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**The peer review of cyanotoxin toxicity criteria and health-based water concentrations to protect human swimmers, dogs, and cattle**

Blooms of cyanobacteria is an emerging ecotoxicological problem and also health risk for humans and domestic animals all over the world. The idea of creating health based criteria to protect people during recreational use of surface water bodies and to protect dogs and livestock should be appreciated. I hope that my comments regarding the development of action levels will be useful.

## **General Approach:**

1. The scope of the assessment was to establish the action levels of four variants of hepatotoxic microcystin, neurotoxic anatoxin-a, and cytotoxic cylindrospermopsin to protect people, dogs and cattle. The cyanotoxins selected by OEHHA have different mechanisms of their toxic action in mammals and induce different toxic effects. **The selection of the most toxic forms of microcystin: LA, -LR, -RR, -YR, anatoxin-a and cylindrospermopsin to determine the action levels is, in my opinion, very relevant.** The four variants of microcystin are similar in structure but they have different water solubility, cell membrane permeability and, as a consequence, their toxicity. However, much more adequate literature is available on the toxicity of microcystin-LR than three other variants of this cyanotoxin. Cytotoxic cylindrospermopsin and neurotoxic anatoxin-a are very commonly produced by many strains of cyanobacteria and their impact on health of many mammalian species is very evident, so action levels for these cyanotoxins should also be developed.

If neurotoxic saxitoxin is found in waters of California, I suggest determination of action levels also for this cyanotoxin, because scientific data indicate that it could also be a serious threat to domestic animals and humans. Neurotoxic saxitoxin is produced by marine dinoflagellates *Alexandrium*, *Gymnodinium* and also by freshwater cyanobacteria such as *Anabaena sp.*, some strains of *Aphanizomenon*, *Cylindrospermopsis* and *Planktothrix* very commonly found in the freshwater environment.

**I agree that the correlation between cyanobacteria cell count and the cyanotoxin level is not consistent and cell count is not efficient basis for action level.** The WHO developed guidelines for health protection on the basis of : 1) low probability of adverse health effects from water  $\leq 20\ 000$  cells/mL or  $10\ \mu\text{g}$  chlorophyll-a/L where cyanobacteria are the dominant species, 2) moderate probability of adverse health effects from waters with  $100\ 000$  cells/mL or  $50\ \mu\text{g}$  chlorophyll-a/L, in case of bloom formation on the water surface. These guidelines are rather based on cell concentrations but not on toxin concentrations. In a cyanobacterial bloom toxigenic (cyanotoxin-producing) and non-toxigenic strains of the same species of cyanobacteria that can exist together. By using light microscopy cyanobacteria cell count, it is not possible to determine the quantity of toxigenic or non-toxigenic strains in a sample. Even if a sample contains only toxigenic strain of cyanobacteria one cannot predict the amount of cyanotoxin produced because the same number of cyanobacteria can contain different amounts of cyanotoxins. The same strain produces various amounts of cyanotoxins depending on certain unknown conditions, and some strains can be more toxic by producing microcystins simultaneously with some other cyanotoxins, such as anatoxin-a (Sivonen 1996).

The best basis for developing the action levels would be determination of concentration of cyanotoxin in the cyanobacterial extract by HPLC and LC/MC methods. Recently the ELISA techniques for some algal toxins have been developed. The ability to produce cyanotoxins can be determined by PCR-techniques.

Some health-protective guidelines for recreational water levels in the USA exist. Vermont Department of Health ([http://healthvermont.gov/enviro/bg\\_algae/bgalgae.aspx](http://healthvermont.gov/enviro/bg_algae/bgalgae.aspx)) suggests that in case of a visible cyanobacterial scum in a recreational water reservoir and microcystin-LR (equivalent) and anatoxin-a concentration in the water is above 6 and 10 µg/l respectively the beaches should be closed.

### **Toxicity criteria**

2. One of the toxicity criteria is cancerogenesis. Microcystin-LR is considered as ‘possibly cancerogenic to humans’, a potent chemical stimulating proliferation of hepatic tumor at low doses but the mechanisms of this effect is still unclear. Some authors associated inhibitory activity of microcystin on protein phosphatases 1 and 2 with tumor promotion. Microcystin at moderate and high concentrations is not directly genotoxic (does not form DNA adducts), but causes production of reactive oxygen species inducing DNA damage and lipid peroxidation leading to formation of liver tumors. It is also suggested that the microcystin-induced oxidative stress is the cause of liver apoptosis. Many short-term studies revealed possible pro-cancerogenic influence of microcystin-LR on hepatocytes, however there is a need for more appropriate long-term studies which is difficult to perform because time-consuming lifetime bioassays should be used. Currently, there are no adequate dose-response results on carcinogenesis induced or promoted by microcystins to use them as the basis for action level development. Some bioassay studies were planned by the National Toxicology Program to expose rats and mice for 24 months to a mixture of microcystins LR and LA but the results are currently not available yet. **As there is a lack of adequate studies for computation of a criterion based on tumor promotion the reference dose for microcystin-LR should be based on liver toxicity.**

3. Data on the toxicity of purified or pure cyanotoxins is limited. Most of the toxicological studies on the acute toxicity of cyanotoxins were performed with the use of cyanobacterial extracts and the some results suggest that cyanobacterial extracts induce more severe toxic effects than purified or pure cyanotoxins. Therefore, reference doses based on the extract

toxicity would be even more health-protective. A cyanobacterial extract used in toxicological studies usually includes the cyanotoxin of known concentration, however it may also contain some other toxic compounds such as lipopolisaccharides or substances of unknown identity even more active than the known toxin or potentiating its toxic effects. Cyanobacterial extracts used in toxicological studies may simulate more adequately natural conditions than a solution of purified or pure cyanotoxin. On the other hand use of extract may not reflect the toxicity of the single cyanotoxin.

**A lack of data on acute toxicity of purified toxins and more severe toxic effects induced by cyanobacterial extracts than purified cyanotoxins suggest that results from studies based on cyanobacteria extracts with known concentration of a toxin are an adequate basis to develop the acute reference doses for microcystin and cylindrospermopsin in domestic animals.**

#### **Exposure assessment**

**4. The scientific data to predict air concentration of microcystins is too limited and more sufficient studies are needed.** Some approach was made recently by Backer et al. (2008) who determined the concentrations of microcystin in water, aerosol of bloom-free lake and blood of 97 people recreating near the lake. The cyanotoxin was found at low concentrations in water (2-5 µg/l) and the aerosol samples (0,1 ng/m<sup>3</sup>). Blood levels of microcystins for all patients were below the limit of detection (0,147 µg/l). The study was performed when the water and aerosol concentrations of the toxin were very low, however it can be assumed that microcystin even when it is at low level in water it can be aerosol-borne and inhaled from during water skiing or from other water activities. Moreover, other scenarios of cyanotoxin inhalation for recreating people should be also considered. For example, dried cyanobacterial cell debris remaining on the shores and beaches of recreational lakes may contain high amounts of cyanotoxins that could be airborne and inhaled or digested when swallowed.

**5. In my opinion, estimation of water amount ingested via gulping during swimming should be also included in the assessment of the canine exposure.** Some amounts of cyanotoxin-contaminated water can be ingested by dogs during gulping and also afterwards, via licking the coat. The amount of absorbed water and cyanobacterial scum seems to be dependent on the length of a dog's hair. The longer hair of a dog, the more water is retained and higher doses of toxic cyanobacteria could be absorbed and then ingested. Assumption that

the water forms a 2 mm layer on the coat may not be applicable to all dog breeds. In case of a small dog with long hair such as Yorkshire terrier the surface of cyanotoxin absorbance would be larger and given that average body weight is smaller in comparison to other dog breeds, the suspected toxic effects would be more pronounced. However, it should be also taken into account that dogs have a natural ability to get rid of the water and cyanobacterial scum by rapid shaking the water off. As a result of this action, the total amount of water ingested during grooming would be smaller. For developing the action levels some other ways of dog exposure to cyanotoxins during exercises should also be considered: via skin, especially for some skin-penetratable cyanotoxins such as anatoxin-a and by inhalation of aerosols or dried cyanobacterial debris containing cyanotoxins when exercising at the edge of water.

### **Microcystin Ecotoxicology**

6. Cyanobacterial toxin-positive blooms are very frequently found in many water reservoirs abundant in many species of fish. Toxicological studies show that these aquatic animals are sensitive to cyanotoxins. Development of action level for those animals seems to be very important issue since these organisms play an essential ecological role and they are also essential for human consumption. However, it is rather impossible to develop the action levels for cylindrospermopsin and anatoxin-a in fish, because there is too little data on the toxicity of these cyanotoxins. On the other hand, there are many toxicological results on the influence of frequently detected microcystin-LR on different endpoints of fish health, such as growth rate, osmoregulation, heart rate, behavior, liver, intestine, kidneys, heart, spleen and gills. Data on microcystin developmental toxicity and immune system also exist. Microcystin toxicity to fish depends on the exposure route. In most studies on acute toxicity in fish this cyanotoxin was administered intraperitoneally and this way of exposure is not natural. The cyanotoxin given into the body cavity usually is more toxic, it is absorbed faster and has different pathways of metabolism. In a number of studies fish were also administered orally freeze-dried cyanobacterial cells and results would be most sufficient for the determination of action levels. Other natural routes of intoxication should also be considered, such as uptake of microcystin directly from water by immersion. In natural conditions the transfer of algal toxins by the food web with zooplankton, crustaceans and smaller fish is also possible. In a such scenario the absorbed doses of cyanotoxins could be much higher in comparison to

direct exposure from water. **I suggest consideration of developing action levels for fish for one cyanotoxin: microcystin-LR** for two reasons:

1. Fish are a very important taxonomical group of animals for human consumption and play an important role in aquatic water ecosystems.
2. It seems that there are enough data for developing the reference dose of microcystin-LR for fish. Consideration of some new results could also be useful for developing the reference dose: such as dietary threshold for microcystin-LR in quart medaka (Deng, 2010).

### **A broader perspective of the scientific issues**

a) Analysis and the development of human and animal action levels is a complex scientific effort. Possible teratogenic and dermatotoxic effects induced by some cyanotoxins were not considered in this report. These are essential endpoints of the toxicity criteria, however, there are too little adequate studies on possible teratogenic effects of cyanotoxins. Some teratogenic influence of anatoxin-a at sublethal dose was found by Astrachan et al (1980) on hamsters such as fetal stunting but not on rats and mice. No teratogenic effects were also found in mice and toads exposed to microcystin-LR however development of African clawed frog (*Xenopus laevis*) eggs was altered. Microcystins and cylindrospermopsin are assumed to be not able to penetrate the skin, however some authors suggest that these cyanotoxins could induce skin toxicity such as allergy or skin irritation. Microcystin-LR was documented to cause eye irritation, it is also an allergenic agent but only at very high concentrations (1,5 mg/ml). Experimental studies revealed that cylindrospermopsin induces delayed-contact hypersensitivity reactions in Balb/c mice. Some reports also suggest highly irritant potency of cylindrospermopsin.

There is a great need for adequate studies to develop the action levels for poultry and currently no toxicological results are available. Possible drinking of cyanotoxin-contaminated water could be a great risk for this essential group of animals used for human consumption. Tissue accumulation and possible further transfer of the cyanotoxins with food to humans should be also considered. The results could be also useful for development of the action levels for birds living in the wild.

b) Action levels are based on commonly used methods of toxicity and exposure assessment. However, I recommend considering some some new results that have been published during recent 2 years.

c) HPLC and LC/MS are very common techniques used for determination of water concentrations of various cyanotoxins. There are some new methods that have been developed recently. ELISA tests for the detection of cylindrospermopsin, microcystins, nodularin and saxitoxins in different media, including water samples and human serum. A good method for determination of microcystin toxicity is colorimetric test measuring protein phosphatases inhibition.

There are methods used to monitor the cyanotoxin production. The antibody-based methods (CQ-ELISA) can be used for the detection of cyanobacterial strains producing cyanotoxins. The ability of a strain to produce cyanotoxins could be determined by the use of PCR-based techniques by which the presence of genes coding certain cyanotoxins can be detected and quantified. The Fluorescent in Situ Hybridization (FISH) allows to localize the cyanotoxin genes in mixed phytoplankton populations. These methods are early warning system that allows to obtain results very quickly, within 1-3 hours.

#### **Additional comments**

1. There is a sentence in the draft on page number 52 that there are 70 congeners of microcystin. Currently, over 80 variants of this chemical have been described.
2. Anatoxin-a is an alkaloid and it has a similar chemical structure to cocaine. Considering the determination of swimmer exposure this cyanotoxin could be not only from the stomach and intestines but also sublingually and from mucous membranes in the mouth.

#### **List of useful reference:**

I added the list of reference that I used in my review.

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