of non-consumer,RiskLognorm(0.00044963,0.00077965,RiskShift(0.00000526553),RiskName("Consumption of Tilapia (kg/kg BW.day)_Consumer")),0)	0.00	0.00031	0.00012	+ ∞

Table 3: Probabilistic dietary exposures (mg/kg BW.d) associated with consumption of different fish contaminated with formaldehyde determined by using spectrophotometric and HPLC methods.

Descriptive Level	Exposure due to formaldehyde contaminated fish consumption									
	Spectrophotometric								HPLC	
	Indian major carp rui		Minor carp		Chinese Carp		Tilapia		Tilapia	
	TP	Cons.	TP	Cons.	TP	Cons.	TP	Cons.	TP	Cons.
Min.	0	0	0	0	0	0	0	0	0	3.92E-05
Mean	2.41E-04	4.87E-04	4.61E-05	9.24E-05	1.64E-04	3.25E-04	3.64E-04	7.30E-04	1.45E-03	2.91E-03
SD	5.79E-04	7.54E-04	1.04E-04	1.30E-04	2.88E-04	3.33E-04	9.78E-04	1.28E-03	3.70E-03	5.08E-03
P_{50}	5.77E-06	2.36E-04	0	5.40E-05	0	2.20E-04	0	3.57E-04	9.03E-05	1.46E-03
P_{75}	2.34E-04	5.47E-04	5.39E-05	1.10E-04	2.20E-04	4.40E-04	3.60E-04	8.00E-04	1.46E-03	3.19E-03
P_{90}	6.65E-04	1.17E-03	1.30E-04	2.05E-04	5.16E-04	7.51E-04	9.70E-04	1.66E-03	3.86E-03	6.54E-03
P_{95}	1.15E-03	1.80E-03	2.04E-04	2.96E-04	7.58E-04	9.83E-04	1.65E-03	2.55E-03	6.50E-03	1.01E-02
P_{99}	2.79E-03	3.67E-03	4.49E-04	6.07E-04	1.33E-03	1.57E-03	4.14E-03	5.84E-03	1.63E-02	2.23E-02
Max.	1.73E-02	1.73E-02	2.93E-03	3.12E-03	3.56E-03	4.20E-03	4.73E-02	4.73E-02	0.12	0.28

'TP' and 'Cons.' Refer to Total Population and Consumers, respectively.

Table 4: Margin of exposures associated with consumption of different fish contaminated with formaldehyde determined by using spectrophotometric and HPLC methods.

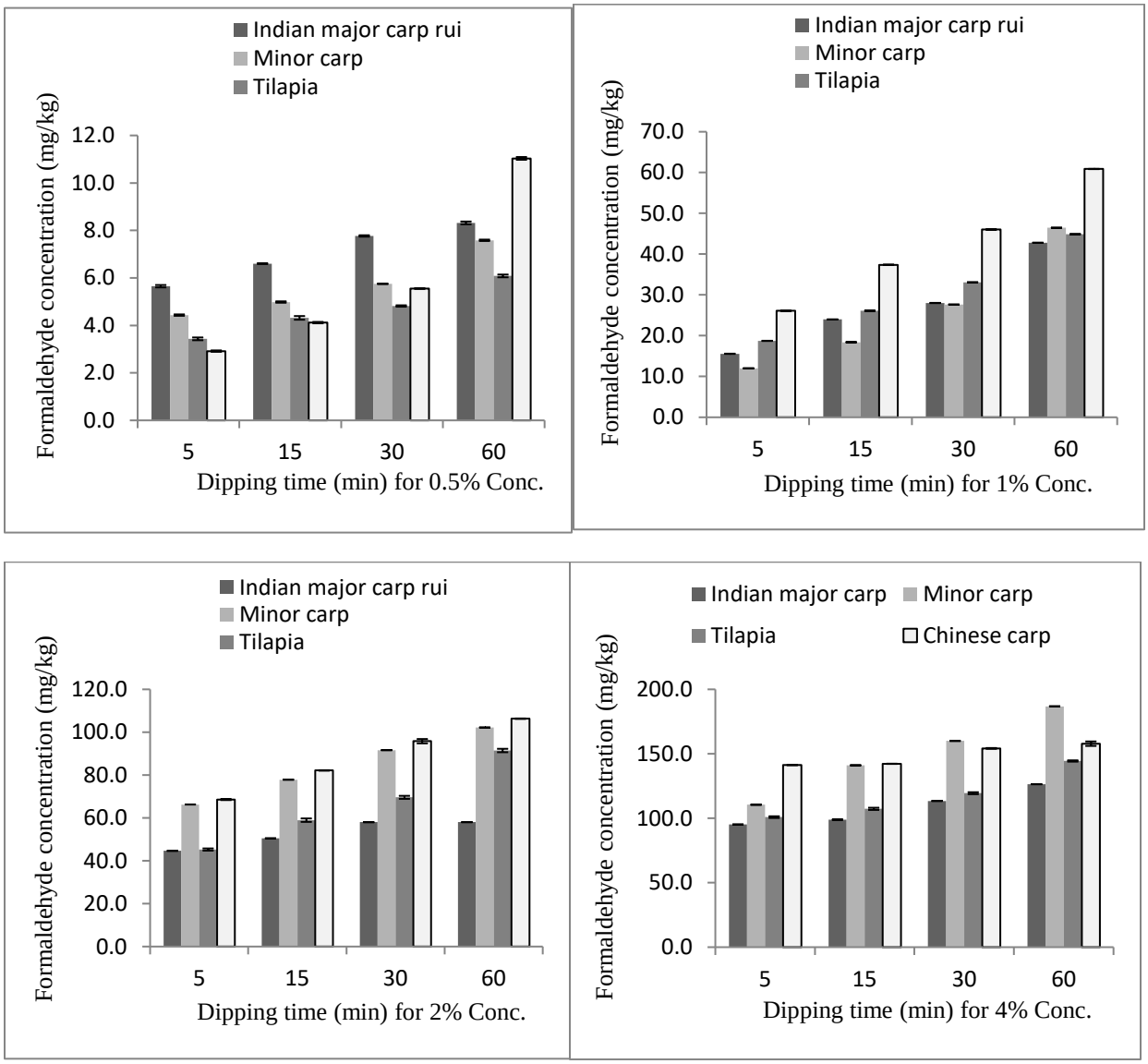
Different Percentile	Margin of Exposure									
	Spectrophotometric						HPLC			
	Indian major carp rui		Minor carp		Chinese Carp		Tilapia		Tilapia	
	TP	Cons.	TP	Cons.	TP	Cons.	TP	Cons.	TP	Cons.
P_{50}	3.99E+06	9.76E+04	N/A	4.26E+05	N/A	1.05E+05	N/A	6.45E+04	2.55E+05	1.57E+04
P_{75}	9.85E+04	4.20E+04	4.27E+05	2.10E+05	1.05E+05	5.23E+04	6.39E+04	2.87E+04	1.57E+04	**7.20E+03**
P_{90}	3.46E+04	1.97E+04	1.77E+05	1.12E+05	4.45E+04	3.06E+04	2.37E+04	1.38E+04	**5.96E+03**	**3.52E+03**
P_{95}	2.00E+04	1.28E+04	1.13E+05	7.77E+04	3.03E+04	2.34E+04	1.39E+04	**9.03E+03**	**3.54E+03**	**2.28E+03**
P_{99}	**8.24E+03**	**6.27E+03**	5.13E+04	3.79E+04	1.73E+04	1.47E+04	**5.56E+03**	**3.94E+03**	**1.41E+03**	**1.03E+03**

Values exceeding the MoE with high priority (MoE<10,000) are shown in bold.

'TP' and 'Cons.' Refer to Total Population and Consumers only, respectively.

'N/A' referred to Not Available

724



725

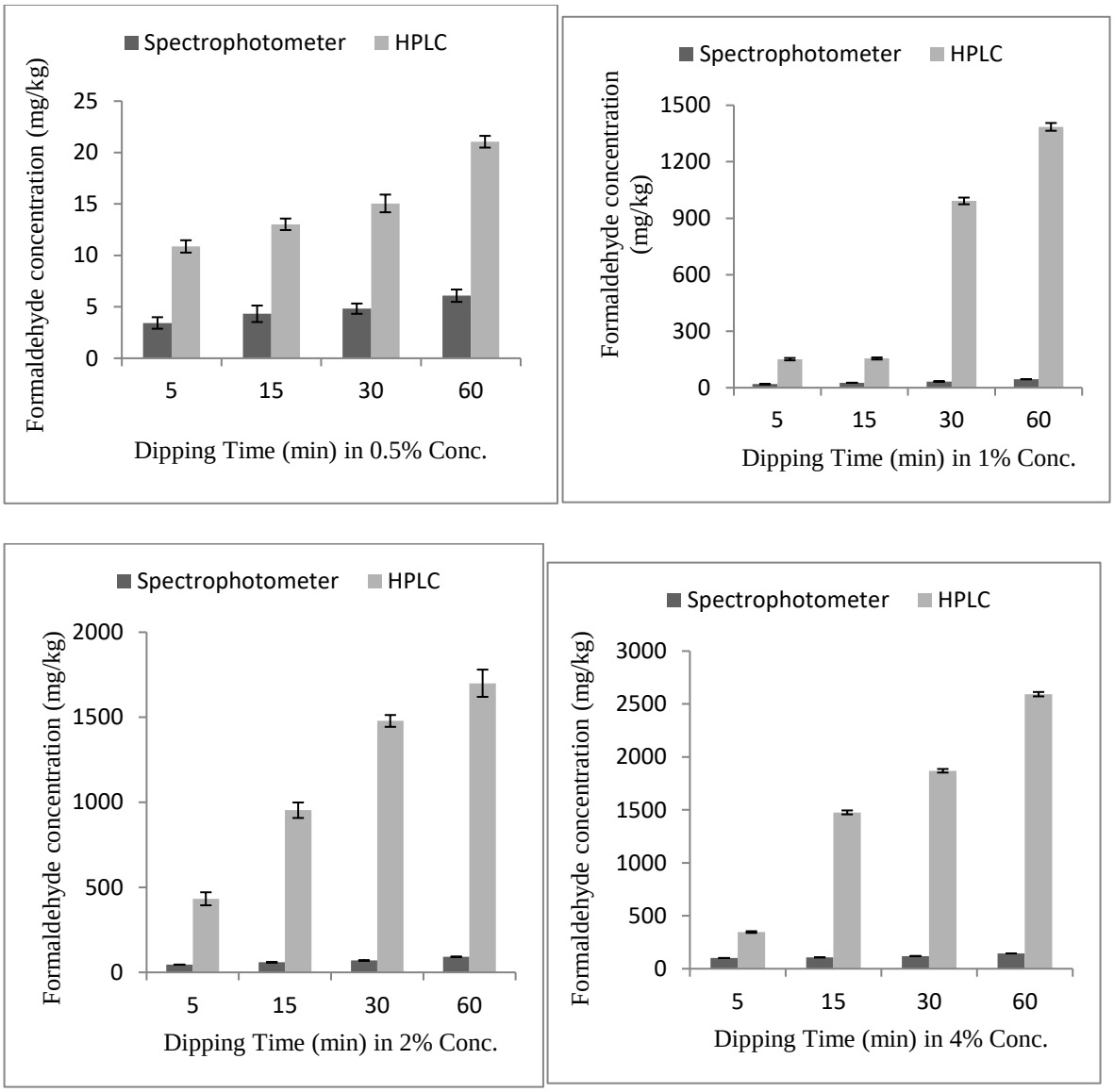
726

727 Figure 1:

728

729

730



731

732 Figure 2:

733

734

735

736

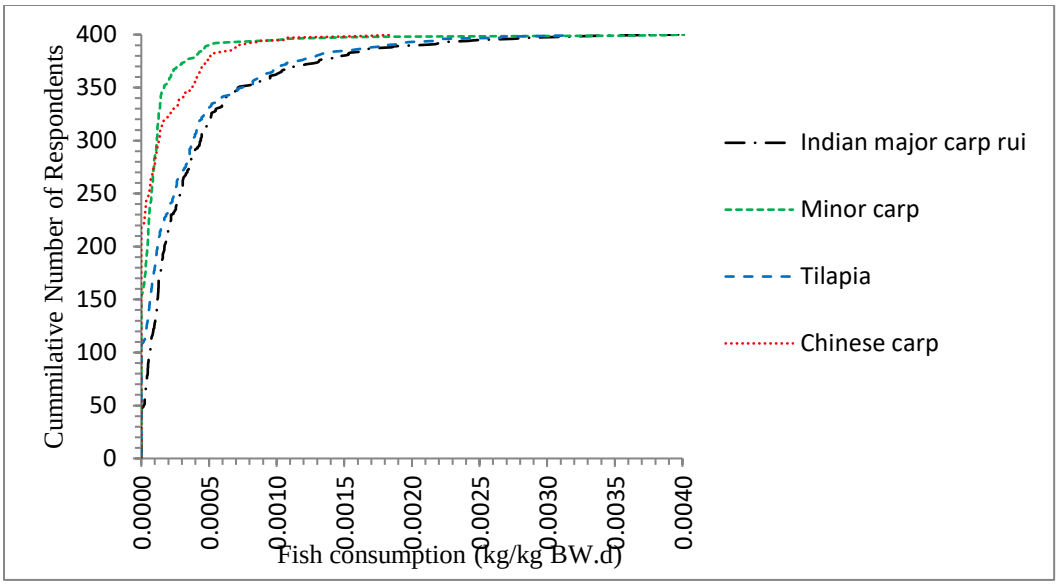


Figure 3:

Figure Captions:

Figure 1: Formaldehyde concentration (mg/kg) in different fish treated with different concentration of formaldehyde and dipping time at laboratory condition measured by using spectrophotometric method. Means ± standard deviation (n=3).

Figure 2: Formaldehyde concentration (mg/kg) in tilapia fish treated with different concentration of formaldehyde at different dipping time at laboratory condition measured by using spectrophotometric and HPLC method. Means ± standard deviation (n=3).

Figure 3: Cumulative consumption (kg/kg BW.d) of different fish by total respondents (non-consumer and consumer).

Highlights

- Natural occurrence of formaldehyde in fish is species and source dependent
- Increased concentration and exposure time increases formaldehyde acquisition
- HPLC provides better quantification of formaldehyde in fish than spectrophotometry
- HPLC confirms risk associated with consumption of formaldehyde treated fish
- Formaldehyde exposure through tilapia consumption is a public health concern