

Harmful cyanobacterial toxic blooms in waste stabilization ponds

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Abstract A coccoid picocyanobacterium *Synechocystis* sp. (0.6-2 µm of cell diameter) was found to be dominant during summer period in the experimental wastewater stabilisation pond of Marrakesh. The taxonomy of this isolated strain was confirmed by electron microscope study. The general patterns of ultrastructure and the mode of cell division resemble *Chroococcales*. The cyanobacterium strain was axenic and cultured on both inorganic Z8 and BG13 media. Mammalian toxicity was confirmed by mice bioassay. The major symptom of poisoning was severe diarrhoea. Histopathological study shows a slight hepatotoxicosis associated with a pronounced change in the intestinal mucosa which shows swelling and destruction of villi epithelium and shedding of enterocytes into the lumen. Although slow, these kinds of poisoning are comparable to those induced by okadaic acid intraperitoneal mice injection (diarrhetic shellfish poisoning "DSP" toxins). By using the enzyme-linked immunosorbent assay (ELISA), the amount of hepatotoxins "microcystins" was determined. The result shows that *Synechocystis* can produce a small amount of total microcystine [an average of 15 µg⁻¹ dry weight corresponding to 20 ng(10⁹cell)⁻¹]. These findings lead us to consider *Synechocystis* as both a potent neurotoxin and hepatotoxin producer. Because of the confirmed cyanobacterium toxicity, an eventual ecological implication should be considered. However, a toxic chronic test experiment on *Daphnia* was simultaneously carried out. Juvenile *D. magna* (less than 24 hours old), were fed three concentrations (10⁴, 10⁶, 10⁸ cells / ml) of *Synechocystis*. A group of organisms fed with *Chlorella vulgaris* (3. 10⁵ cells/ml) and another group without food, were studied as control treatments. Only animals cultured with 10⁴ cells/ml of cyanobacterium survived at 80% until the end of the test (21 days). Reproduction and normal growth occurred in control treatments fed with *Chlorella vulgaris* and the group fed with the lowest concentration of *Synechocystis*. One-way ANOVA statistical analyses show significant differences in *Daphnia* survival and growth, between treatments with and without *Synechocystis* and between treatments with and without food. In terms of this study, there is evidence that toxic picocyanobacteria blooms occurring in wastewater stabilization ponds of Marrakesh, could have harmful repercussions on zooplanktonic, bacteria and other algae communities. Consequently, this will constitute a possible hindrance for sewage self-purification process and system treatment performance. In addition, the reuse of such treated wastewater effluent for irrigation will constitute an additional, potent, health hazard for animals and human's.

Keywords *Chlorella*; *daphnia*; microcystins; picocyanobacteria; *synechocystis*; wastewater

Introduction

The assimilative capacity of stabilization ponds systems depends on the interaction between the ecosystem's various biotic and abiotic factors. Among the biotic factors, algae play an essential role in the self-purification process. During biological sewage purification using stabilization ponds or other biological treatment systems, microalgae can operate and affect the treatment process in many ways (Wong and Tam, 1998). Although the quality of wastewater effluent and climatic conditions may vary, some algal genera are common to

wastewater stabilization ponds. In Marrakesh sewage ponds, the picocyanobacterium *Synechocystis* sp. is of particular interest (Mezrioui and Oudra, 1998). The picocyanobacteria flora, essentially *Synechococcus* and *Synechocystis* genera, can account for a significant fraction of phytoplankton productivity and biomass in various aquatic systems (Lincoln and Hill, 1980; Maeda *et al.*, 1992; Mezrioui and Oudra, 1998). There are advantages to using eukaryotic microalgae rather than prokaryotes (cyanobacteria) for biological treatment purposes. Toxic cyanobacteria are now recognised as a hazard to human and animal health. The bloom-forming cyanobacterium *Microcystis* seems to be the most isolated toxic strain studied and the most widely distributed among toxigenic blue-green algae (Watanabe *et al.*, 1996). The taxonomic connection between *Synechocystis* and *Microcystis* (Chroococcales) leads one to think about their potential toxicity and toxin production. In addition, the toxicity of some of *Synechocystis* and *Synechococcus* isolated strains has been confirmed (Lincoln and Carmichael, 1981; Mitsui *et al.*, 1987; Oudra *et al.* 1998a). So far, toxic picocyanobacteria blooms have not been seriously taken into consideration. Therefore, much interest should be given to these kinds of blue-green algae, which could develop nuisance blooms. Current literature dealing with cyanobacteria-zooplankton interaction reports that there is evidence of a negative effect of toxic cyanobacteria on aquatic herbivorous invertebrate zooplankton survival, growth and reproduction, as Daphnids (Reinikainen *et al.*, 1995; Sbiyyaa *et al.*, 1998) and rotifers (Gilbert, 1996) and fish (Bury *et al.*, 1996). Some observations concerning the possible effect of *Synechocystis* blooms on the disappearance of cladoceran populations (e.g. *Miona micrura*) from the experimental wastewater stabilisation pond of Marrakesh, have been reported by Tifnouti and Pourriot (1989). According to these findings, it is evident that toxic picocyanobacteria blooms occurring in sewage treatment ponds, could present a possible hindrance to the self-purification process and will constitute an extra sanitary hazard for wastewater reuse. The aim of this work was to investigate the toxicological aspects of this isolated picocyanobacterium *Synechocystis* and its eventual ecological implications (whether the toxins present an acute and chronic hazard to Cladocera, *Daphnia magna*).

Materials and methods

Cultivation of cyanobacterium and toxicological tests

For mammalian toxicity (mice bioassay), the isolation and purification of the cyanobacterium strains were performed using 2.5% agar BG13 solid medium (Ferris and Hirsch, 1991). The strain was axenic, according to the method previously described (Oufdou *et al.*, 1998). The cultures were routinely maintained in 1000 ml of BG13 medium in 2 litre conical flasks, and were constantly illuminated by cool white fluorescent light, which give a measured irradiance of $62 \mu\text{E m}^{-2} \text{s}^{-1}$ (Quantameter Li-cor 185A). The room temperature was kept at 26°C . The culture flasks were constantly agitated by magnetic stirring. The cells were harvested by centrifugation at the end of the logarithmic phase of growth. The lyophilized cells in 0.9% NaCl, were then assayed to determine the intraperitoneal (i.p) lethal dose for 50% of the animals tested (LD_{50}) using male Swiss mice 18-22g. After injection, the animals were observed continuously for four hours. Symptoms and survival times were recorded. For confirmation of poisoning signs, histopathological examination of target organs (liver and intestine) was performed. By application of the highly sensitive enzyme-linked immunosorbent assay, the content of microcystins toxins produced by *Synechocystis* strain, was determined (An and Carmichael, 1994) using the Millipore plates kits "EnviroGrad™ Microcystins Plate Kit SDP222S4".

For the Daphnid toxicity test, the cyanobacterium non-axenic strain was cultured under $24 \mu\text{E m}^{-2} \text{s}^{-1}$ fluorescent lights cool-white continuous illumination, at 20°C room temperature in 5-l Erlenmeyer flasks containing 3l of the inorganic medium Z8 (Kotai, 1972). The

cells were harvested during the exponential growth phase and concentrated by centrifugation to obtain the highest cells concentration (10^{12} cell)/ml, algal suspension, from which culture dilutions (10^8 , 10^6 , 10^4 cells/ml) were prepared. The algal food suspensions were stored in the dark at 4°C, all the *Synechocystis* aliquot suspensions were changed each week. The direct cells count was determined by the Haemocytometer-Mallassez, 0.2 mm deep.

Cultivation of Cladoceran (*Daphnia magna*)

For the experiment, female *Daphnia magna* were obtained by keeping mature individuals (picked from culture maintained as a clone at Department of Zoology, Faculty of Science, Porto). The Daphnids were cultured in standard medium for toxicity testing M4 medium (Elenet and Bias, 1990). They grew under favourable conditions (20°C temperature and 14/10-light/dark cycle) and were fed with $3 \cdot 10^5$ cells/ml of *Chlorella vulgaris* (green algae growing on Z8 medium). After some days, juvenile animals (0-24 hours old) were picked for the experiment. An appropriate number of juveniles were randomly separated into similar conditions. Five replicates were used for each treatment concentration (one animal in 50 ml of M4 medium). The daphnids used for the chronic test were fed daily with cyanobacteria cells suspensions and the culture medium was renewed daily. Control experiment series were kept without food or fed with *Chlorella vulgaris*. During the experiment time (21 days) and for each treatment, the *D. magna* size was estimated by measuring via animal exuvia, the first exopodite of the second antenna which was significantly correlated to body length ($r^2 = 0.99$). This method was previously described and statistically discussed by Barros (1994). The experiments reported above were run with a new-born *Daphnia* with around 1.5 mm body length. The experiments include also the estimation of survival time of *Daphnia* exposed to various concentrations of cyanobacteria strain and reproduction behaviour in each experimental series.

Results and discussion

Morphology and taxonomy of the isolated cyanobacterium strain

Among the smallest known planktonic cyanobacteria are the unicellular coccoid, ovoid and rod-shaped picoplanktonic species (cell ranging in linear dimension from 0.2 to 3 µm) that compare with the Eubacteria in both shape and size (Pearl, 1988). It appears that the picocyanobacteria bloom occurring periodically during the hot period, was one of the peculiarities of the Marrakesh sewage pond operating under an arid climate. The picocyanobacteria bloom was in major part constituted by the chroococcoid *Synechocystis* genus, the dominance of which induce is an apparent change of the color of the pond effluent which turns a brilliant green (Mezrioui and Oudra, 1998). A similar phenomenon was observed and described in large outdoor algal cultures used for protein production (Lincoln and Hill, 1980). According to *in situ* growing wild strain size (0.6-1.2 µm of cell diameter), the species identified most closely with, although *S. parvula* Perfiliew as described by Stramach (1966). The cultured laboratory strain had a larger cell diameter, which ranged from 1.4 µm to 2.6 µm. This last change leads us to refer to the genus *Synechocystis*, without species designation. This genus occurs widely in both marine and freshwater habitats. The electron micrograph shown in Figure 1 pictures the major fine structural components of the prokaryote chroococcaeal cells with polyphosphate and cyanophycin granules forming a circular cluster in the centre of the cell and with the photosynthetic thylakoides entirely peripheral.

Toxicological study on the cyanobacterium

An axenic cyanobacterial culture was harvested at the end of the logarithmic growth phase.

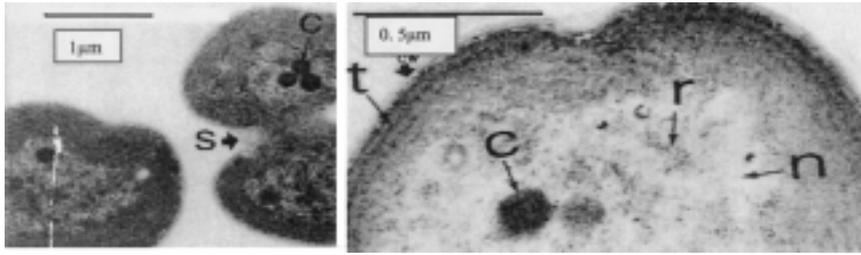


Figure 1 Electron micrograph of *Synechocystis* cells magnified x11000 and x19000 showing ultrastructure of picocyanobacterium (S: septum formation, C: cyanophycin granule, n: nucleoplasm, r: ribosomes, t: thylakoids (photosynthetic lamellae), cw: cell wall).

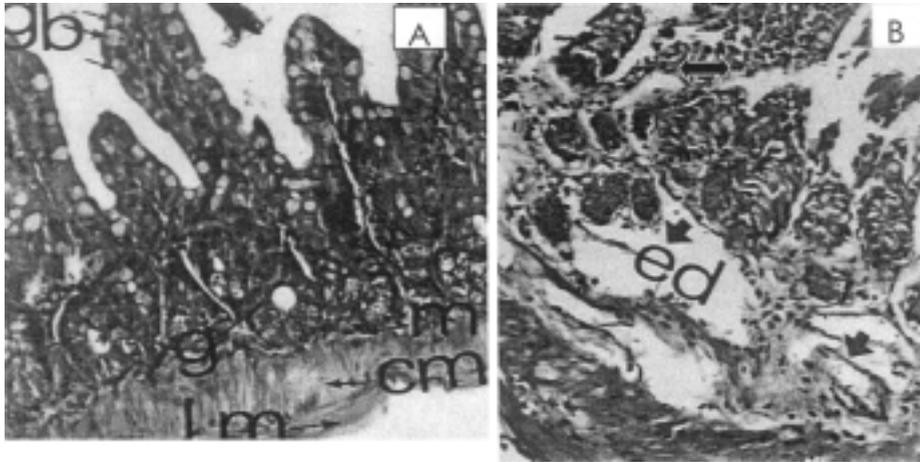


Figure 2 Histological examination of the small intestine of mice (E&H stain). A: normal structure of the villi of control intestine with normal goblet cells (gb), intestinal gland in crypt region (g), muscularis mucosae (m) and circular and longitudinal muscle (cm, lm) (x200). B: mice small intestine acutely poisoned by i.p injection of *Synechocystis* aqueous extract, showing intestinal damage with shedding of epithelium of the villi (↔) and destruction of the crypt region by oedema (edm) (x400).

Concerning the mammalian toxicity assessment, the results show that an intraperitoneal mice injection (i.p) of lyophilised material at 650 mg/kg was lethal in all cases. The calculated 24-h LD₅₀ ranged from 150 to 350 mg/kg. The survival time at the lethal dose was about 7-12 hours. The major poisoning symptoms manifested were ataxia, paleness, piloerection, “salivation” and severe diarrhoea, which appears 15-30 min after injection. Histopathological examination showed slight liver damage, with some extensive haemorrhaging and sinusoid capillary destruction. Nevertheless, this observed liver injury was less drastic than that induced by (i.p) injection of *Microcystis* cells (Oudra *et al.* 1998b). In order to investigate the poisoning symptoms, histopathological examination on the small intestine was also performed. The results showed a pronounced change in the intestinal mucosa with swelling and destruction of epithelium of the villi and shedding of enterocytes into the lumen, resulting in a complete collapse of the villi architecture. There was also visible damage of the crypt region (Lieberkühl glands) and an inflammatory reaction was also observed (Figure 2).

The results showed that *Synechocystis* could induce two kinds of poisoning with hepatotoxic and gastrointestinal disturbance symptoms. The content of hepatoxins “microcystins” was determined by application of ELISA technique. The result shows that *Synechocystis* can produce a small amount of microcystine (cyclic heptapeptide), On average 15 µg/g dry weight. This estimated content of toxins seems to be very slight in

comparison with *Microcystis* Moroccan isolated strain, which contains 600 µg/g dry weight (Oudra et al., submitted). It is recognized that “microcystins” were considered the principal hepatotoxic toxins produced by *Microcystis* strains (Watanabe et al., 1996). The hepatotoxic effect of a *Synechocystis* sauvageau sp. isolated from a large outdoor algal culture used for protein production has been also confirmed by Lincoln and Carmichael (1981). Nevertheless, these authors reported that mice poisoning symptoms were essentially hepatotoxic without any signs of diarrhoea. The reported that the general effects of *Synechocystis* sauvageau toxin are indicative of a peptide of the anatoxin-c type, although the survival time was longer than the 1 to 3 hours typical of anatoxin-c. In our case, we could not suggest the existence of anatoxin-c type with a survival time longer (7 to 12 hours). Working with *Synechocystis* strain, which induce diarrheal effects as a major mice poisoning symptoms, led us to compare the intestine damage with that induced by okadaic acid (OA) and dinophytoxins (DTX) in suckling mice and rats, respectively, described by Tero et al. (1986) and Lange et al. (1990). The enterotoxic activity of OA and DTX was also observed by Hamano et al. (1986) by means of suckling mice bioassay. They confirmed the destructive effect on epithelium of small intestine with oedema of *lamina propria* of villi. In this present investigation, the poisoning effect caused by *Synechocystis* on the small mice intestine appears to be as drastic as that described by Tero et al. (1986) and Hamano et al. (1986). According to these findings, we suspected the existence of a mixture of microcystins and other diarrheagenic toxins (like DSP toxin compounds).

Toxic chronic effect of *Synechocystis* on *Daphnia* (ecological implication)

The first results obtained from this experiment to test the chronic toxicity effect of *Synechocystis* on *Daphnia magna*, show that without an alternative adequate food source, the ingested cyanobacterium cells are very toxic to juvenile *D. magna* at a high concentration level (10^8 cells/ml). In comparison with control treatments (Daphnids without food and fed with *Chlorella vulgaris* (3×10^5 cells/ml) the survival time of animals feeding with 10^8 cells/ml and 10^6 cells/ml of *Synechocystis* did not exceed 7 days. Only animals cultured with 10^4 cells/ml of the cyanobacterium survived at 80% until the end of the experiment (21 days) (Figure 3A). The result of one-way ANOVA analysis (Table 1) showed a highly significant effect of food nature levels on *Daphnia* survival ($P < 0.05$). The Newmann-Keuls test, confirmed that concentrations of cyanobacteria influence strongly the animal's survival time. Concerning the toxic effect of ingesting cyanobacterial cells on *Daphnia* reproduction, it appears that normal reproduction only occurred in the control treatment with *Chlorella vulgaris* (Table 2). However, *D. magna* cultured with the lowest and sub-lethal concentration of *Synechocystis* (10^4 cells/ml), showed a delay in its reproduction cycle. The results confirm that toxic ingested cells of *Synechocystis* could inhibit or delayed the *Daphnia* reproduction.

The experiments show a significant effect of cyanobacterial ingested cells on development and growth of these animals. There was a significant difference in *Daphnia* size between treatment with and without *Synechocystis* and between treatment with and without food (Figure 3B). According to each experiment conditions, the estimated *D. magna* growth rates were remarkably different (Table 2). It is evident that the growth rate of *D. magna*, feeding on sub-lethal *Synechocystis* dilution was more important than that recorded in the control experiment without food. The toxic effect of *Synechocystis* reduced *D. magna* growth rate by 29% - 75% of controls fed with *Chlorella vulgaris*. *Daphnia* starvation reduces the growth at 45%. The results of a one-way ANOVA analysis, show a significant effect of higher cyanobacteria concentration on daphnid growth ($P < 0.05$). The Newman-Keuls test, showed that each food quality has a specific action on daphnia growth (Table 3). Toxins produced by several species of cyanobacteria are a potentially serious

environmental problem. These substances can be acutely toxic as evidenced by the death of livestock and other animals that have been exposed to them. Although, the mammalian toxic effects of cyanobacterium *Synechocystis* genus have been confirmed its effect on Cladocera was scarcely reported (Arnold, 1971). Our results showed that *Synechocystis* ingested cells are toxic to the Cladocera, *D. magna*. The same results were found by many authors working with *Microcystis*, *Oscillatoria* strains (Nizan *et al.*, 1986; Demott *et al.* 1991; Sbiyyaa *et al.*, 1998). The exposure of *D. magna* to high concentrations of *Synechocystis* without an alternative food, induced acute toxicity which caused total mortality within 120 hours of culture time.

Daphnids fed with sub-lethal amount of *Synechocystis* (10^4 cells/ml), had a long survival time, but the reproduction and growth were slightly affected. We have recent evidence that *D. magna* is influenced by nutritional quality of food and cyanobacterial toxins can directly impact Cladoceran production, growth and survival. Consequently, *D. magna* cannot survive on a *Synechocystis* diet. This toxicity may have resulted essentially from cyclic heptapeptide “microcystins” which was determined among other unidentified compounds in this cyanobacteria strain culture (around 15 μ g/g dry weight). These products

Table 1 Chronic effect of *Synechocystis* ingested cells on survival of *Daphnia*. Results of one way ANOVA analyse (5%) and Newmann–Keuls test.

Food	%survival	Homogenous groups	
Chl	100	A	
No	100	A	
Syn1	88	A	
Syn2	38	B	
Syn3	19	C	
	Df	F	P
Total variation	9.9		
Food	4	53.48	0.00
Residual variation	95		

Table 2 Computation of *Daphnia* global growth rate and total number of viable new-born Daphnids, according to food level and quality. (*Growth rate calculated from only 5 days of survival time).

Nature of food	No	Chl	Syn1	Syn2	Syn3
Growth rate (day-1)	0.11	0.24	0.18	0.07	0.11*
Cumulus of new-born	0	133	5	0	0

Table 3 Chronic effect of *Synechocystis* ingested cells on Growth of *Daphnia*. Results of one way ANOVA (5%) analyse and Newmann–Keuls test .

Food	Mean growth	Homogeneous groups	
Chl.	3.73	A	
Syn1	3.03	B	
No	2.47	B C	
Syn2	2.15	C	
	Df	F	P
Total variation	79		
Food	3	11.64	0.00
Residual variation	76		

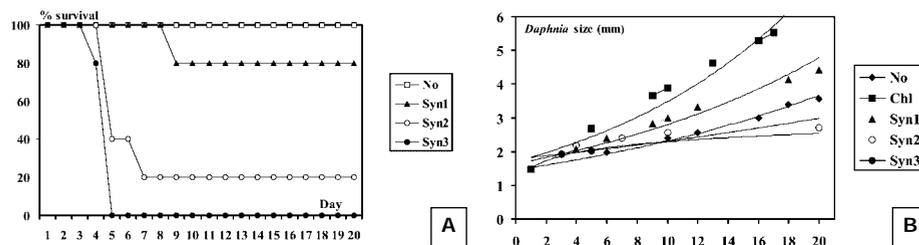


Figure 3 A: Effect of *Synechocystis* ingested cells on *Daphnia magna* survival. B: Variation of *Daphnia magna* growth size according to food quality. (Syn1: 10^4 cell/ml, Syn 2: 10^6 cell/ml and Syn 3: 10^8 cell/ml of *Synechocystis*. Chl: Concentration of *Chlorella vulgaris* cells ($3 \cdot 10^5$ cell/ml). No: Without food.)

realised essentially by *Microcystis* strain have been reported as toxicant to zooplankton in particular Cladoceran species (Watanabe *et al.*, 1996).

Conclusions

The first part of results obtained in this study confirms the toxicity of one strain of *Synechocystis* genus isolated from wastewater oxidation ponds. Hepatotoxic effect's were induced by "microcystins" toxins produced by *Synechocystis* strain. Diarrheal effects associated with hepatotoxicosis confirm that *Synechocystis* could produce a mixture of various toxins. Results of the chronic toxicity on *Daphnia* (Cladocera) test show the eventual ecological implication of this picocyanobacteria bloom in reducing *Daphnia* population. This toxic picocyanobacterium was found dominant during the summer period in the experimental wastewater stabilization ponds of Marrakech. This strain has been also recognised as a biotic selective agent against bacterial populations, in particular pathogenic bacteria growing in wastewater effluent (Mezrioui *et al.*, 1994). In addition, harmful cyanobacterial toxins could affect all aquatic organisms in the food chain (Kotak *et al.*, 1996), particularly rotifers (Gilbert, 1996), and fish (Bury *et al.*, 1996), and even the photosynthetic activity of plants (Abe *et al.*, 1996). In stabilisation ponds, self-purification process could not be complete without a positive microalgae, bacteria and zooplankton interaction. Generally, green algae present many advantages, whereas toxic cyanobacteria pose technical problems to this biological treatment system.

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