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Toxic concentrations of metronidazole to *Microcystis protocystis*

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Abstract

Antimicrobials are among the most commonly used drugs and have become a class of contaminants with great environmental importance. Metronidazole is an antimicrobial used for the therapeutic management of several human diseases. The toxicity of antimicrobials on aquatic species may affect sensitive microorganisms and reduce metabolic processes. Cyanobacteria is a group of organisms that are of great ecological importance in aquatic environments. Studies indicate that cyanobacteria are very sensitive to some antimicrobials. Therefore, it is necessary to evaluate the effects of metronidazole contamination on phytoplankton. The aim of this study was to investigate the effects of metronidazole on the growth of the cyanobacterium *Microcystis protocystis* and to evaluate the stability of this antimicrobial agent in the culture medium over a period of 96 hours. *M. protocystis* was resistant to growth inhibition by metronidazole. The EC50 of this antimicrobial for *M. protocystis* was 117.3 mg L^{-1}. Under the growth inhibition test conditions, neither a significant change in the MNZ concentration nor the presence of drug metabolites or degradation products was observed. These results indicate low cellular uptake of the antimicrobial agent and its persistence in the culture medium.

Keywords: antimicrobials, toxicity, metronidazole, cyanobacterium, *Microcystis protocystis*.

Concentrações tóxicas de metronidazol para *Microcystis protocystis*

Resumo

Antimicrobianos estão entre os fármacos mais usados e tem se tornado uma classe emergente de contaminantes com grande importância ambiental. A toxicidade dos antimicrobianos sobre as espécies aquáticas podem afetar microrganismos sensíveis e reduzir seus processos metabólicos. O Metronidazol é um antimicrobiano usado para o manejo terapêutico de várias doenças humanas. Cyanobacteria é um grupo de organismos de grande importância ecológica no ambiente aquático. O estudo visou investigar os efeitos do metronidazol- MNZ sobre o crescimento da cianobactéria *Microcystis protocystis* e avaliar a persistência do antimicrobiano no meio de cultivo durante 96h. *A M. protocystis* mostrou-se resistente à inibição de crescimento pelo MNZ. A $EC_{50\%}$ do MNZ para a espécie foi 117,3 mg L^{-1}. Nas condições do teste de inibição de crescimento não foi observada variação significativa da concentração de MNZ nem a presença de metabólitos ou produtos de degradação do fármaco, indicando baixa captação celular do antimicrobiano e sua persistência no meio de cultivo.

Palavras chave: antimicrobiano, toxicidade, metronidazol, cianobactéria, *Microcystis protocystis*.

In recent decades, pharmaceuticals have become a growing class of organic contaminants with great environmental importance. Several studies have detected the presence of pharmaceutical residues in wastewater, surface water, and even treated water (Kümmerer, 2001; Yamamoto et al., 2007; Zuccato et al., 2010).

Antimicrobials are among the most commonly used drugs, and they are a major concern from an environmental point of view. The purpose of these compounds is to interrupt the growth of their biological targets, however, in aquatic environments, they may also affect non-target organisms and compromise the survival of these species.

The widespread use of antimicrobials and their inappropriate disposal have been postulated to be predisposing factors for the increase in antimicrobial resistance (Mokracka et al., 2012; Pauwels and Verstraete, 2006; Zuccato et al., 2010). The contamination of surface water by antimicrobials increases the selection of resistant microorganisms and the transfer of genetic material between species. These effects may lead to the emergence of human pathogens that are resistant to clinically available therapeutic agents (Pauwels and Verstraete, 2006).

The uses of antimicrobials for the clinical treatment of infections in humans and animals and for increasing animal productivity are the usual sources of pharmaceutical residues in the environment (Zuccato et al., 2010). Sewage from houses, hospitals, and animal farms are the most significant sources of pharmaceuticals found in the environment.

After entering the water system, organic compounds typically begin the natural processes of depuration, particularly biodegradation. The toxicity of antimicrobials on aquatic species may affect sensitive microorganisms and reduce metabolic processes. As a result, these antimicrobials persist in the environment.

Metronidazole (MNZ) [2-methyl-5-nitro-1*H*-imidazole-1-ethanol] (Figure 1) is an important drug for the therapeutic management of several human diseases caused by protozoa, anaerobic bacteria, and periodontal processes (Tally and Sullivan, 1981; Bendesky et al., 2002; Yu et al., 2009; Freeman et al., 1997). This antimicrobial is also widely used for the treatment of animals, particularly for parasitic infections in fish (Sakamoto et al., 2011; Zeleny et al., 2009). Because it is inexpensive, effective and safe, MNZ is often used both in the clinic and for the management of animals.

It should be noted that the bactericidal effect of MNZ on anaerobic bacteria is due to its reduction to a highly reactive intermediate in the bacterial intracellular environment, which causes DNA double strand breaks (Bendesky et al., 2002). This process is mediated by a ferredoxin system that is characteristic of anaerobic microorganisms. However, the antibiotic is also active against protozoa and other cytotoxic and mutagenic effects of MNZ are known (Bendesky et al., 2002) and can affect the sensitivity of exposed microorganisms (Lanzky and Halling-Sørensen, 1997).

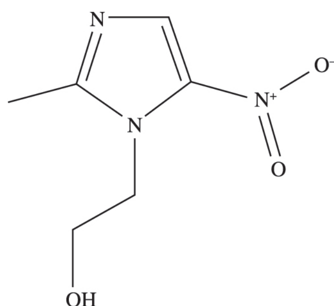

Figure 1. Chemical structure of Metronidazole – MNZ.

Cyanobacteria are a group of organisms that are of great ecological importance in aquatic environments. They are primary producers, therefore they have the ability to fix atmospheric nitrogen and store phosphate in their biomass, and they significantly participate in the cycling of the major organic elements carbon, nitrogen, and phosphorus (Lovelock et al., 2010; Scott, 2010). These species may play an important role in the environmental depuration of xenobiotics by using organic compounds as a carbon source, leading to the mineralisation of xenobiotics or promoting the biotransformation of these agents.

Among the cyanobacteria, the genus *Microcystis* is often found in polluted aquatic environments. This characteristic suggests that these species have efficient mechanisms of cellular protection, such as a physical barrier against penetration by xenobiotics, biodegradation, bioaccumulation, or mineralisation of contaminants, which justifies studying their role in the bioremoval of organic contaminants and the elucidation of their resistance mechanisms.

The available ecotoxicity studies indicate that cyanobacteria are very sensitive to some antimicrobials (Fent et al., 2006). Therefore, it is necessary to evaluate the effects of MNZ contamination on phytoplankton.

The aim of this study was to investigate the effects of MNZ on the growth of the cyanobacterium *Microcystis protocystis* cultures in the laboratory and to evaluate the stability of this antimicrobial agent in the culture medium over a period of 96 hours.

1. Materials and Methods

The drug used was a generic injectable metronidazole solution at a concentration of 5 mg mL^{-1}(0.5%) manufactured by the pharmaceutical company Isofarma Industrial Farmacêutica Ltda –Ce, Brasil.

The chemical reference for metronidazole was provided by the Brazilian Pharmacopeia with 99.6% drug purity.

The reagents, solvents, and chemicals used in this study were of analytical or chromatographic grade. All solutions were prepared using Milli-Q water (Millipore®, USA).

Culture The cyanobacterium *Microcystis protocystis* was isolated from water samples collected in the Dom Helvécio lake located in Rio Doce State Park, Minas Gerais, Brazil. The species is maintained in culture in the cyanobacteria bank of the Laboratory of Limnology and Aquatic Ecology at the Biological Science Institute of the Federal University of Minas Gerais (LIMNEA-ICB-UFMG).

Culture medium The medium used to culture *Microcystis protocystis* was ASM-1 (Gorham et al., 1964). In total, 750 mg L^{-1} of 3-(N-morpholino) propanesulfonic acid (MOPS), pKa = 7.2 at 25 °C, was added to freshly prepared and sterilised medium. The pH was adjusted to 7.0 with either 0.1 mol L^{-1} HCl or NaOH solution.

Experiments *Microcystis protocystis* in ASM-1 medium was incubated at 25 °C ± 2, irradiance of 98 mmoles.m^{-2}.s^{1} and 12 hour photoperiod until a cellular density of 10^6 cells mL^{-1} was obtained. The culture was distributed into Erlenmeyer flasks in triplicate with increasing concentrations

of MNZ (50-150 mg L^{-1})The tests and controls were maintained with stirring on a shaker table at 25 °C ± 1 and continuous light. After 96 hours, the biomass was estimated by optical density, and the chlorophyll concentration was measured (OECD, 2006).

Optical Density (OD) After homogenisation of the culture, 5 mL aliquots were removed and transferred into 10 mm cuvettes. The absorbance of the sample was determined at a wavelength of 680 nm.

Chlorophyll dosage After homogenisation of the culture, vacuum-filtered 20 mL aliquots were removed. The membrane (Whatman GF/C 0.45 mm) was macerated, and the sample was transferred to 15 mL tubes. Acetone was added as the extracting agent, and the final volume was adjusted to 10 mL. The mixture was protected from light and stored in a refrigerator. After 2 hours, the mixture was centrifuged for 20 minutes at 3000 rpm. The supernatant was transferred to a 10 mm quartz cuvette, and spectrophotometric readings were taken at four different wavelengths, 630 nm, 647 nm, 664 nm and 750 nm. The chlorophyll *a* concentration was calculated using the Jeffrey and Humphrey method (Jeffrey and Humphrey, 1975) and expressed in mg L^{-1}(APHA, 1999).

Determination of the EC_{50} of *M. protocystis* The cyanobacterium growth rates in the tests and controls were calculated by the chlorophyll *a* yield as a function of time. The EC_{50} was measured from the linear equation of the growth inhibition rates as a function of the MNZ concentrations (OECD, 2006).

Extraction of mucilage After 21 days of growth, the *M. protocystis* culture was centrifuged, and the biomass was separated and dried at 60 °C. Two volumes of ethanol were added to the supernatant. The mixture was stored for 2 hours in a refrigerator to separate the mucilage. The mucilage was separated by centrifugation at 4000 g for 20 minutes at 15 °C and then dried in an oven at 60 °C until a constant weight was reached (Torres et al., 2007). The raw yield was calculated as the ratio of the cyanobacteria dry biomass to the dry weight of the mucilage.

Extraction of MNZ from the cultures After culture homogenisation, a 5 mL aliquot was removed and filtered (Millex, PVDF, 0.45 mm, 33 mm). Then, 0.10 mL of the filtrate was transferred to a volumetric flask, and the volume was adjusted to 10 mL. Three hundred microlitres of this solution was removed and transferred to a 2.0 mL microtube, and 1.0 mL ethyl acetate was added. The mixture was centrifuged at 12,000 rpm for 10 minutes at 5 °C after agitation for 30 seconds in a vortex mixer. Eight hundred microliters of this solution was transferred from the organic phase to a microtube, and the solvent was removed by vacuum centrifugation for 25 minutes at 60 °C. The pellet was dissolved in 300 µL mobile phase.

Analysis of MNZ stability in the culture medium MNZ concentrations were determined by high-performance liquid chromatography (HPLC), and detection was performed by spectrophotometry in the ultraviolet region (UV). Twenty microliters of the samples was injected into a Thermo Scientific modular chromatographic system with a PDA Plus detector and autosampler at 5 °C. The separation was performed using a Shimadzu column (150 mm × 4.6 mm, 5 mm) at 30 °C, and the mobile phase consisted of 10 mM monobasic sodium phosphate pH 3.0 and acetonitrile (80:20 v/v) with a 1 mL min^{-1} flow rate. The run time was 3.5 minutes, and the chromatograms were obtained with detection at 317 nm.

2. Data analysis

The initial and final concentrations of metronidazole in the culture medium were compared by analysis of variance for paired samples (ANOVA) using the Statistica 7 program. The differences between the treatments were considered significant when $p < 0.05$.

To determine the EC_{50}, the concentrations used to study the growth inhibition of *M. protocystis* by MNZ were defined based on the EC_{50} values of microalgae previously studied (Lanzky and Halling-Sørensen, 1997) and preliminary assessments. The concentrations for the toxicity test ranged from 50 mg $L^{-1}$to 150 mg L^{-1}. The EC_{10} and EC_{50} were 57.2 and 116.88 mg L^{-1}, respectively (Figure 2).

The EC_{50} for MNZ against *M. protocystis* was greater than the EC_{50} for the green algae (Chlorophyta), *Chlorella* sp. (12.5 mg L^{-1}), and *Selenastrum capricornutum* (40.4 mg L^{-1}), as determined by the production of chlorophyll (Lanzky and Halling-Sørensen, 1997). These results suggest the possibility of change in the richness and dominance of the phytoplankton community towards favouring species that are resistant to contamination by xenobiotics.

The growth inhibition of *M. protocystis* exposed to increasing concentrations of MNZ as measured by the chlorophyll *a* yield is shown in Figure 3.

Chlorophyll is the primary photosynthetic pigment, and it is widely used to estimate the phytoplankton biomass in surface freshwaters (APHA, 1999). The productivity of chlorophyll *a* as a function of MNZ concentration indicates that the photosynthetic system of the species is affected by lower concentrations of the drug than those necessary to inhibit the growth of the species. However, it should be noted that the spectrophotometric method

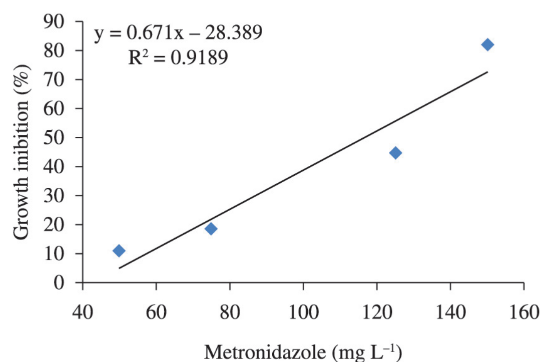

Figure 2. Percent growth inhibition of *M. protocystis* by MNZ.

for measuring chlorophyll is subject to interference from processes such as pigment decomposition. This optical method may either under- or overestimate the concentration of chlorophyll due to its degradation or overlap with the absorption of pigments, variability may also occur in the individual intracellular levels if this pigment, which may not reflect the levels in the entire biomass (Šoštariè et al., 2009; Sharma et al., 2011).

Analytical assessment of the xenobiotic concentration in the culture medium is essential to ensure the validity of the toxicity data and to evaluate the role of this species in bioremoval of the pollutant.

The cultures used in the growth inhibition tests were examined by HPLC/UV. To validate this method, the following values for MNZ were determined: the lower limit of quantification (100 mg L^{-1}), linearity (100-1600 mg L^{-1}), selectivity, precision (0.16%), accuracy (97.79%), and recovery (96.55%). The developed and validated method showed satisfactory parameters for MNZ quantification in the culture medium of *M. protocystis*.

MNZ concentrations were analysed initially and after 96 hours (final time) to monitor the stability of the drug under the physical conditions of testing (temperature and irradiation) and its biodegradability.

The retention time for MNZ was 2.75 minutes. The sample concentrations were obtained from the calibration curve that was prepared and analysed during the same analytical run by interpolating the value of the area obtained.

The differences in the MNZ concentrations over 96 hours of drug exposure were not statistically significant (Table 1).

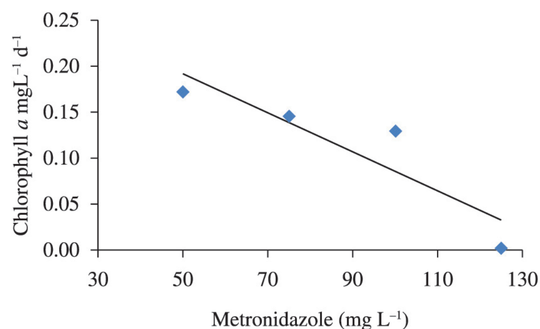

Figure 3. *Microcystis protocystis* growth as monitored by chlorophyll *a* during a 96-hour metronidazole toxicity test.

The analytical determinations on extended runs did not have a peak that indicates the presence of MNZ metabolites or photodegradation products of this drug.

Drugs in the aquatic environment can be degraded by hydrolysis, photolysis, interaction with other compounds in the medium, or the action of microorganisms. In the tests performed with the *M. protocystis* culture, no reduction in MNZ concentration was observed in the control with ASM-1 medium (50 mg L^{-1}). This finding indicates that under these conditions, the drug was resistant to abiotic degradation.

By contrast, one of the most efficient methods of xenobiotic detoxification is inactivation by cellular enzymatic systems. In humans, MNZ is extensively metabolised by the hepatic cytochrome P_{450} leading to the oxidation of its side chain with the formation of two metabolites, one of which is active (Bendesky et al., 2002; Lanzky and Halling-Sørensen, 1997). *M. protocystis* was resistant to high MNZ concentrations, and no metabolites or antimicrobial biotransformation products were observed, indicating that the species is not a significantly strong metaboliser of MNZ over 96 hour of drug exposure.

Although some characteristics of MNZ, such as low molecular weight and lipid solubility, allow this molecule to easily penetrate cells by passive diffusion in both anaerobic and aerobic organisms (Tally and Sullivan, 1981), the results suggested that there was no significant drug uptake by the cells.

One possibility is that the dense mucilaginous sheath that surrounds the cells *of M. protocystis* might protect it against penetration by MNZ.

The mucilaginous sheath isolated from *M. protocystis* corresponded to 41% of the dry weight of this cyanobacterium. The mucilaginous sheaths are composed of polysaccharides, and alginic acid is the main component. These sheaths are hydrophilic in the presence of polar and ionized groups and may function as a barrier to xenobiotic penetration. According to Sabra et al. (2001), alginates do not have a unique function in the cell but provide a plurality of protective properties against several adverse environmental conditions.

MNZ showed no significant toxicity to *M. protocystis,* indicating that this cyanobacterium has considerable resistance to this antimicrobial drug. The role of the mucilage sheath in protecting the intracellular environment from the action of this drug may explain the high resistance of

Table 1. Metronidazole concentrations from a culture of cyanobacteria *Microcystis protocystis* after 96 hours of exposure (repeated measures ANOVA, p>0.05).

Nominal concentrations	[MTZ] $T_{initial}$	[MTZ] T=96 h
1000 (MNZ Control)	765.78 ± 51.64	776.59 ± 30.25
500	444.63 ± 41.59	417.97 ± 12.89
750	734.43 ± 71.24	666.64 ± 15.95
1250	1,045.05 ± 92.68	1,043.81 ± 10.83
1500	1,307.4 ± 74.13	1,327.34 ± 32.09

Values (mg L^{-1}) represents mean ± SD, n=3.

M. protocystis and should be elucidated to better understand their behaviour in a polluted environment.

The persistence of MNZ indicates the need for studies investigating adequate methods for its inactivation before residues of this antimicrobial are released into bodies of water.

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