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Variable Exposure and Responses to Cyanotoxins in Cattle Grazing on Pastures in the Coastal Zone of the Baltic Sea: A field Study

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ABSTRACT: Cyanobacteria blooms are common in Baltic Sea and their intensity have been increased due to anthropogenic eutrophication. In this study we investigated the cyanotoxin levels in the water samples collected from four different locations in the Baltic Sea at three different seasons including summer 2011. Protein phosphatase 2A (PP2A) inhibition assays, Enzyme-linked immunosorbent assays (ELISA) and liquid chromatography-mass spectrometry (LC-MS) were employed to detect cyanotoxin variants. Microcystin-LR equivalents (MCE) were detected in a number of the water samples collected at site A (0.4 to 0.64 µg MCE/ L) and at site B (0.24 to 0.44 µg MCE/ L). Cyanotoxin concentrations, as measured by ELISA, ranged between 0.98 -7.45 µg MCE/L in samples collected at site A and between 0.12 to 0.68 µg MCE/L, in samples collected at site B. By using LC-MS one of the molecules present in the samples from site A was determined to be nodularin (0.213 to 0.524 µg/L) whereas samples from site B did not contain this toxin nor did they contain any of the most toxic microcystin species mentioned. The data obtained show good correlation with the MC concentration changes measured in samples and these concentrations were relatively higher during warmer months. In addition we also investigated the adsorption of toxins from water into the circulation of grazing cattle and the results show no measureable liver damage resulting from cyanotoxin poisoning.

Key words: Cyanotoxins, ELISA, LC-MS, Grazing cattle, Baltic coastal zone

INTRODUCTION

The Baltic Sea (BS) is one of the largest brackish water bodies in the world and exhibits probably the world's largest cyanobacteria blooms (Hansson, 2006).Their intensity and frequency have been increased due to anthropogenic eutrophication (Finni *et al.,* 2001; Kononen, 2001).Obviously, this is one of the most acute problems in the BS (Nehring, 1992; Rosenberg *et al.,* 1990). In the BS the dominant species of cyanobacteria are *Microcystis*, *Anabena*, *Aphanizomenon* and *Nodularia* species which commonly produces several forms of microcystin (MC) and nodularin (NOD).Usually the blooms are intense during warm windless summer periods, (Kononen *et al.,* 1998; Sivonen and Moselio Schaechter, 2009).Thus there is a link between global warming and frequency of water blooms.

As cyanobacteria disintegrate, they release toxins into the surrounding water that can be potentially lethal, since several toxins block intracellular phosphatases in liver and other organs (Puschner *et al.,* 1998).The total toxicity is dependent on the mixture and type of toxin present since toxicity varies with molecular structure. Cattle are poisoned when they drink from water bodies where cyanobacteria have accumulated and have no aversion against BS which in most areas has salinity below 1%. In some cases, affected cattle die within a few hours of exposure; but in sub-acute cases, death may occur later (Schweikhardt, 2010).

It can be estimated that about 14.8% of the total Swedish bovine population live in the coastal zone. Since a large part of the cyanobacteria blooms appearing in the BS, also contain toxins (Kononen *et al.,* 1998; de Figueiredo *et al.,* 2004; Sivonen and Moselio Schaechter, 2009) there is a great risk that the animals become exposed. However, the frequency of actual intoxications or subclinical consequences are largely unknown. One major reason for this is that it

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is too cumbersome and expensive to send samples for analysis/bodies for autopsy. A few trials have been focused to determine the safe levels of intake of cyanobacterial cells (toxins) in domestic animals, but the levels of toxins causing sub-clinical problems in livestock are poorly characterized.For several reasons such as animal health and welfare as well as food security it is important to know the potential adverse effects associated with natural exposure to cyanotoxins.

The aim of this study was to investigate the prevalence of contaminated BS water outside of coastal pastures and absorption of these toxins into the circulation of grazing cattle and eventually a physiological response in terms of biomarkers of liver damage.

MATERIALS & METHODS

Four pastures, harboring grazing cattle were selected as study sites in the present study. They are located in the coastal zone directly facing the BS, where water blooms often occur. Three sites were located in Åland in the communities of A; Lumparland B; Finström and D; Jomala and a fourth site C was situated on the Swedish east coast in the community of Västervik (Fig 1.).On each site six cows or heifers were selected for the study. The main inclusion criterion was that the animals were kept on coastal pasture for the entire grazing season (May-October) with sea water as their main source of drinking water.

Water samples were collected continuously at approximately weekly intervals, but samples from site C was taken only in connection with the three blood sampling events. Samples were frozen at -20^{o}C until analyzed in the late autumn of 2011.

Blood sampling was performed on three occasions to cover the entire grazing season. Thus, in each animal (n=24) one sample was taken in the beginning of Period 1 (15.5.2011 to 30.6.2011, spring) another in the beginning of Period 2 (1.7.2011 to 31.7.2011, summer) and a final sample in the end of period 3 (08.08.2011 to 15.10.11, autumn). Venous blood was taken from tail vein and serum was prepared by first allowing blood to coagulate for 30-60 minutes after which it was centrifuged at 5000 rpm for 20 minutes and the serum was separated before it was frozen and kept at -20 ^{o}C until analyzed within 2 months.

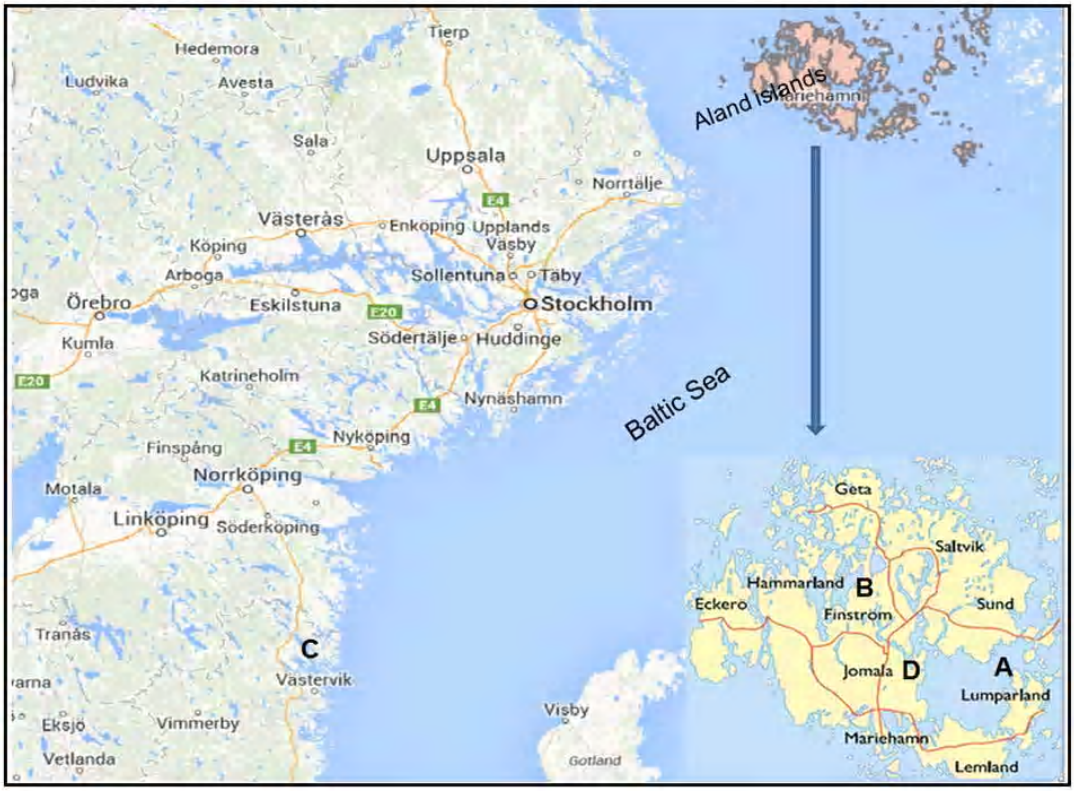

Fig. 1.Map showing the collection sites of the study area in Baltic Sea. [A] Lumparland [B] Finström [C] Västervik and [D] Jomala. Sites A,B and D are located in Aland islands and site C is located on the Swedish east coast

MCs/NODs PP2A Kit (Abraxis, Pennsylvania, USA) was used to detect phosphatase inhibition activity due to presence of cyanotoxins in water.MCs/NODs PP2A Kit is based on the phosphatase activity inhibition by cyanotoxins like MCs.Under normal conditions the phophatase enzyme PP2A is able to hydrolyse a specific substrate that can be detected at 405 nm. Samples containing MCs or other cyanotoxins will inhibit the PP2 activity proportionally to the amount of toxin contained in the sample. All assays were conducted in triplicate. The standard inhibition curve was drawn using pure Microcystin-LR (MC-LR) as reference at final concentrations of 0.25 –2.5 ppb.

The Abraxis (Pennsylvania, USA) microcystins kit based on antibodies against the amino acid ADDA (Fischer *et al.,* 2001) was used by following the manufacturer's instructions. Since ADDA is a common part of the molecular structure of most MCs and NODs, this method is considered a congener-independent assay. Calibrations were done with pure MC-LR at a final concentration of 0.15 –5.0 ppb provided with the kit, by plotting %B/Bo against logC.

The Abraxis ELISA for Serum (microtiter Plate) (Pennsylvania, USA) kit was used to analyze the ADDA containing cyanotoxins (MC/NOD) in serum samples.The test is a direct competitive ELISA in serum, and is based on the recognition of all MCs, NODs and their congeners, containing ADDA, by a monoclonal antibody. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run. Pure MC-LR was used as standard. The limit of quantification (LOQ) for MC on the ELISA plate is 0.175 µg/L. The limit of detection (LOD) for MC or NOD on the ELISA plate is 0.147 µg/L. Samples were run on a Molecular Devices Corp. (Palo Alto, CA), Vmax kinetic microplate reader.

Serum samples collected from cattle were thawed.Then 1 mL aliquots were transferred to 15 mL Corex glass tubes; 10 mL of methanol was added to each tube, mixed and centrifuged at 9000 rpm for 30 min.The supernatant was decanted into 20 mL vials. The pellet was resuspended with 5 mL of MeOH, centrifuged as before, and added to scintillation vials.Five mL of hexane was then added to each vial. The vials were then capped and vortexed. The hexane layer was discarded and the methanol layer was washed three more times with 5 mL hexane. Samples were dried under vacuum in a Speed VAC Concentrator at 40 °C and then taken up in 2 mL of 5% HOAc. After this step, water samples were also prepared by using the same procedure. This solution was passed through an Oasis HLB solid phase extraction (SPE) cartridge (Waters, Milford, MA, USA). The HLB SPE cartridge was conditioned with 1 column volume of MeOH and 1 column volume of water.The SPE cartridge was washed with 5 mL of 30% (v/v) MeOH in water.The cyanotoxin fraction was then eluted with 5 mL of MeOH and dried. Samples were resuspended in 1 mL of 10% (v/v) MeOH in water and centrifuged at 10200 rpm through a YM-10 (Millipore, Bedford, MA) molecular weight cutoff filter. These extracted serum and water samples were analyzed for cyanotoxins by using LC-MS and the results were expressed as MC-LR equivalents.

MC-LR, RR, YR and NOD were analyzed by LC-MS (HP 1100 Series, Agilent Technologies Inc., Palo Alto, CA) equipped with an autosampler, quaternary gradient pump, thermostatted column compartment kept at 40 ÚC and single quadrapole mass analyzer (G 1946D) controlled by Chemstation Rev.B.04.01 software. Chromatographic conditions and parameters in MS were based on the methods of (Maizels and Budde, 2004) and (Takino and Kyono, 2000) optimized with modifications. Sunniest C18 column (10 cm length, 2 mm i. d. and 5 µm particle size) connected to a SunShell C18 Guard Filter 4mm i.d. and 4 mm thickness (ChromaNik Technologies Inc., Osaka, Japan), was used to analyze the toxins. The mobile phase used was composed of 0.1% formic acid in water (A) and acetonitrile (B).The gradient run was from 30% B to 65% B over 10 min, then to 30% B over 20 min. ESI -MS analyses was performed at the optimized settings; vaporizer temperature 350°C, drying gas temperature 350 °C at a flow rate of 1.8 L/min, nebulizer pressure at 60 psi, corona current 8µA, capillary voltage at 4000 V and fragmentor voltage at 70 V. Total ion current of mass spectra were recorded in the mass range *m/z* 100–1000.

For quantification, the mass spectrometer was operated in the positive ion mode to using selected ion monitoring (SIM) for MC-LR at m/z 498, YR at m/z 523, RR at m/z 520 and for NOD at m/z 825. The calibration curves of this method were constructed by injecting the standard solutions across 3 different concentrations (0.25, 2.5 and 25 ng/20 µL) for MC-LR, RR, YR and NOD. In the case of water samples, selected ion was also monitored at m/z 397 in both standards and test samples, as the response equally with m/z 825.

The analyses were performed according to established methods at the Clinical Chemistry Laboratory (SLU, Uppsala, Sweden). The following 14 variables were analyzed: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), bile acids, total bilirubin, cholesterol, urea, creatinine, total protein, albumin, triglycerides and glucose.Significant

differences between periods were tested using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons in the statistical and graphics program Graphpad Prism Version 5.02 (Graphpad Software, Inc., La Jolla, CA, USA). The significance level was set at $p < 0.05$.

Recovery experiments were carried out in quadruplicate spiking in serum and water samples with mixed cyanotoxins solution of the three commercial standards (MC-LR, MC-YR, MC-LR and NOD) at 1 µg/ mL. The extraction and analysis was performed as described previously, and the recovery and the relative standard deviation of the analytical method were calculated.

RESULTS & DISCUSSION

During the summer of 2011 the cyanobacteria blooms at study sites were generally weak.Only occasionally could the naked eye see traces of water blooms. Accumulation of cyanobacteria in the water could not be seen in site C during mid-July.At the study sites in Åland no visible water blooms were reported. However, intermittent blooms occurred further out to sea, as seen in distribution maps monitoring of algal surface distribution in the BS from Swedish metrological institute (SMHI, Norrköping, Sweden).From the same maps initial signs of cyanobacteria blooms were observed in late June in the Gulf and southern part of the Baltic Proper. In late August, there were still some minor surface water accumulations in the Gulf of Bothnia. The last monitoring date was 09/08/2011.

The cyanotoxin concentrations in water as analyzed by the PP2A inhibition assay results and their relationship to water temperature are shown in Fig. 2.Cyanotoxins were detected in 2 out of 4 study sites and the concentrations ranged from 0.4 to 0.64 and 0.24 to 0.44 µg MCE/ L from pasture A and B respectively. The highest mean (± SD) concentration was 0.92 ± 0.19 µg MCE/L in samples collected from pasture A in period 3. No cyanotoxins were detected from study sites C and D during the study period. It is evident from the results that a number of water samples originating from study sites A and B were contaminated with cyanotoxins during the period of investigation (May – Sep 2011), with the highest concentrations in the later phase of this study period.It seems that the increase in concentration of cyanotoxins is associated with high water temperature.This is consistent with findings that water temperature is the most significant factor related to cyanobacteria biomass (Chen *et al.,* 2001; Rapala *et al.,* 1997; Sivonen, 1990).

The seasonal variation of the cyanotoxin concentrations as estimated by ELISA are shown in Fig. 3. Measurable cyanotoxin levels were detected in water samples with concentrations ranging from 0.98 to 7.45 from site A and 0.12 to 0.68 µg MCE/L from site B. Especially high ELISA responses (7.45µg MCE/L) were obtained as cyanotoxins from site A in period 3. The data from water samples by PP2A and ELISA not only exhibit the cyanotoxin contents at sites A and B but also illustrate the variations in concentrations of cyanotoxins in water samples collected in the same position (Fig. 3). Water samples originating from study sites A and B were found to contain cyanotoxins and their concentrations ranged between 0.24 to 0.64 µg MCL/L by using PP2A assay. The presence of cyanotoxins in these samples was further confirmed in the ELISA assay which in many cases showed considerably high levels compared with the PP2A assay.The LC-MS analyses specifically showed that one of the active toxins in the water samples from site A was NOD, whereas this toxin could not be demonstrated in samples from site B.

The results obtained from PP2A and ELISA assays demonstrate thus that water samples from the A and B sites contained cyanotoxins with a varying range of concentrations.The differences in the concentration levels between the PP2A and ELISA assays that we found in this study may be explained by differences in analytical principle. Thus, is has to be remembered that ELISA estimates the total amount of toxin present containing the amino acid ADDA and PP2A estimates total toxic activity which differs considerably between toxin species.

In order to get information of what toxin species are present in PP2A or ELISA positive samples LC-MS analyses was carried out. Due to its high specificity and sensitivity, LC-MS has become the method of choice for quantitative determination of analytes in biological and water (environment) samples (Chen *et al.,* 2002). Because cyanotoxins are cyclic peptides with a high proton affinity, using LC/MS with an ESI ion source can obtain best sensitivity and accuracy. Several analytical applications of this have been reported in the literatures (Hilborn *et al.,* 2005; Meriluoto, 1997; Yuan *et al.,* 2006). Similarly, in the present study we used ESI LC-MS to detect cyanotoxins in the serum and water extracts and obtained a cleaner MS spectrum with good reproducibility of the fragment ions by using standards (Fig. 4).In view of this it can be claimed that the combination of assays and analyses is necessary for a thorough safety assessments of in drinking water.

The results of the blood samples analyses by ELISA assay revealed the presence of toxins in 6 individual animals that were kept at sites A and B.Concentrations ranged from 0.52 to 0.125 and 0.14 to

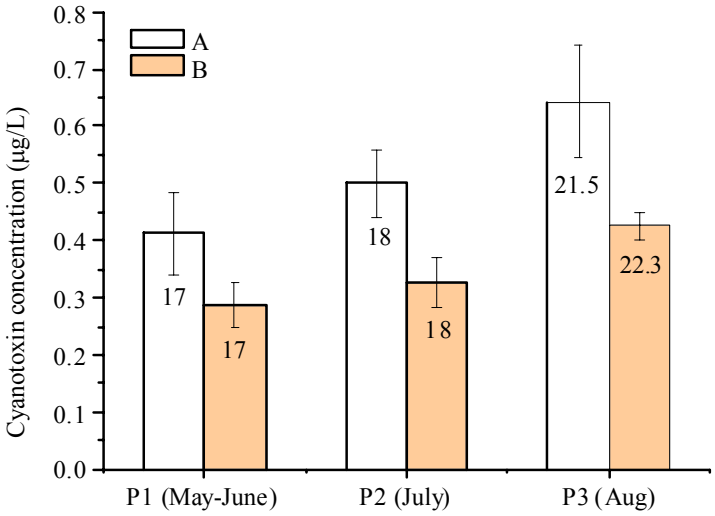

Fig. 2. Seasonal variations in the levels of total cyanotoxins by PP2A at pasture A and B of the Baltic coastal zone

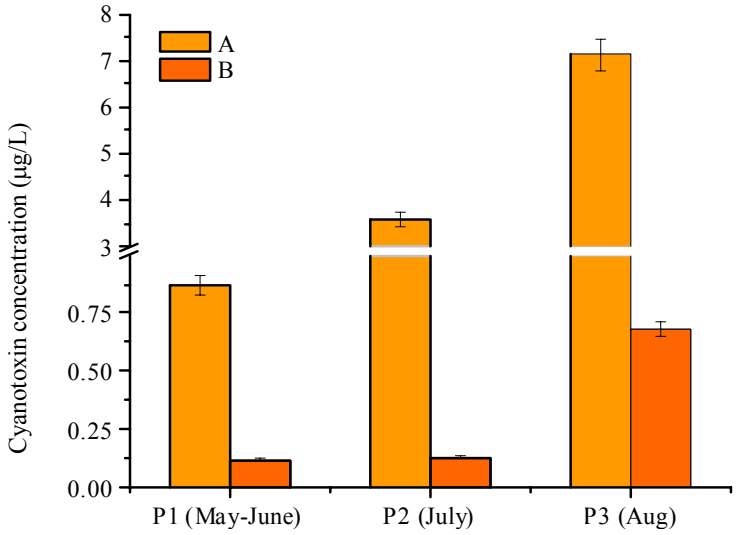

Fig. 3. ELISA results of the total cyanotoxin levels in water samples at pasture A and B

0.102 µg/L in samples originating from site A and B, respectively.Blood samples collected from sites C and D did not show any measurable levels of cyanotoxins. NOD was only detected in water samples from site A in period 2 and 3, and the concentrations were ranging from 0.213 to 0.524 µg/L (Fig. 5 and Fig. 6).However, no NOD was detected in any of the water samples originating from study sites B, C and D. In addition no serum samples from any of the study sites showed presence of MC-LR, RR, YR and NOD.

Accordingly maximum concentration of cyanotoxins was observed from those samples by PP2A and ELISA in the same period. In this point of view, PP2A, ELISA and LC-MS based determination of MCs gave satisfactory agreement between the obtained results.On other hand the values determined by ELISA were not only higher than values from the PP2A assay but also higher than those determined by LC-MS. This could be due to the fact that our cyanotoxin standards contained only the four most toxic species, and not other cyanotoxins.

Now it might be asked how the concentrations of cyanotoxins in water samples that we found in the present study relates to previous measurements of BS water. In the present study ELISA reveals 7.5 µg/L of cyanotoxins and LC-MS results 0.5 µg/L of NOD from pasture A in period 3. These concentrations are very low when compared to the cyanotoxin concentrations (20-40 µg/L) reported earlier from Östra Kyrksundet in Åland (Lindholm and Meriluoto, 1991). Similarly, higher levels of NOD were reported as 90 -18135 µg /L in BS (Mazur and Pliński, 2003), 90 µg/L in the Gulf of Finland.

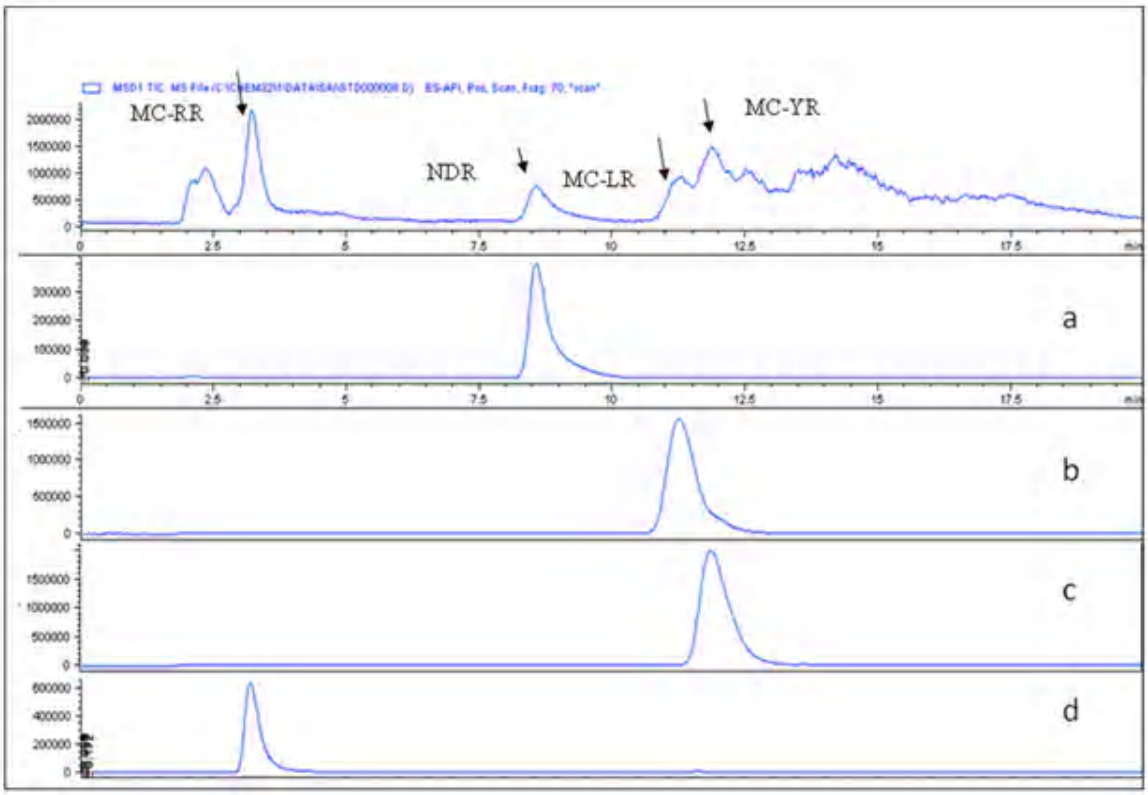

Fig. 4. LC-MS Chromatogram of the Separation of Microcystins-LR, YR, RR and Nodularin standards (20 µL injected). Selected Ion Monitoring (SIM) (a) NDRL – *m/z* 825 (b) MCYR- *m/z* 523 (c) MC- LR- *m/z* 498 (d) MC-RR- *m/z* 520

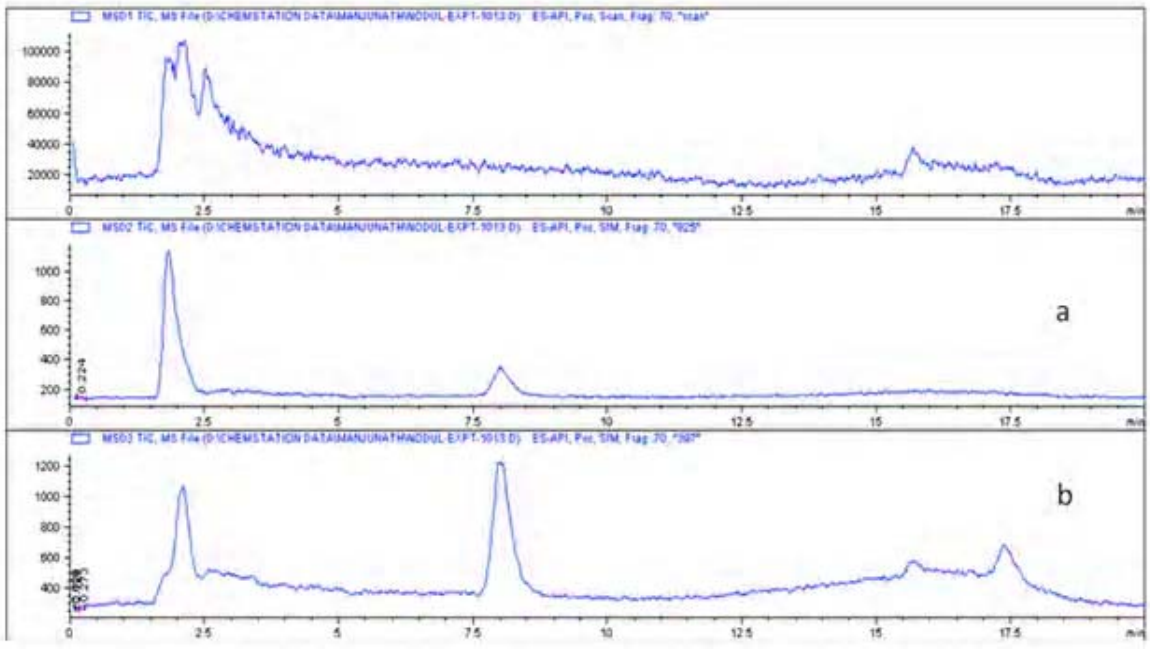

Fig. 5. LC-MS chromatogram of nodularin in water sample from Site A in period 2 (20 µL injected). Selected Ion Monitoring (SIM) for Nodularin (a) *m/z* 825 (b) *m/z* 397

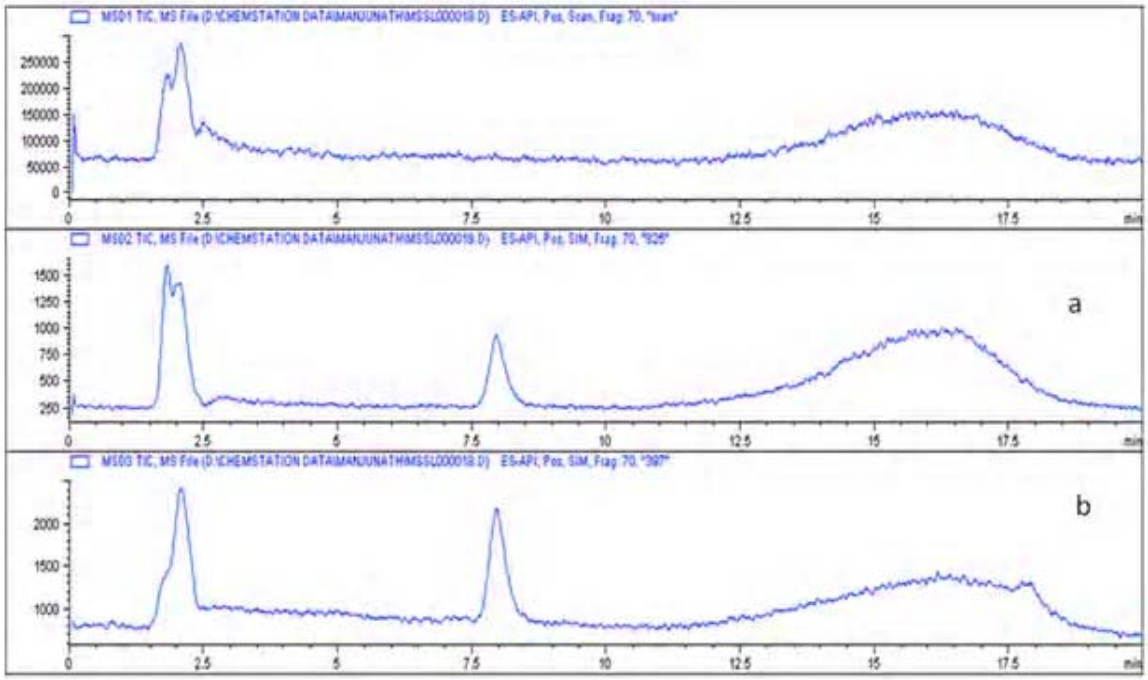

Fig. 6. LC-MS chromatogram of nodularin in water sample from Site A in period 3 (20 µL injected). Selected Ion Monitoring (SIM) for Nodularin (a) *m/z* 825 (b) *m/z* 397

A comparison was made between cyanotoxins exposed and unexposed individual animals with respect to biomarkers of liver damage and no significant differences were detected (Data was not shown).

Serum samples from 6 out of 12 animals that were kept at study sites A and B and that were collected in period 3, showed detectable levels of cyanotoxins by ELISA, about 0.052 to 0.125 µg MCE/L.It is of noteworthy that the maximum concentration of cyanotoxins in water samples was observed from these sites in the same period. In view of this it is surprising that the presence of cyanotoxins in the circulation of exposed animals was not reflected in significant elevations of biomarkers of liver damage.However, this is most certainly due to the fact that LC-MS analysis of serum samples showed absence of most potent liver toxic variants MC-LR, RR, YR and NOD and it also means that the ELISA assay must have recognized less toxic variants than those mentioned. The fact that the changes in serum biomarkers of liver function were generally small must be a reflection of low level exposure of cyanotoxins and no exposure to the most toxic ones. This in turn is a reflection of the fact that water blooms occurred in a very small scale during the summer of 2011.Another explanation can be found in the fact that the large population of microflora of the fore stomach (rumen) of cow has a capacity to degrade algae toxins. Previous *in vitro* investigation in our laboratory reveals that the rumen microbial flora of cow showed a capacity to degrade MC-LR, YR, RR and NOD. However, the degree of degradation was both dose and time dependent (Manubolu *et al.*, unpublished).

In the literature analysis of serum from cows poisoned by cyanotoxins appears relatively rarely performed in cases of acute poisoning because most studies deal only with the macroscopic and histological necropsy findings rather than serum assay results. However, a study by Orr *et al.* (2003), where over a period of 28 days, cattle exposed to low doses (1 x 10^5 cells/mL) of *Microcystis aeruginosa* and where one could not see the changes in serum parameters. Our present field results cannot be compared with the earlier laboratory studies by (Orr *et al.,* 2003) and (Frazier *et al.,* 1998), because of the huge variation in the exposure levels in the experimental animals.

CONCLUSION

In the present study, only two (site A and B) local water bodies were found to contaminated with cyanotoxins among the four selected sites, which were water sources for cattle. Further, they resist low levels of cyanotoxin exposure during shorter periods without major negative changes in biomarkers of liver function. However, the effect of high and prolonged exposure to cyanotoxins remains to be elucidated. Future directions of the present study is to extended the study period for screening of cyanotoxin levels in water

bodies and to follow the uptake into blood of naturally exposed cattle.

Abbreviations

Microcystins (MCs), Nodularins (NODs), Microcystin-LR (MC-LR), Microcystin-RR (MC-RR), Microcystin- YR (MC-YR), Protein phosphatase 2A (PP2A), Enzyme-linked immunosorbent assays (ELISA), World Health Organization (WHO), intra peritoneal injection (i.p.), ochratoxin A (OTA), AFB1(Afaltoxins-B1), Mass Spectrum (MS), Liquid Chromatography Mass Spectrometry (LC-MS), Electron spray Ionization (ESI), Selected Ion Monitoring (SIM), Standard Error (SE) and Tolerable Daily Intake (TDI), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH).

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