USES OF ALGAE IN THE CONTEXT OF PROBLEMS OF THE ENVIRONMENT AND OF PUBLIC HEALTH

4rd part

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ALGAL TOXINS IN FRESHWATER

The phenomenon of algal blooms in freshwater used for drinking or for bathing is a reason for alarm and the subject of continuous scientific research worldwide and also in Italy. The reasons can be attributed to the following (Bruno, 2002).

a - The blooms recorded are often toxic and the toxins most frequently identified are microcystins; these cause major hepatic damage, pulmonary and renal haemorrhages and have been evaluated as an oncogenic risk. The key role of microcystins in the promotion of the primary human hepatic tumour has been shown on the basis of epidemiological studies recently carried out in China. Therefore, the environmental destination of these toxins should be followed with a certain attention, as it may concern the aquatic food chain. Acute intoxications with a fatal result for man have been reported in Brazil in the states of Bahia (1988) and of Pernambuco (1996). The extermination of aquatic fauna should therefore be interpreted not only as the consequence of the anoxia of the aquatic medium (caused by the decay of the algal blooms) but also by the possible presence of algal toxins. Hence the need to include in the tests of the waters qualitative and quantitative inspections of the algae and their products concerning the search for algal toxins in addition to the usual parameters of dissolved oxygen and colimetry (Decree of the President of the Republic 24th May 1988, no. 236).

b - The algal blooms of eutrophic waters are mainly due to some taxons of Cyanophyceae, but their taxonomic identification is still very uncertain in many cases, requiring more detailed specialist knowledge. Furthermore, the Cyanophyceae are the algae which are most frequently toxic: no less than 40% of the species produce toxins: hepatotoxins (microcystins and nodularins in freshwater and brackish water and cylindrospermopsin in tropical waters), neurotoxins (anatoxin-a, anatoxin-a(s)), Paralytic Shellfish Poisons, PSP, and dermatotoxins (serious acute dermatitis in bathers).

c – A third of the blooms identified to date in Italy are of a toxic nature and the toxic algal species most frequently involved are the following Cyanophyceae:

- cystis aeruginosa (types of microcystins: -LR; -RR; -YR).
- Planktothrix (ex Oscillatoria) rubescens (type of microcystin: -YR; -RR; the hepatotoxic substance has provided a DL50 = 100 mg/kg p.c.).
- Anabaena flos-aquae or Aphanizomenon flos-aquae (which release respectively anatoxin-a and saxitoxins).
- Anabaena affinis.
- Anabaena planctonica (an algae first known as not toxic, but which has then proven to be toxic due to the presence of anatoxin-a).
- Anabaena lemmermannii (produces anatoxina-a(s)).
- Planktothrix (ex Oscillatoria) aghardii.

c - The phenomenon appears serious and on the increase, judging from the frequency of reports. For example, in 1999 the reports recorded by 98 Italian health structures located in 9 northern regions (46 structures), 5 central regions (22 structures) and 6 southern regions (30 structures), were respectively equal to 25, 29 and 10, i.e. more than in 1993 (the first year of activity of the data base established by the Higher Health Institute).

d -There is a shortage of structures and skills for the management of this specific health risk and the reclamation of the environment concerned. This makes training in the public or semi-private territorial units of control and management for the introduction of water into the aqueduct networks essential.

e -Systems of purification are still inefficient.

The cystins form a family of monocyclic heptapeptides the basic structure of which is cyclic(-D-ala-X-D-b-MeisoAsp-Y-Adda-D-iso-Glu-Mdha-); this represents the sequence of the following seven amino acids, inter-linked to form a cycle (Dawson, 1998; Sivonen, 1996; Takai e Harada, 2000):

- D-Ala is L-alanine (position 1);
- X and Y are two L-amino acids (respectively in positions 2 and 4);
- D-b-MeisoAsp is D-eritro-b-methyl-isoaspartic acid (position 3);
- Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (position 5);
- D-iso-Glu is D-glutamic acid (position 6);
- Mdha is N-methyldehydroalanine (position 7).

The two L-amino acids, X and Y, are used in the common denomination system adopted for the cystins; for example, when X = L-leucine and Y = L-arginine, the toxin is called cystin-LR or MCYST-LR (figure 12). Other variants with different amino acids are shown with the common name or its acronym preceded by square brackets which contains the replacement amino acid with a number in apex position which indicates the position it occupies in the conventional formula of the modified cystin. For example, with reference to figure 12, the following notations can be explained:

- [D-Asp3]MCYST-LR: means that the amino acid in position 3 of the formula of MCYST-LR is replaced by D-aspartic acid;
- [D- Asp3, D- Dha7]MCYST-LR: means that the positions 3 and 6 of the amino acids of the formula of MCYST-LR, are occupied respectively by D-aspartic acid and by dehydroalanine.

A list of 48 cystins is provided by Sivonen (1996) and Takai and Harada; the molecular weight (between 900 and 1200), the structural formula, the producing algal species and the bibliographical references are shown for each of these.

The cystin that is most frequently found is MCYST-LR; this is also the most toxic (DL50 i.p. in the mouse = 50 mg/kg, Takai and Harada, 2000; 36 – 122 mg/kg in rats and mice by various ways of administration, including aerosol inhalation, Dawson, 1998).

Nodularin or NDLRN (figure 13), produced by Nodularia spumigena is, on the other hand, a monocyclic pentapeptide (Takai and Harada, 2000; Sivonen, 1996), the basic structure of which is cyclic(-D-b-Me isoAsp-L-Arg-Adda-D-isoGlu-Mdhb), where:

- D-b-MeisoAsp is D-eritro-b-methyl-isoaspartic acid (position 1);
- L-Arg is L-arginine (position 2);
- Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (position 3);
- D-iso-Glu is glutamic acid (position 4);
- Mdhb is N-methyldehydrobutirric acid (position 5).

Figure 12. Structural formula of cystin-LR.

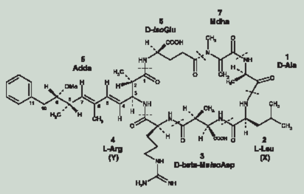


Figure 13. Structural formula of nodularin.

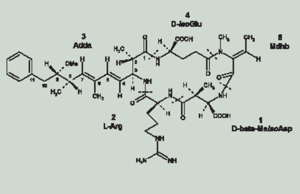


Figure 14. Structural formula of cylindrospermopsin.

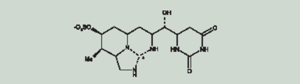
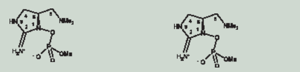


Figure 15. Structural formula of anatoxin-a(S) and anatoxin-a.



NDLRN is therefore structurally analogous with MCYST-LR, from which it differs in the absence of two L-amino acids, one in position 1 (D-Ala) and the other in position 2 (X), and the diversity of the group in position 7 (figures 12 and 13). Only a few variants of NDLRN have been identified, unlike the cystins which number about 60. Amongst the minor nodularins can be mentioned: [DMAdda3]nodularin, [(6Z) Adda3]nodularin, [DAsp1]nodularin and [L-Val2]nodularin; the last-mentioned, known as motuporin, has been found in the marine sponge Theonella swinhoel.

The unusual amino acid, Adda, present both in cystins (position 5) and in nodularins (position 3), is responsible for the biological activity of these toxins (Takai and Harada, 2000).

Active cystins and nodularins are hepatotoxic; they inhibit the serine-threonine protein phosphatases 1A and 2A in the liver with a consequent destruction of the cytoskeleton and serious hepatic haemorrhages (Dawson, 1998). The cystins are powerful promoters of tumours and carcinogens (Falconer and Buckley, 1989; Niskiowaki-Matsushima et al., 1992), whilst the nodularins can be carcinogenic (Ohata et al., 1994).

The algae of species belonging to the Nostocales and Stigonematales orders are, on the other hand, producers of cylindrospermopsin (figure 14). Cases of hepatoenteritis were recorded in 1979 in subjects (139 children and 10 adults) who had drunk the water from a dam on Palm Island (Queensland, Australia), in which the presence of Cylindrospermopsis raciborskii (order: Nostocales) was ascertained; subsequently a new hepatotoxin was isolated which was given the name of cylindrospermopsin was given (Bourke et al., 1983; Moore et al., 1993). DL50 i.p. in the mouse was equal to 2.1 mg/kg at 24 hours and 0.2 mg/kg at 5 – 6 days (Othani et al., 1992). Cylindrospermopsin inhibits the synthesis of glutathione (Runnegar et al., 1994, 1995) and the synthesis of protein (Terao et al., 1994).

Anatoxina-a is the secondary 2-acetyl-9-azabicyclic(4,2)-non-2-ene ($C_{10}H_{15}NO$; PM = 165) amina, found in Anabaena flos-aquae, Anabaena lemmermannii, Oscillatoria sp., Aphanizomenon sp., Cylindrospermum sp. and also Anabaena planctonica Brumnthaler in Sardinia (Bruno et al., 1994). DL50 i.p. in the mouse is 200 – 250 _g/kg (Devlin et al., 1997; Carmichael et al., 1990).

The replacement of acyl by propionyl in the formula of anatoxin-a gives homo-anatoxin-a which has been found in Oscillatoria formosa. The formula is $C_{11}H_{17}NO$ (PM = 179) and DL50 i.p. in the mouse is 250 _g/kg.

Anatossina-a(s) is the phosphoric ester of cyclic n-hydroxyguanine ($C_7H_{17}N_4O_4P$; PM = 252; figure 15; DL50 i.p. in the mouse = 20 _g/kg). It has been found in Anabaena flos-aquae.

Algal blooms in eutrophic lakes are not only unsightly for the environment and unpleasant and annoying particularly for bathers but they are a serious risk for human health due to the algal toxins that can be found in drinking water. Cyanobacteria, producers of hepatotoxins (MYCST-LR and –RR) and neurotoxins (anatoxin-a) are generally dominant in the biomass. The environmental conditions favouring the formation of algal blooms in freshwater include (Skulberg et al., 1984):

- from moderate to high levels of nitrogen and phosphorus ;
- water temperature between 15 °C and 30 °C;
- calm or stagnant water;
- pH between 6 and 10.

Protection of an essential asset: drinking water

Algae are one of the many causes (decomposing organic material, vegetation, mineral substances, hydrogen sulphide) that give rise to unpleasant tastes and odours of drinking water. For example, Moncrieff (1951) already associated characteristic odours to some genii of algae:

-Asterionella, smell of geraniums and fish (if present in considerable quantities)
-Synedra, smell of earth;
-Anabaena, smell of grass and even the horrible characteristic smell of pigsties if present in considerable quantities;
-Ceratium, smell of fish;
- Anuroea, smell of fish;
-other algae, smell of nasturtiums, watermelon and melon.

More recently, the substances that are responsible for giving unpleasant tastes and odours to drinking water have been identified, mainly geosmin and 2-methyl-isoborneol (Volterra, 1997; Fontani and Cucchi, 1998; Bruno and Nusca, 2000):

Geosmin $C_{12}H_{22}O$ — 2-Methyl- isoborneol $C_{11}H_{20}O$

With reference to algae in particular, there are two aspects that can have a direct reference with whether water is drinkable:

- the organoleptic characteristics that can be altered in the odour and taste as a consequence of the release of particular substances due to the cell decay of algae in water reservoirs and in the distribution of drinking water;
- the presence of algal toxins that can cause potential intoxication for consumers with the syndromes clearly described in literature (table 5).

Amongst the characteristics of drinking water, that of being free of unpleasant odours and tastes is fundamental for the purposes of organoleptic acceptability for consumption. The growing trend of the habitual consumption of mineral water by consumers can very probably be attributed in some way to a less than full acceptance of the organoleptic quality of the nearest reservoir or the supply of the area of residence. In any case, in this trend, there is the reflection of the general sense of mistrust deriving from greater knowledge of the health risks linked with environmental pollution and the conviction of the unsuitability of the measures of prevention or reclamation implemented by local authorities. Hence the greater trust and security of mineral waters presented for consumption with all the guarantees of naturalness and organoleptic pleasantness.

In order not to make the organoleptic evaluation of a drinking water subjective, the European Union issued Directive 80/778 according to which it is compulsory to test the substances used to make the containers and transport system of drinking water, regulating the technical examinations necessary (European Standard prEn 1420-1, established by the CEN).

- WHO: water is defined acceptable for human consumption when it is found to be without taste and odour for 90% of consumers.
- EPA: waters which, diluted 3 times, present organoleptic characteristics which are only just perceptible are admitted for consumption.
- European Union: water is considered drinkable if, when diluted 2 – 3 times it does not have any unpleasant odours and tastes at standard temperature (12 °C and 25 °C).
- Italy: the Decree of the President of the Republic of 24th May 1988 no. 236 on the quality of water for human consumption established the requisites evaluated on the basis of values and indications relative to the parameters as per Attachment I. The expression of the results of the organoleptic evaluations, both of the odour (to be compared with determinations of taste) and taste (to be compared with the determinations of odour) foresee in both cases a dilution rate of 0 (guiding value, VG) and a maximum admissible concentration (CMA) of 2 or 3, respectively at 12°C or 25°C.

The evaluation of the organoleptic characteristics of water that has been made drinkable is carried out by special analyses (Bruno and Nusca, 2000):

a) Analysis of the profile of the flavours (Flavour Profile Analysis) performed by adequately selected and trained people to smell samples of duly treated water (with the addition of 10 mg/L of mercury chloride to prevent bacterial growth) in standardized conditions, also concerning the temperature (cold: 20 °C for the WHO and 40 < °C for the APHA; warm: 58 °C for the WHO and 60 °C for the APHA). The measurement of the odour is made according to :

- intensity (facility of detecting the odour which can be expressed on various scales);
- measurability in the "Threshold Odour Number, TON), which expresses the number of progressive dilutions of the sample (all brought to 200 ml) until the apparent disappearance of the odour;
- quality (expressed according to previous experience and olfactory associations of pleasantness or unpleasantness which can be referred to a scale of odours of reference);
- acceptability (evaluated according to the psycho-sensory reaction of pleasantness or unpleasantness).

b) Determination of the taste by sampling (without swallowing) of 15 ml of sample in a 50 ml beaker at 40 °C, making reference to a scale of principal typical flavours (28 on the I.A.W.P.R.C. scale; D'Arca et al., 1972). The same method is also used for the release of odours and tastes from containers made of various materials.

The various causes responsible for giving unpleasant tastes and odours to drinking water include:

- the same treatments for making water drinkable which during the phases of ozonization and pre-chlorination form alomethanes which give a taste of chlorine or a medicinal taste which can be detected in tap water;
- the action of the monochloramine on the amino acids drained from the water of washout of the soil due to the formation of aldehydes (2-methylpropanale; 2-methylbutanal; 3-methylbutanal and phenylacetaldehyde) with a fruity odour;
- the presence of actinomycetes (more fre-

Substances	Characteristics	Producing algal species
Geosmin*	Intermediate intracellular product of the synthesis of carotenoids and chlorophyll that is released with decay and death of the cell. Oleous substance that boils at 270 °C, with a characteristic odour of earth. Levels < 30 ng/L are not perceptible and > 45 ng/L cause complaints by users. The maximum quantity is found in algal tricomes.	Anabaena macrospora Anabaena flos-aquae Anabaena circinalis Oscillatoria brevis Oscillatoria bornetii Planktothrix aghardii Aphanizomenon flos-aquae
2-methyl-isoborneol*	Intermediate product of the biosynthetic system of isoprenoids. Gives off an odour of mould. Levels > 8 – 10 ng/L cause complaints by users. The maximum quantity is found in algal tricomes.	Cianoficee: Phormidium tenue Oscillatoria tenuis
b-cyclocitral**	Moderate odour of tobacco smoke	cystis sp. M. aeruginosa
Hexanal and heptanal trans-4-heptanal and trans, trans-2,4-heptadienal	Odour of fish and rancid fat.	Uroglena sp. Dinobryon sp.
1-penten-3-one	Rancid odour	Khieri et al., 1995
Decanal		Uroglena sp. Dinobryon sp Asterionella formosa
Octatriene		Asterionella formosa Fragilaria crotonensis

*By means of chromatographic sniffing, geosmin and 2-methyl-isoborneol can be detected at concentrations of 7 and 4 ng/L respectively (Kenefick et al., 1992).
** The limit of detectability in GC-FIDis of 0.5 _g/L (Kenefick et al., 1992).

Table 7. Main chemical substances produced by algae responsible for unpleasant characteristics of water (Bruno and Melchiorre, 2000; Bruno and Nusca, 2000).

quently of the genii Actinomyces, Streptomyces, Actinoplanes, monospora, Nocardia, Streoptospora and Rhodococcus), environmental filamentous bacteria commonly to be found in water due to the washout of soils (with concentrations that can reach 105 – 3x106 individuals/g of mud and 2x104 – 103 individuals/ml of water); they grow slowly, taking about two weeks, and their spores settle in the dead points of the pipes (reaching values of 180,000 ufc/ml) causing characteristic odours of fish, grass and hay in the aerobic phase and of wood, potatoes and earth in the anaerobic phase;
- the presence of fungi (mainly of the genii Phialophora, Acremonium and Penicillium) conveyed by the washout waters which produce odorous substances, especially trichlorophenol (which is also formed during the chlorination of water) which in the presence of metal is transformed into 2,4,6-trichloroanisol;
- algae as they themselves produce odours and tastes but also because on the death of the cells they are a nutritive substratum for actinomycetes, bacteria, fungi and invertebrates which in turn produce odours and tastes.
From the scientific evidence available for algae (Bruno and Melchiorre, 2000) the following can be concluded:
- Almost all the algal phyla can produce odours and tastes:
- Cyanophyceae 45 % of the species
- Chrysophyceae, Cryptophyceae and Dinophyceae 14 % of the species
Chlorophyceae and Bacillariophyceae 21 % of the species:
- The cellular density required to produce a quantity of odorous substance perceptible by users is species-specific and ranges from a few thousand to a few million cells per 100 ml of water.
- The substances most frequently identified are: geosmin, 2-methyl-isoborneol, β-cyclocitral, saturated and unsaturated aliphatic hydrocarbons, aldehydes, chetones (table 7).
No correlation has been shown between the presence of cystin-LR and odorous substances in samples of algal biomass (up to 500 _g/g). In other words, the identification of odorous substances in reservoirs of freshwater cannot represent proof of the presence of cystins (Kenefick et al., 1992), as these are odourless and tasteless.
Strains of Planktothrix agardhii produce geosmin and a hydrosoluble hepatotoxin (Skulberg and Skulberg, 1985). Cystis aeruginosa produces MCYSTs and not geosmin and 2-MIB; but various other cystis produce _-cyclocitral (Hayes and Burck, 1988).

As a consequence of algal blooms in reservoirs of freshwater has to be conditioned in order to make it drinkable, using suitable treatment which allows the removal of the toxins (including cystins, nodularins, anatoxins, saxitoxins). Guiding values considered for drinking waters as far as cystin-LR is concerned are:
A – Provisional values of the WHO (European Conference of Bad Elster of 23rd-25th April 1997):
-1 µg/L for acute toxicity; 0.3 µg/L for chronic toxicity with promotion of tumours and potentially carcinogenic;
-according to the toxic production per cell found on average, 0.30 µg/L correspond more or less to 1,785,000 cells/L;
B - Circular on bathing by the Ministry of Health of 31st July 1998:
-limit of ingestion : 0.84 µg/L of cystins (5,000,000 cells/L);
-prohibition of bathing in freshwater: 5,000,000 cells/L;
-prohibition of bathing in seawater: more than 10,000,0000 cells/L;
-supervision of any cases of dermatitis or complaints and ascertainment of toxicity: between 100,000 and 5,000,000 cells/L in the case of freshwater and between 1,000,000 and 10,000,000 cells/L in the case of seawater with ascertainment of the toxicity between 100,000 and 1,000,000 cells/L;
-usual tests under 100,000 cells/L.
The prohibition of water contaminated by toxic algal blooms includes:
- use as drinking water that can entail acute and chronic intoxication;
- use in hospitals, due to the presence of toxins in products used for dialysis;
- bathing, due to acute intoxications and contact dermatitis;
- sprinkler irrigation, because the toxins are also harmful for plants. In addition, the algal cells dry on the leaves and may be swallowed with the vegetable, maintaining their toxic power for 24 – 48 hours;
- fishing, as fish species, if they do not have the possibility of leaving the area of blooms, are intoxicated in their turn and accumulate toxins in their internal organs and muscles, which are naturally self-purified in about 60 days;
- hunting in areas where the only sources for drinking are bodies of water with bloom (the prohibition preliminarily requires a histological and instrumental test of the presence of toxins in the tissues and organic fluids).
The Decree of the President of the Republic no. 236 of 24th May 1988 concerning the quality of water for human consumption, in Attachment I shows the relative requisites or parameters of quality (A. Organoleptic parameters; B. Chemical and physical parameters; C. Parameters concerning undesired substances; D. Parameters concerning toxic substances; E. biological parameters) the control of which guarantees in general the quality of drinking water. The possibility of keeping other parameters (other than those considered in Attachment I, letters A-E) which may represents factors of risk for the populations is considered only in the "Warning". "Algae" appear in the first position in the list of parameters.
More in general, the problem of the protection of the surface sea water and subterranean water is specifically regulated by the aforementioned Legislative Decree 152/1999 (and subsequent corrective amendments with Legislative Decree no. 258 of 18th August 2000) as republished in the form of an updated text in Official Journal no. 246 of 20th October 2000 (ordinary supplement no. 172). The objectives and also the temporal limits for their achievement have already been shown in Part One of this series of articles and shown below. Referring to the decree and the study of the 63 articles of the text, only some of the general aspects and objectives are reported here:
art. 4, art. 5, art. 6, art. 7, art. 9, art. 10, art. 12, art., art. 15
- identification, for the purposes of the protection and reclamation of surface and subterranean waters of :
- "minimum objectives of environmental quality" for significant bodies of water, defined according to the capacity of the bodies of water to maintain the natural processes of self-purification and to support large and highly diversified animal and plant communities. The regions identify for each significant body of water, or part of it, the class of quality corresponding to one of those shown in Attachment I and adopt the relative measures necessary. 31st December 2016 is the date established to reach the state of "good" quality for each body of water, but by 31st December 2008, each body of water must achieve the requisites of the "sufficient" state (definitions if Attachment I).
- "objectives of quality by specific destination", which identify the state of the bodies of water suitable for a particular use by man, life of fish and molluscs. Water with a specific functional destination is:
a) surface freshwater for the production of drinking water, which is classified (according to the characteristics in Attachment 2, table 1/A) in the categories A1 (simple physical treatment and disinfecting), A2 (normal physical and chemical treatment and disinfecting) and A3 (advanced physical and chemical treatment, refining and disinfecting);
b) waters for bathing (meeting the requisites as per the decree of the President of the Republic no. 470(a) of 8th and amendments;
c) freshwater that requires protection and improvement in order to be suitable for the life of fish (requisites in attachment 2, table 1/B). The following are privileged: a) courses of water that cross the territory of national parks and natural reserves of the State, as well as natural and artificial regional parks; b) natural and artificial lakes, ponds and other bodies of water located in the aforementioned territories; c) surface freshwater included in the marsh areas declared "of international importance" d) surface fresh water (not included in the previous categories) which show great scientific, naturalistic, environmental and productive interest) as they represent the habitat of rare animal or plant species or ones in danger of extinction, or the seat of complex aquatic ecosystems deserving preservation or the place of ancient and traditional forms of fish production;
d) water for the life of molluscs in coastal and brackish seawater (requisites in attachment 2, table 1/C).

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