pH-DEPENDENT HYDROPHOBICITY OF THE CYANOBACTERIA TOxin MICROCYSTIN-LR

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(First received October 1997; accepted May 1998)

Abstract—Although the presence of hepatotoxins in surface and drinking water is regarded as an eco-toxicological and human health problem, limited knowledge exists on the fate of these compounds. Therefore the n-octanol/water distribution ratio (Dow) of the hepatotoxin microcystin-LR (MC-LR) was determined in order to enable the prediction of the fate of this compound in the aquatic environment. The presence of two free ionizable carboxyl groups and one free ionizable amino group in MC-LR suggest that its speciation and partitioning between octanol and water is pH dependent. This was experimentally studied and confirmed in the present study. The log Dow of MC-LR decreased from 2.18 at pH = 1 to −1.76 at pH = 10. From the low log Dow values, especially at the basic pH region at which the toxin producing cyanobacteria flourish, a low tendency to bioconcentrate from water in biota can be expected.

Key words—microcystin-LR, cyanobacteria, n-octanol/water distribution ratio, pH, bioconcentration

INTRODUCTION

Microcystins are cyclic peptides containing 7 amino acids which are produced as secondary metabolites by cyanobacteria such as Microcystis aeruginosa, Microcystis viridis, Nostoc spp., Oscillatoria agardhii and Anabaena flos-aquae (Tsuji et al., 1994). Over 50 microcystins have been identified so far (Harada et al., 1996). Accumulation of microcystins may lead to acute death due to liver necrosis and haemorrhages in both mammals and fish (Tencalla et al., 1994) or may lead to chronic effects such as liver cancer (Carmichael, 1994).

The most abundant microcystin making up between 45.5 and 99.8% of total microcystin concentration in natural blooms is microcystin-LR (MC-LR) (Vasconcelos et al., 1996) which is shown in Fig. 1. For MC-LR the two variable amino acids are leucine and arginine which have the single letter abbreviations L and R, respectively (Carmichael et al., 1988).

Microcystins usually remain in cyanobacteria and are only released in the water due to cell lysis (Lahti et al., 1997). The fate of the toxins once released in the water has not been studied often in detail so far. What is known is that the toxin is chemically stable in water (Tsuji et al., 1994). Only in the presence of algal pigments disappearance by photolysis was measured (Tsuji et al., 1994). In times of bloom, photolysis can be seen as one of the detoxification routes. The major route of detoxification of MC-LR is probably biodegradation (i.e. Bourne et al., 1996; Takenaka and Watanabe, 1997; Lahti et al., 1997). However, low water temperature, and low MC-LR concentrations may lead to half-lives of 9 days or more (Lahti et al., 1997).

The partitioning of the toxins between water, sediment and air has not been studied earlier. The lack of data on aqueous solubility, octanol/water partition coefficient (Kow) and Henry’s law constant prevent proper predictions of this partitioning, using existing distribution or fate models (Mackay, 1991). Therefore, the contribution of this study is the determination of the Kow of the most abundant hepatotoxin, MC-LR, in order to enable prediction of the partitioning of this toxin in the environment.

The Kow of persistent neutral organic compounds highly correlates with the tendency of a molecule to concentrate in the lipids of organisms and the organic carbon of sediments and soils (Karickhoff et al., 1979; Mackay, 1982). For ionic compounds the use of Kow-values is complicated by the dependence on both pH and type and concentration of counterions. MC-LR contains two ionizable carboxyl...
groups and one ionizable amino group (Fig. 1) that are not part of peptide bonds that make up the cyclic peptide structure (Rudolph-Bo¨hner et al., 1994). The pK_a-values of these groups are reported to be 2.09, 2.19 and 12.48, respectively (Chang, 1981). These pK_a-values are measured in free amino acids and can be slightly different for amino acids incorporated in a peptide. Based on the reported pK_a-values, different MC-LR species are dominant at different pHs (Table 1).

Besides the dominant species, other species are present at the different pH ranges in water. The term octanol/water distribution ratio (D_{ow}) instead of octanol/water partition coefficient (K_{ow}) is therefore used, because more than one species is present and each contributes to the partitioning between octanol and water (Jafvert et al., 1990; Schwarzenbach et al., 1993; Escher and Schwarzenbach, 1996). In the very small pH range that is estimated between 2.09 and 2.19 the zwitter ion with no net charge is dominant. This is the most “neutral” species of MC-LR and it is this species which therefore may be expected to have the highest tendency to partition into octanol. In the pH range 6–9 at which cyanobacteria may flourish (Carmichael, 1994) lower D_{ow}-values are expected.

In order to test the influence of pH on D_{ow} of MC-LR, the latter is measured at a large pH range with the emphasis on the pH range which the cyanobacteria favor.

### MATERIALS AND METHODS

**Chemicals**

Microcystin-LR (MC-LR >98%) was a kind gift from Linda A. Lawton of the School of Applied Sciences, Robert Gordon University, Aberdeen, U.K. Methanol (>99.8%) was purchased from Merck (Darmstadt), 1-octanol (>99.5%) was purchased from Fluka (Buchs), Acetonitrile (ACN >99.8%) was obtained from Biosolve, (Barneveld). Trifluoroacetic acid (TFA 99%) was obtained from Aldrich (Steinheim).

#### pH-dependent D_{ow} of MC-LR

In order to determine the pH dependency of D_{ow} of MC-LR stock solutions of 0.1 M NaCl in distilled water were prepared that were calibrated to the selected pH with NaOH or HCl.

For pH 5, 6, 7, 8.5 and 10, glass centrifugation tubes were filled with 5 ml water at the selected pH in triplicate. To the aqueous phase a spike of 5 µg of MC-LR dissolved in 30 µl methanol was added leading to a methanol concentration of 0.6% (v/v). The MC-LR did not change the pH of the water. To each tube 1 ml of octanol was added. Subsequently, the centrifugation tubes were horizontally shaken (225 rpm) in a thermostated water bath (Julabo SW-21C, Seelbach) at 25 ± 0.4 °C for 72 h. Then, the tubes were centrifuged for 20 min at 900 g (Megafuge 1.0, Heraeus sepatech).

Of the octanol phase 0.7 ml was transferred to another centrifugation tube. The rest of the octanol and 1 ml water from the top were discarded. The remaining water was analyzed without further treatment.

The 0.7 ml octanol sample was extracted with 0.7 ml water (pH = 12) and centrifugated for 20 min at 900g. This was done because octanol cannot be directly injected on the HPLC because it does not mix with the eluents. The octanol and the upper 0.3 ml of the water were pipetted of and discarded. Both the concentrations in the remaining octanol and water samples were determined in a HPLC system as described below. The D_{ow} was determined by dividing the MC-LR concentration in octanol by the concentration in water.

Additional single measurements were done at pH 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 6.6. Spike and octanol were added as described above. For these only the water samples were measured on HPLC. The D_{ow} was deter-

<table>
<thead>
<tr>
<th>pH</th>
<th>Dominant species</th>
<th>Net charge of MC-LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH &lt; 2.09:</td>
<td>(COOH)$_2$(NH)$_2$</td>
<td>+</td>
</tr>
<tr>
<td>2.09 &lt; pH &lt; 2.19:</td>
<td>(COO$^-$)(COOH)(NH)$_2$</td>
<td>0</td>
</tr>
<tr>
<td>2.19 &lt; pH &lt; 12.48:</td>
<td>(COO$^-$)(NH)$_3$</td>
<td>$-$</td>
</tr>
<tr>
<td>pH &gt; 12.48:</td>
<td>(COO$^-$)$_2$(NH)</td>
<td>$-$</td>
</tr>
</tbody>
</table>

### Table 1. Dominant species of MC-LR at different pH-value
Recoveries and mass balances

The extraction efficiency of MC-LR from octanol into water (pH = 12) was determined by direct spiking to octanol and was found to be $94 \pm 3\%$ (S.E.M.) ($n = 3$). The concentrations in octanol were corrected for this determined extraction efficiency. The mass balance, defined as the percentage of the amount of MC-LR spiked that was retrieved from the octanol and water phase together, was not influenced by the pH and was $81.0 \pm 1.3\%$ (S.E.M.) ($n = 18$).

MC-LR was found to be stable at all tested pHs at the used contact times. The pH of the sample at the moment of HPLC injection was found not to influence the retention or response.

Analysis

The water and octanol samples were injected in a 20 µl loop connected with a HPLC system with a Merck-Hitachi L-6200 Intelligent pump which was supplied with a Chrompack ChromSpher C18-column connected to a Merck-Hitachi L-4000 UV detector. Quantitation was done with a Merck-Hitachi D-2500 Chromato-Integrator. The column was eluted isocratically with ACN + 0.05% TFA/millipore water + 0.05% TFA (50/50) at 0.4 ml/min. The selection of the eluents was based on Lawton et al. (1994).

RESULTS AND DISCUSSION

The determined log $D_{ow}$ at the different pHs are shown in Fig. 2. It can be seen that the log $D_{ow}$ ranges from 2.18 at pH = 1 to $-1.76$ at pH = 10. For the pHs in which the log $D_{ow}$ was determined in triplicate the 95% confidence limit is plotted around the means (Fig. 2). The range of the confidence limit increases with decreasing log $D_{ow}$ due to increasing variation in the determined concentration in the octanol phase which is close to the detection limit at log $D_{ow} = -2$. The grey shade shows the range at which cyanobacteria normally flourish (Carmichael, 1994).

No big changes in log $D_{ow}$ are found near the $pK_a$-values of the carboxyl groups. The pH dependency is more characterized by a gradually decreasing log $D_{ow}$ with pH. No constant log $D_{ow}$ levels are found at certain pH ranges as has been found for other ionic compounds such as pentachlorophenol (Jafvert et al., 1990). This could mean that during the entire pH range the speciation of MC-LR is changing significantly. It can be deduced that the tendency of the $(COOH)_2(NH_2^+)$-species to partition into octanol is much higher than that of the $(COO^-)_2(NH_2^+)$-species. This may be due to a higher stability of the counter ion–MC-LR complex or a higher intrinsic capacity to accumulate in octanol for the former species, but further studies should reveal this.

Based on the determined low log $D_{ow}$ values and the relationships reported between log bioconcentration factor ($K_c$) and log $K_{ow}$ (e.g. Mackay, 1982), only limited bioconcentration direct from water into biota by passive diffusion can be expected. Another reason for the expected low bioconcentration is the large size of the MC-LR molecule, which may prevent passive diffusion across the cell membrane to occur (Opperhuizen et al., 1985). Gastrointestinal uptake via the food is therefore expected to be a much more relevant route of uptake which is supported by laboratory studies reported in the
literature (Eriksson et al., 1989; Tencalla et al., 1994; Laurén-Määttä et al., 1995). Uptake via the bile acid transport system may lead to faster uptake in the gastro-intestinal tract than expected from the low hydrophobicity of MC-LR (Runnegar et al., 1981).

CONCLUSIONS

The log $D_{ow}$ of MC-LR decreased from 2.18 at pH = 1 to −1.76 at pH = 10. From the low log $D_{ow}$ values, especially at the basic pH region at which the toxin producing cyanobacteria flourish, a low tendency to bioconcentrate from water in biota can be expected.

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REFERENCES


