Bangladesh J. Zool. **45**(1): 1-10, 2017 ISSN: 0304-9027 (print)
2408-8455 (online)

EFFECTS OF TOXIC *MICROCYSTIS AERUGINOSA* BLOOM ON LIVER OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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Abstract: The effects of toxic *Microcystis aeruginosa* on the liver of Nile tilapia, *Oreochromis niloticus* were investigated in the laboratory. Four treatments were setup into aquariums for 15 days to investigate the histopathological changes exposed to *Microcystis aeruginosa* bloom. Fishes of treatment A_1 were treated as a control group and given artificial feed. In the three treatments (A_2, A_3 and A_4), the *M. aeruginosa* cell concentration were 35×10^2, 72×10^2, 149×10^2 colony/ml, respectively. In the control group, no histopathological change was observed. In exposed fish, histopathological alterations were characterized by swollen and granular cytoplasm, vascular proliferation, bile stasis, fatty change and focal necrosis. Histopathological changes were observed within five days of exposure when fish exposed to moderate (72×10^2 colony/ml) to high (149×10^2 colony/ml) bloom concentration, whereas it took 15 days in lower concentration (35×10^2 colony/ml). Histopathological changes proved that intake of toxic *M. aeruginosa* bloom by aquatic animals, particularly Nile tilapia has significant effects on its internal organs that may cause of massive mortality. Accordingly, cyanotoxin accumulation in fish tissue my pose a risk to human health through the food chain.

Key words: Microcystins, Histopathological effects, *Microcystis aeruginosa* bloom, Liver, *Oreochromis niloticus*

INTRODUCTION

Tilapia, *Oreochromis niloticu*s is a commercial aquaculture species in Bangladesh contributing nearly 11.28% of total inland fish production (FRSS 2016). Culture of Tilapia in eutrophic pond is a common practice in Bangladesh. This paper deals with the histopathological effects of toxic *M. aeruginosa* bloom on *O. niloticus*.

Cyanobacterial toxins (Hepatotoxins) are the most frequently found toxins in fresh and brackish waters all over the world (WHO 1999). Microcystins (MCs), the cyclic heptopeptide, are produced by different cyanobacterial genera such as *Microcystis*, *Planktothrix*, *Nostoc, Anabaena*, *Oscillatoria*, *Anabaenopsis* and *Hapalosiphon* (Carmicheal and Li 2006). Among 80 different variants of these microcystins, microcystin-LR is the most common and toxic form which occurs more often in cyanobacterial blooms (Dawson 1998). Exposure to cyanotoxins

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 DOI: http://dx.doi.org/10.3329/bjz.v45i1.34189

represents a health risk to aquatic organisms, wildlife, domestic animals and humans through drinking, ingestion or contact with either cyanobacteria or toxins from the water (Dietrich and Hoeger 2005). The toxicity of microcystins is due to inhibition of the catalytic subunits of protein phosphates 1 and 2A (PP1, PP2A). Inhibition of PP1 and PP2A dephosphorylate phosphoseryl or phosphothreonyl proteins lead to hyper phosphorylation of cytoskeletal proteins resulting deformation of hepatocytes (Runnegar *et al.* 1981). Additionally, tumor promotion and liver injury caused by oral consumption of microcystins and poses serious health risk (Falconer 1991). Indirect evidence of possible promotion of primary liver cancer, associated with the contamination of surface drinking water supplies by MCs producing cyanobacteria have been reported by Ueno *et al.* (1996). Moreover, accumulations of MCs in aquatic animals have been reported by many workers (Mohamed *et al.* 2003, Xie *et al.* 2005, Deblois *et al.* 2008, Lei *et al.* 2008). Fish consumption is the potential route of human exposure to microcystins, as they stand at the top of the aquatic food chain which may create high risk to human health. Fish histopathology is widely used as a biomarker to evaluate water quality and potential hazard. Different laboratory studies have shown the effects of microcystins in different organ of fishes by histopathology (Fisher *et al.* 2000, Fisher and Dietrich 2000, Gupta and Guha 2006, Atencio *et al.* 2008, Ferrira *et al.* 2010). Fish liver is an excellent organ to study the environmental quality biomarkers, due to its role in the specimen's metabolism, which include the production of proteins, the oxidation, conjugation, methylation, inactivation or detoxication of substances, or rather the excretion of pollutants (Brusle and Anadon 1996). Histopathological change has been observed in liver of tilapia (*Oreochromis* sp.) exposed to a single intraperitoneal (i.p.) injection of the pure standards (MC-LR and MC-RR) at a dose of 500 µg/kg and changes are megolocystsis, necrotic process, and micro vesicular steatosis in liver (Atencio *et al.* 2008). Hepatic tumor and strong hepatic hemorrhages have also been reported (Tencalla *et al.* 1994, Fisher *et al.* 2000).

Most studies have done by intraperitoneal injection (extracted microcystins) or oral exposure (freeze-dried cyanobacterial cell). However, IP injections of microcystins are not analogous to field exposures since the toxin is absorbed faster and metabolized differently when administrated into the abdominal cavity (as with the IP route) as compared to oral administration (Ibeling *et al.* 2007). An intraperitonial injection dose of 550 µg MC showed mortality in rainbow carp whereas a same oral dose showed no mortality and minor pathological change has been identified (Tencalla *et al.* 1994). Lower and higher severity has been observed in histopathological changes with application of 300 and 500 µg/kg IP

MC-LR while 400 µg/kg oral dose of MC-LR resulted in the same change (Fisher and Dietrich 2000). Not only exposure route, susceptibility of fish to the toxins is also different. In comparison to the pathological events in salmonids exposure to MCs, where a slower development of pathology and primarily necrosis cell death prevails, and the pathology in carp rapidly develops in lower toxins (Fisher *et al.* 2000). Therefore, specific toxicity thresholds and effects are different in fishes.

MCs may find their way into human body through various aquatic food chain including finfish and shellfish. However, the extend of hazard caused by the consumption of fish and shellfish has not yet been assessed in Bangladesh. The potential health hazard through consumption of contaminated fish (aquatic food) should be seriously taken since cooking of such food does not deactivate the said toxins (Zhang *et al.* 2010). So, this experiment was conducted to know the toxic effects of *M. aeruginosa* bloom on the liver of Nile tilapia *O. niloticus*.

MATERIAL AND METHODS

Collection of fish and cyanobacteria bloom: Tilapia, *O. niloticus* was collected from Babul Fish Farm, located at Chittagong Road, Dhaka. The average weight of fish was 10.23 ± 1 g (mean ± Sd). Unialgal bloom of *Microcystis aeruginosa* was collected from Nazira Bazar Pond, Old Dhaka city (23º43'26''N and 90º24'24''E).

Experimental design: Four aquarium of 100 litre each designated as A_1, A_2, A_3, A_4 were setup for the experiments. Fish were acclimated for 7 days prior to experiment. Aquariums were containing dechlorinated tap water, temperature was maintained at 24 ± 1ºC, pH 7.8 ± 2 and dissolved oxygen was 7.5 mg/l, photoperiod of 12 hrs and continuous aeration was given with submerged pumps. Fish were feed with artificial commercial fish feed during acclimatization period.

Extraction and determination of MCs: M. aeruginosa bloom was initiated in February and the highest cell density (95%) was observed in June, 2015. The bloom sample was collected with plankton net (no. 55 bolting silk plankton net). The concentrated samples were filtered through a 0.45 µm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60 - 70ºC. The GF/C filters were extracted with 2.0 ml water/methanol (50 : 50; v/v) per filter by ice-cooled sonication for 4 min with an ultrasonic probe GM 70 (Bandelin, Berlin, Germany). The extract was sonicated for another 15 min in an ultrasonic bath. Extracts were centrifuged (10000 g, 15 min) and the supernatants were filtered using 0.22 µm nylon syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to the high performance liquid chromatography (HPLC).

The HPLC/uv determination of microcystins was carried out following the method of Lawton *et al.* (1994) with some modifications. Separation was performed using a C18 column (Phenomenex prodigy, ODS (3), 250 × 4.6 mm, 5 µm) and acetonitrile/water/0.05% TFA) as the mobile phase. Microcystins were detected using an UV detector (Shimadzu SPD-10AV; λ = 238 nm). HPLC/MS-MS analyses were applied to confirm the identity of the toxins. HPLC-MS/MS was performed using Shimadzu HPLC LC-20A coupled to an ABSciex 4000 QTrap with an electrospray interface. Analytes were separated on a Phenomenex Gemini 5 µm C18 column (150*3 mm) with a guard column and a mobile phase consisting of 5 mM ammonium acetate in water/acetic acid (99 : 1; v/v) (eluent A) and 5 mM ammonium acetate in methanol/water/acetic acid (97 : 2 : 1; v/v/v) with a flow rate of 0.4 ml/min. Elution started with 60% eluent A and 40% eluent B.

Since reference materials for desmethylated microcystins were not available commercially, determination of the concentrations of desmethyl-MCs, [D-Asp3, Dha7] MC-LR, and [Dha7] MC-LR, was performed using the standard calibration curves of MC-LR. Reference standards of microcystin-RR, -LR, -YR, -LA, LF and –LW were purchased from calbiochem/novabiochem (La Jolla, CA, USA). Acetonitrile and methanol obtained from VWR (Leuven, Belgium) were HPLC grade. All chemicals used were analytical grade.

Exposure of O. niloticus to M. aeruginosa cells: After acclimatization period, fishes were released into aquariums. In the control group (75 litre water) fish were feed with formulated feed during the experiment. Aquaria A_2, A_3 and A_4 tank were stocked with 35 × 10^2, 72 × 10^2 and 149 × 10^2 colony/ml concentration of cells of *M. aeruginosa*, respectively. No artificial feed was given. Aquariums were supplied continuous aeration. Temperature was maintained at 24 ± 1ºC, pH 7.8 ± 2, dissolved oxygen 7.5 mg/l with a photoperiod of 12 hrs darkness. Every alternate day 20% of water was exchanged and added same concentration of *M. aeruginosa* bloom. Total exposure period was 15 days. Five fishes were sacrificed by anesthesia (0.02% clove oil; Hilltech Canada Inc. Vankleak Hill, Ontario, Canada) at five day intervals from four different aquaria. Exposed fish liver samples were taken for histological study.

Histopathology: Liver tissues were fixed washed in physiological saline and preserved in Bouin's fluid for 18 hrs. The samples were dehydrated with ethyl alcohol, cleaning with xylene, impregnated and embedding in paraffin and cut into 3 - 4 µm thick sections by a microtome machine (KD 2258, Kedee, China). Fixed and prepared slides were held overnight. The sections were stained with hematoxylin and eosin (HE), then mounted with PBX and observed under a light microscope (Olympus CX41 co-observation microscope; Humansan 1997).

RESULTS AND DISCUSSION

Microcystis toxins characterization: HPLC analysis of *Microcystis aeruginosa* extract showed six peaks, the retention time of which agreed well with standard MC-RR, MC-YR, MC-LR, MC-LA, MC-WR, dm-MC-RR (Fig. 1). The results of HPLC-MS revealed the identification of six variants of microcystins, according to their corresponding molecular weight MC-LR (at m/z 950.0 $(M+H)^+$), MC-RR (at m/z 519.5 $(M+2H)^{2+}$), MC-YR (at m/z 1045.0 $(M+H)^+$), MC-LA, MC-WR, dm-MC-RR. In *M. aeruginosa* sample, the concentration of MC-RR was the highest (240 µg/g) followed by MC-YR (5.6 µg/g), MC-LR (30 µg/g), MC-LA (12 µg/g), MC-WA (16 µg/g) and dm-MC-RR (15 µg/g; Table 1).

Table1. Characterization and concentration of microcystins in *Microcystis aeruginosa* bloom sample

	Level of microcystin (µg/g)												
Types of micro-cystins	MC-RR	MC-YR	MC-LR	MC-LW	MC-LF	MC-LA	Nodu-larine	MC-WR	dm MC-RR	dm MC-LR	MC-HtyR	MC-LY	Total
Total	240	5.6	30	<0.6	<1.9	12	<0.2	16	15	<1.3	<1.7	<1.3	318.6

Control fishes: The liver of fish comprises a continuous cells mass of large hexagonal hepatic cells (hepatic parenchyma). Hepatic cells are of polygonal shape containing more or less spherical nucleus with a single prominent nucleolus. They are located among sinusoids forming cord like structures known as hepatic cords. Bile canaliculus is centrally located in each cord. There is no clear division of hepatic cells into lobules (Brusle and Anadon 1996, Fig. 2a). No abnormality was observed in the liver cell of controlled fish.

Five days exposure: After five days of exposure in low concentration bloom (35×10^2 colony/ml), cells were normal, no structural change was observed. In moderate concentration of bloom (72×10^2 colony/ml), cells showed changes in the structural organization. Accumulation of bile in the lining of endothelium cells of liver was observed. Accumulation of fat in the cells was also observed. Liver of fish exposed in higher concentration of bloom (149×10^2 colony/ml) was swollen and cytoplasm granular. Vascular proliferation developed with the comparison of control fish. Occasional bile stasis and mild focal necrosis were found.

Ten days exposure: After ten days of exposure, more advanced tissue abnormalities were detected. Bile stasis, fatty change, vascular proliferation showed in larger area of fish liver exposed in moderate (72×10^2 colony/ml; Fig.

2c) and high concentration (149 × 10^2 colony / ml) of bloom. Cell transition was normal in low concentration (35 × 10^2 colony / ml).

Fifteen days exposure: Tissue damage was highest in fish when exposed over 15-day duration in different concentrations of bloom. Moderate vascular proliferation and cellular bile stasis was developed in the intra sinusoidal space of liver exposed in lower concentration of bloom (35 × 10^2 colony / ml). Bile stasis in endothelial cells, fatty liver was observed in fish exposed in moderate bloom concentration (72 × 10^2 colony / ml). In the higher bloom concentration (149 × 10^2 colony / ml) hepatocytes were markedly swollen with granular appearance of the cytoplasm (Fig. 2b). Highest vascular proliferation was observed with huge bile accumulation (Fig. 2e). Cells showed minimal hepatic necrosis (Fig. 2f).

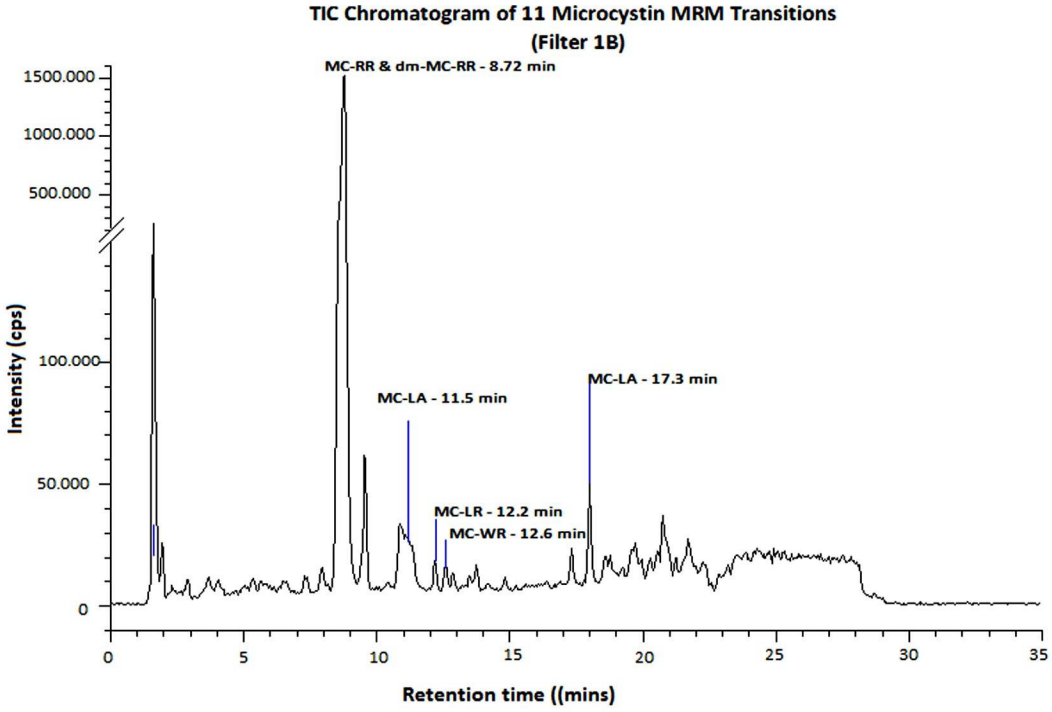

Fig. 1. TIC chromatogram of microcystins MRM transition (bloom filter).

The cells have been found swollen and cytoplasm was cloudy and granular in this study. At the dose of 1000 µg MC-LR / kg, hepatocytes have been found with condensed cytoplasm and lost their granular appearance with chromatin clumping and condensation (Li and Xie 2009). Similar observation was reported by Gupta and Guha (2006) that histopathological changes like hepatocytes swelling, dissociation of hepatocytes in liver of *Heteropneustes fossilis* (0.1 ml MCs extracted from natural bloom: 24 hrs after treatment).

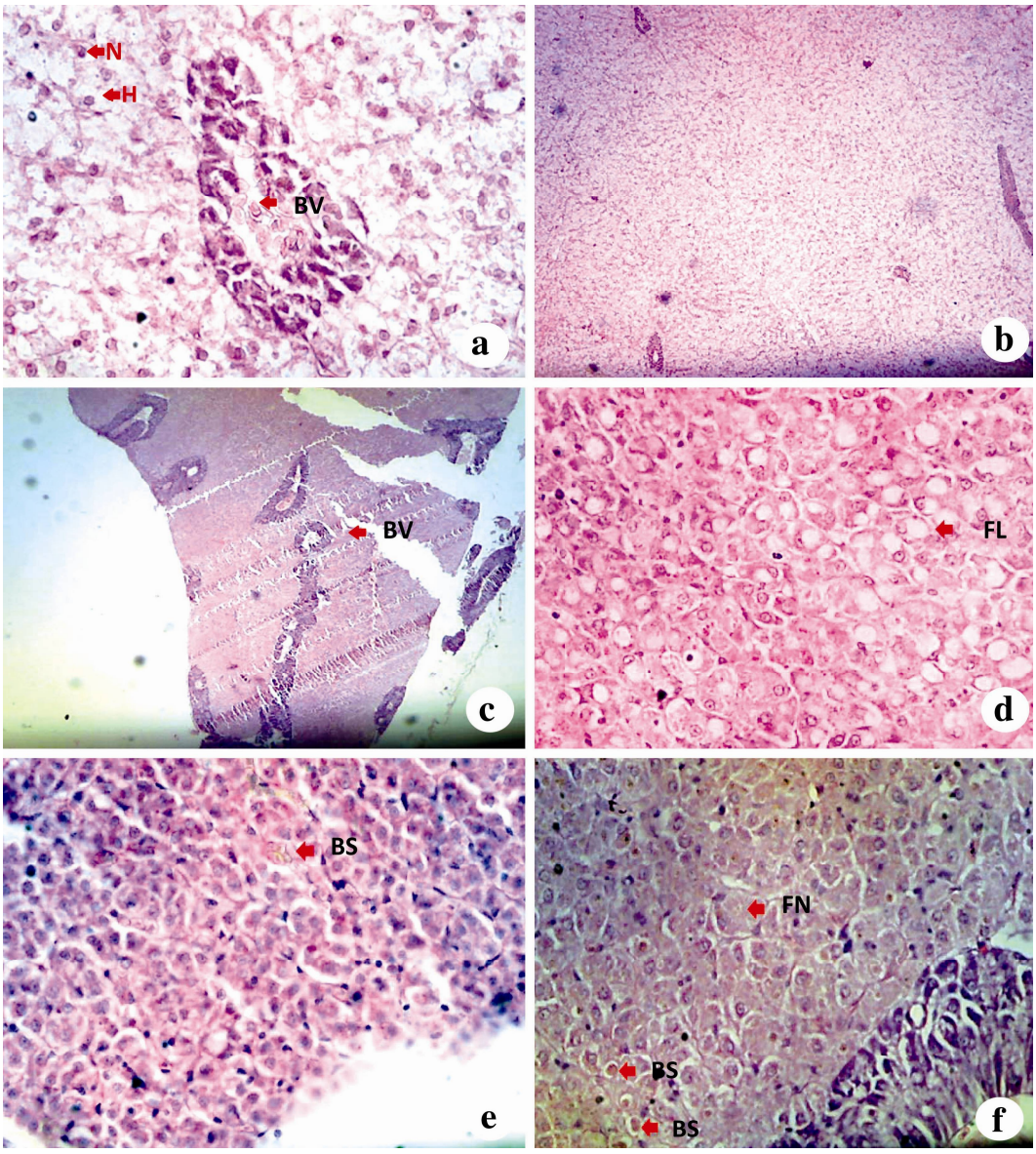

Fig. 2. Section of liver of *Oreochromis niloticus*. a. control group showing normal nucleus and hypatocytes. H, hepatocytes; N, nucleus; BV, blood vessel; 10×45 (H & E stain). b. Experimented group (149 × 10^2 colony/ml: 5 days after treatment) showing condense cytoplasm and swelling in hepatocytes; 10×10 (H&E stain). c. Experimented group (149 × 10^2 colony/ml: 15 days after treatment) showing vascular proliferation. BV, Blood vessels; 10×10 (H & E stain). d. Experimented group (72 × 10^2 colony/ml: 10 days after treatment) showing fatty liver; 10×45 (H & E stain) e. Experimented group (149 × 10^2 colony/ml: 15 days after treatment) showing accumulation of bile in the endothelial cell; 10×45 (H & E stain) f. Experimented group (149 × 10^2 colony/ml: 15 days after treatment) showing focal necrosis and bile stasis; BS, bile stasis; FN, focal necrosis.

Formation of new blood vessels is known as angiogenesis (Brem 1976). Angiogenesis is essential for tumor growth, invasion and metastatic spread (Stefansson *et al.* 2006). The rapid growth of tumor explants is dependent on the development of new blood vessels (Algire *et al.* 1945) and the growth of malignant tumors depends on the process of angiogenesis (Folkman 1971). There are several angiogenic markers for assessing metestic spread and prognosis in malignant tumor. Vascular proliferation is a meaningful variable in assessing the angiogenic phenotype of endometric carcinoma (Stefansson *et al.* 2006). Vascular proliferation has seen in the liver of treated fish, so it could be predicted that cell showed primary response to develop tumor.

Bile stasis is a condition where bile cannot flow from the liver to the duodenum which was also observed in this study. Under a microscope, the individual hepatocytes will have a brownish-green or yellow-brown granules within the cells representing bile that cannot get out of the cell (Pacheco and Santos 2002). This accumulation of bile indicates possible damage to the hepatic metabolism (Fanta *et al.* 2003).

Fatty degeneration is the excessive accumulation of fat in the cytoplasm and is often accompanied by nuclear atrophy. In mammals, fatty liver (hepatic lipidosis, hepatic steatosis, lipoid liver disease, fatty degeneration of the liver) is the term to describe liver that contain more visible lipid in hepatocytes than one expects to see in that organ (Kelly 1993). Accumulation of fat can result in of either toxic exposure or nutritionally induced. Increased hepatic glycogen has been demonstrated histologically, histochemically and biochemically in *Oryzias latipes* (medaka) and *Poecilia reticulata* (guppy) those were exposed to bis (tri-n-butyltin) oxide (TBTO) and di-n-butyltindichloride (DBTC) (Wester *et al.* 1990). Microvesicular steatosis has also been reported in tilapia exposed in MC-LR (Atencio *et al.* 2008).

Response of the fish liver to toxins is hepatocyte necrosis. The most characteristic reaction to toxicity is an apoptotic type of single cell death (Boorman *et al.* 1997). Focal necrosis has been developed in this study. Pathological change includes necrosis and apoptosis have been observed in liver of rainbow trout, *Oncorhynchus mykiss* induced in microcystin-LR (Fisher *et al.* 2000). In both mammals and fish, microcystins can cause damage to cytoskeletal elements of hepatocytes, possibly via inhibition of protein phosphatases (Tencalla and Dietrich 1997). It is assumed that focal necrosis is the primitive stage of cell destruction followed by severe necrosis, hemorrhagic shock resulting total loss of architecture of organ causing death of animals (Kotak *et al.* 1996). The precise dozes of those cyanotoxins are still an open issue which should be solved in order to prevent possible health risks.

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(Manuscript received on 5 January, 2017; revised on 19 February, 2017)