

Alterations in the intestine of Patagonian silverside (*Odontesthes hatcheri*) exposed to microcystin-LR: Changes in the glycosylation pattern of the intestinal wall and inhibition of multidrug resistance proteins efflux activity

Flavia Bieczynski^{a,*}, Walter D.C. Torres^b, Julio C. Paineñfilu^a, Juan M. Castro^a, Virginia A. Bianchi^a, Jimena L. Frontera^d, Dante A. Paz^d, Carolina González^e, Alejandro Martín^e, Silvina S.M. Villanueva^c, Carlos M. Luquet^a

^a Laboratorio de Ecotoxicología Acuática, INIBIOMA – (CONICET-UNCo), CEAN- Ruta 61 km 3, Paraje San Cabao, 8371, Junín de los Andes, Neuquén, Argentina

^b CEAN- Ruta 61 km 3, Paraje San Cabao, 8371, Junín de los Andes, Neuquén, Argentina

^c Instituto de Fisiología Experimental, IFISE-CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570, 2000, Rosario, Santa Fe, Argentina

^d Laboratorio de Biología del Desarrollo, IFIBYNE-CONICET, Universidad Nacional de Buenos Aires, Pabellón II, Ciudad Universitaria, 1428, Ciudad Autónoma de Buenos Aires, Argentina

^e Dirección Técnica y de Desarrollo Tecnológico, Agua y Saneamientos Argentinos, Av. Figueroa Alcorta 6081, 1425, Ciudad de Buenos Aires, Argentina

ARTICLE INFO

Article history:

Received 29 February 2016

Received in revised form 15 July 2016

Accepted 22 July 2016

Available online 25 July 2016

Keywords:

Abcc transporters

Cyanotoxin

Glycoconjugates

Intestinal sacs

Lectin-histochemistry

Multixenobiotic resistance

Odontesthes hatcheri

ABSTRACT

Accumulation and toxicity of cyanobacterial toxins, particularly microcystin-LR (MCLR) have been extensively studied in fish and aquatic invertebrates. However, MCLR excretion mechanisms, which could reduce this toxin's effects, have received little attention. The Patagonian silverside, *Odontesthes hatcheri*, is an omnivorous-planktivorous edible fish, which has been shown to digest cyanobacterial cells absorbing MCLR and eliminating the toxin within 48 h without suffering significant toxic effects. We studied the effects of MCLR on glycoconjugate composition and the possible role of multidrug resistance associated proteins (Abcc) in MCLR export from the cells in *O. hatcheri* intestine. We treated *O. hatcheri* with 5 µg MCLR g⁻¹ body mass administered with the food. Twenty four hours later, the intestines of treated and control fish were processed for lectin-histochemistry using concanavalin A (ConA), *Triticum vulgaris* agglutinin (WGA), and *Dolichos biflorus* agglutinin (DBA). MCLR affected the distribution of glycoconjugates by augmenting the proportion of ConA-positive at the expense of WGA-positive cells. We studied MCLR effects on the transport of the Abcc-like substrates 2,4-dinitrophenyl-S-glutathione (DNP-SG) and calcein in ex vivo intestine preparations (everted and no-everted sacs and strips). In treated preparations, CDNB together with MCLR (113 µg MCLR g⁻¹ intestine, equivalent to 1.14 µmol L⁻¹ when applied in the bath) or the Abcc inhibitor, MK571 was applied for one hour, during which DNP-SG was measured in the bath every 10 min in order to calculate mass-specific DNP-SG transport rate. MCLR significantly inhibited DNP-SG transport ($p < 0.05$), especially in middle intestine (47 and 24%, for luminal and serosal transport, respectively). In middle intestine strips, MCLR and MK571 inhibited DNP-SG transport in a concentration dependent fashion (IC_{50} 3.3 and 0.6 µmol L⁻¹, respectively). In middle intestine strips incubated with calcein-AM (0.25 µmol L⁻¹), calcein efflux was inhibited by MCLR (2.3 µmol L⁻¹) and MK571 (3 µmol L⁻¹) by 38 and 27%, respectively ($p < 0.05$). Finally, middle intestine segments were incubated with different concentrations of MCLR applied alone or together with 3 µM MK571. After one hour, protein phosphatase 1 (PP1) activity, the main target of MCLR, was measured. 2.5 µM MCLR did not produce any significant effect, while the same amount plus MK571 inhibited PP1 activity ($p < 0.05$). This effect was similar to that

Abbreviations: ABC, ATP-binding cassette; Abcb1, p-glycoprotein; Abcc, multidrug resistance-associated proteins; Bcrp, breast cancer resistance protein; BSA, bovine serum albumin; calcein-AM, calcein acetoxymethyl ester; CDNB, 1-chloro-2,4-dinitrobenzene; ConA-FITC, fluorescein labeled *Canavalia ensiformis* agglutinin; DBA, biotinylated-*Dolichos biflorus* agglutinin; DNP-SG, 2,4-dinitrophenyl-S-glutathione; DTT, dithiothreitol; GSH, reduced glutathione; GST, glutathione-S transferase; GS-X pumps, ATP-dependent glutathione S-conjugate exporters; MC, microcystin; MCLR, microcystin-LR; MXR, multixenobiotic resistance; Oatps, organic anion transporting polypeptides; pNPP, P-nitrophenyl phosphate disodium salt; PP, protein phosphatases; SPE, solid phase extraction; WGA, biotinylated-*Triticum vulgaris* agglutinin.

* Corresponding author at: LIBIQUIMA (CONICET), Departamento de Química, Facultad de Ingeniería, Universidad Nacional del Comahue, Buenos Aires 1400, (8300) Neuquén, Neuquén, Argentina.

E-mail addresses: 675.bieczynskif@comahue-conicet.gob.ar, flabieczynski@gmail.com (F. Bieczynski).

of 5 μM MCLR. Our results suggest that in *O. hatcheri* enterocytes MCLR is conjugated with GSH via GST and then exported to the intestinal lumen through Abcc-like transporters. This mechanism would protect the cell from MCLR toxicity, limiting toxin transport into the blood, which is probably mediated by basolateral Abccs. From an ecotoxicological point of view, elimination of MCLR through this mechanism would reduce the amount of toxin available for trophic transference.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

The gastrointestinal system mediates the absorption, distribution, metabolism and excretion of a wide array of endogenous and exogenous compounds, including toxicants (Barltrop and Brueton, 1990; Klaassen and Aleksunes, 2010; Luckenbach et al., 2014). The luminal side of the intestinal wall is covered by a mucous layer secreted by goblet cells, which is mainly composed of glycoproteins and by the glycocalyx, formed by glycosylated membrane proteins (mucins) attached to the cell (Linden et al., 2008). These glycoproteins vary in composition, depending on the region of the intestine, dietary habits, age, sex, and species (Díaz et al., 2003). Among other functions, mucins protect the epithelium against proteolysis, microorganisms and constitute the first barrier for the uptake of xenobiotics (Díaz et al., 2003, 2008; Loretz, 1995). The second barrier is composed by membrane proteins, which are involved in the uptake and elimination of physiological substrates as well as many drugs and toxicants. Among these proteins, there is a group of transporters which belongs to the ABC superfamily (ATP-binding cassette) that acts in tandem with biotransformation enzymes, such as glutathione S-transferases (GST) and cytochrome P450 enzymes (CYP) (Bard, 2000; Luckenbach et al., 2014). The P-glycoprotein 1 (Pgp; ABCB1), the multidrug resistance-associated proteins 1, 2 and 3 (MRP1–3; ABCC1–3) and the breast cancer resistance protein (BCRP; ABCG2) have been described as toxicologically relevant transporters (Chan et al., 2004; Klaassen and Aleksunes, 2010; Leslie et al., 2005; Luckenbach et al., 2014; Takano et al., 2006). According to the zebrafish nomenclature, in the present work capital letters denote ABC superfamily in general or specific mammalian ABC proteins while lowercase letters denote non-mammalian proteins. ABC proteins have been first described as conferring multidrug resistance (MDR) in mammalian tumor cells (Gottesman and Pastan, 1993). Nevertheless, these proteins are present in normal tissues of a wide range of species, including fish and aquatic invertebrates. The term “multixenobiotic resistance” (abbreviated here as MXR, although in the literature this abbreviation has also been used with other meanings) is applied to the role of ABC proteins in the protection of aquatic animals against environmental toxicants (Kurelec, 1992). Abcb1 is the most studied transporter in relation to xenobiotic defense, although recent studies show that Abcc proteins also play an important role in MXR (Ferreira et al., 2014; Luckenbach et al., 2014; for a review).

In mammals, ABCB1 is generally localized in apical membranes of polarized cells and transports a broad range of moderately hydrophobic compounds, preferentially small sized and cationic ones, including non-metabolized toxic compounds and phase I biotransformation products (Klaassen and Aleksunes, 2010; Takano et al., 2006). In the intestine, as well as in kidney and liver, ABCC2 is localized to the apical membrane while other ABCCs, such as ABCC1 and 3, have basolateral location. ABCC1–3 export conjugated and unconjugated anionic compounds (phase II products), such as 2,4-dinitrophenyl-S-glutathione (DNP-SG) and bilirubin glucuronides (Fardel et al., 2005; Klaassen and Aleksunes, 2010). ABCG2 is mainly located at the apical membrane of enterocytes and transports some

compounds which are also ABCB1 or ABCC1 substrates (Chan et al., 2004).

Toxic metabolites produced by cyanobacteria (cyanotoxins) represent a threat to life when produced in high concentrations during cyanobacterial blooms. Microcystins (MC) are hepatotoxins usually associated with environmental risk cases and with animal and human intoxication reports (Dietrich and Hoeger, 2005; Dörr et al., 2010; Pavagadhi and Balasubramanian, 2013; Sivonen and Jones, 1999; Wiegand and Pflugmacher, 2005). The characteristic toxic effects caused by microcystin-LR (MCLR), one of the most frequent and toxic MC variants (Codd et al., 2005), are mediated by inhibition of protein phosphatases (PP) 1 and 2A which could affect many cellular functions regulated by protein phosphorylation/dephosphorylation, such as cytoskeletal function and maintenance of hepatocyte ultrastructure (Eriksson et al., 1990; Honkanen et al., 1990; MacKintosh et al., 1995; Runnegar et al., 1995; Williams et al., 1997; among others). Additionally, oxidative stress has also been reported as mediating MCLR toxicity (Amado and Monserrat, 2010). Moreover, the combination of these effects could lead to cytotoxicity and to the activation of tumor promoting cascades (Campos and Vasconcelos, 2010; Carmichael, 1992; Falconer and Yeung, 1992; Hooser et al., 1989; Kuiper-Goodman et al., 1999).

Deleterious effects of MCLR have been studied in many fish species (Atencio et al., 2008; Ferrão-Filho and Kozłowski-Suzuki, 2011; Fischer and Dietrich, 2000; Malbrouck and Kestemont, 2006; Pavagadhi and Balasubramanian, 2013; Sahin et al., 1996; Soares et al., 2004; Williams et al., 1997; Xie et al., 2004). Surprisingly, in spite of being the main site of MCLR uptake in fish, the intestine has received little attention with respect to MCLR effects and detoxification (Bieczynski et al., 2013, 2014; Bury et al., 1998; Tencalla and Dietrich, 1997; Xie et al., 2004).

In general, fish are less sensitive to MC toxicity than mammals but their sensitivity varies over a wide range, depending on, e.g., the species and the exposure route (reviewed by Malbrouck and Kestemont, 2006). For example, 1.7 μg MCLR g^{-1} body mass (bm), applied by oral gavage, caused hepatic damage and mortality in the common carp, *Cyprinus carpio* but did not cause any toxic effect on the rainbow trout *Oncorhynchus mykiss*, while a higher dose, 6.6 μg MCLR g^{-1} bm, caused severe toxic effects and mortality in both species. On the other hand, intraperitoneal injection of 0.55 μg MCLR g^{-1} bm was lethal for both species (Fischer and Dietrich, 2000). Xie et al. (2004) have reported that the phytoplanktivorous silver carp, *Hypophthalmichthys molitrix*, which actively feeds on toxic cyanobacteria, has reduced intestinal absorption of MCLR. Altogether, the results cited above suggest that resistance to MCs is, at least in part, determined by digestion – absorption – detoxification processes in the digestive tract. Several laboratory and field studies have shown that different fish species are able to reduce MC absorption and/or metabolize and eliminate the toxin accumulated in the tissues (Bieczynski et al., 2013, 2014; Sahin et al., 1996; Soares et al., 2004; Williams et al., 1997; Xie et al., 2004). The comprehension of MCs detoxification mechanisms in different species could help to explain interspecific differences in susceptibility to these toxins. Nevertheless, the mechanisms involved in MC transport

across the intestinal wall are far from being fully understood. The organic anion transporting polypeptides (Oatps) have been suggested as the main candidate membrane transporters for the uptake of MC (Boaru et al., 2006; Fischer et al., 2005; Lu et al., 2008; Meier-Abt et al., 2007). There are evidences on the biotransformation of MCLR through conjugation with reduced glutathione (GSH) in a phase II reaction catalyzed by the enzyme glutathione S-transferase (GST) before its excretion (Kondo et al., 1996; Pflugmacher et al., 1998; Sahin et al., 1996). Kondo et al. (1996) also mention the possible production of another metabolite, which is formed by epoxidation (phase I reaction), followed by hydrolysis and conjugation with both sulfate and GSH. However, there are still very few studies about the way in which native MCLR or its metabolites are exported from the cells. Amé et al. (2009), Contardo-Jara et al. (2008) and Lu et al. (2015) have associated Abcb1 and Abcb4 to MCLR excretion in fish and mussels. On the other hand, Ito et al. (2002) and Malbrouck and Kestemont, (2006) have suggested that GS-X pumps (ATP-dependent glutathione S-conjugate exporters) and Abcc, respectively, could mediate the elimination of MCLR conjugates. Additionally, a previous work of our group has shown that Abcc-like transporters localized both in the apical and in the basolateral membrane of *Oncorhynchus mykiss* enterocytes could be involved in the transport of MCLR to the intestinal lumen and to the blood, respectively (Bieczynski et al., 2014).

Odontesthes hatcheri is a highly valued edible fish native to inland waters of Patagonia, Argentina (Hualde et al., 2011). As other members of *Atherinopsidae*, *O. hatcheri* has a short digestive tract with no true acid stomach (Horn et al., 2006). It is a planktivorous-omnivorous fish with opportunistic habits and occasional consumption of cyanobacteria (Hualde et al., 2011; Vila and Soto, 1981). This fish is abundant in hydroelectric reservoirs of Patagonia, where toxic cyanobacterial blooms are frequent (Echenique and Aguilera, 2009; Othaz Brida et al., 2010; Puig, 1992). It is not clear whether *O. hatcheri* actively feeds on cyanobacteria or consumes them incidentally when feeding on zooplankton. However, we have previously reported evidence about the capacity of *O. hatcheri* to digest toxic cyanobacterial cells (Bieczynski et al., 2013).

In this study, we aimed to elucidate the interaction of MCLR with *O. hatcheri*'s intestinal wall. First, we used lectin-histochemistry for detecting possible effects of MCLR in the intestinal epithelium glycoconjugate composition and distribution pattern along the digestive tract. Second, we studied cellular mechanisms, which could participate in MCLR transport involved in the absorption/detoxification processes. We evaluated the effects of MCLR on luminal and serosal transport of the Abcc-like substrates DNP-SG and calcein in ex vivo intestine preparations. Third, we studied the intracellular effects of MCLR on PP1 activity in ex vivo intestine preparations in the presence of a specific ABCC inhibitor.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), Coomassie blue brilliant G250 and MK571 were purchased from Sigma-Aldrich (St Louis, MO). P-nitrophenyl phosphate disodium salt (pNPP) was from Merck (Germany). Calcein acetoxymethyl ester (calcein-AM, Calbiochem, San Diego, CA) was a generous gift from Dr. Amro M. Hamdoun (Scripps Institution of Oceanography, UCSD). Biotinylated-*Triticum vulgaris* (wheatgerm) agglutinin (WGA), fluorescein labeled *Canavalia ensiformis* agglutinin (ConA-FITC), and biotinylated-*Dolichos biflorus* agglutinin (DBA) were purchased from Vector Labs (Burlingame, CA). Streptavidin-rhodamine was purchased from Life Technologies (Gaithersburg, MD). All the used chemicals and

reagents were commercial products of analytical grade purity and were used as supplied.

2.2. Fish and MCLR extract

Adult *O. hatcheri* of both sexes (total $n = 81$; 14.16 ± 0.55 g body mass (g bm), for lectin-histochemistry and, 22.5 ± 7.3 g bm, for ex vivo studies) were obtained from the Centro de Ecología Aplicada del Neuquén (CEAN), Junín de los Andes, Argentina. Fish were maintained in aquaria (8 kg/m^3) with constant flow of filtered water from Chimehuin river and aeration. Temperature was kept at $20 \pm 5^\circ \text{C}$. Fish were fed with commercial pejerrey feed at a ratio of 1% body mass per day. All the maintenance and experimental protocols were approved by the Bioethics Committee, Faculty of Biochemical and Pharmaceutical Sciences, National University of Rosario, Argentina (6060/116).

MCLR was purified from a nonaxenic *Microcystis* sp. culture in BG11 medium. Cyanobacteria cells were disrupted by the addition of acetic acid (reaching pH 3) and sonication for 90 min. The toxin released from the cells was purified and concentrated by solid phase extraction (SPE) with C-18 columns (Phenomenex, 500 mg). Columns were washed with 2 mL of hexane-dichloromethane-methanol-water mixture, and then samples were passed through the column at a flow rate of 4 mL/min. After that, columns were rinsed with 1.5 mL of 20% methanol and were allowed to dry for 30 min. MCLR was eluted twice with 1.5 mL of 90% methanol and the eluates were collected in 5 mL tubes. The combined eluates were evaporated under air flow to a final volume of 1 mL. The resulting extracts were analyzed by full UV scan with a Waters® HPLC system arranged with a Model 600 pump, a Model 996 DDA and Millennium software. Separation was performed using a Supelco column, SUPELCOSIL LC-PAH $5 \mu\text{m}$ ($4.6 \text{ mm i.d.} \times 15 \text{ cm}$), Cat Number 58318, according to Reference Method IRAM 29036. MCLR was the only toxin detected in the extract at a concentration of 318 mg L^{-1} . We did not detect significant peaks at 206 nm, which could indicate the presence of lipopolysaccharides (supplementary Fig. 1).

The MCLR doses used for the experiments can be found in natural blooms. For a 10 g individual, the highest dose applied ($5 \mu\text{g g}^{-1} \text{bm}$) requires $50 \mu\text{g}$ of MCLR, which is equivalent to ca. 2×10^5 cells of *M. aeruginosa* NPJB1 (Azevedo et al., 1994; Bieczynski et al., 2013) or to 64 mL of water from a *M. aeruginosa* bloom in Jacarepaguá Lagoon, Brazil (Soares et al., 2004).

2.3. Lectin-histochemistry

The experimental treatment consisted of the administration of a single sublethal dose of MCLR in the food. The treated group ($n = 2$) received $5 \mu\text{g MCLR gbm}^{-1}$ added to the food, while control fish received the same food without MCLR. Experimental diets (toxic and control) were prepared by crushing commercial pejerrey pellets in a mortar and mixing the powder with distilled water (control) or with the corresponding volume of MCLR extract (previously evaporated under N_2 flow and resuspended in distilled water) to form small pellets. The food ratio was reduced to 0.5% bm to ensure that all the food was consumed. We checked visually that every individual was fed.

Fish were sacrificed by decapitation 24 h after food administration. The entire digestive tube was immediately removed, carefully rinsed with ice-cold saline and cut into three segments: anterior (the first portion of intestine, from the esophagus to the first intestinal curvature), medium (segment between the first and the second curvature) and posterior (from the second curvature to the end of the intestine, discarding the intestinal valve). After dissection, portions of approximately 5 mm of each segment were immediately immersed in Bouin's fixative. The fixed tissues were processed rou-

tinely and embedded in paraffin. Sections of 4 μm were cut with a microtome and mounted on slides.

Fluorescein-labeled lectins were used to identify specific sugar residues in the epithelium and mucous layer of each intestinal segment. Briefly, mounted 4 μm sections were deparaffinized with xylene, hydrated through a graded ethanol series and washed in phosphate-buffered saline (PBS). Subsequently, these sections were incubated with different lectin combinations ($n=2$ for each combination). The characteristics and full names of lectins used are detailed in Table 1.

WGA-ConA: Samples were incubated for 16 h with WGA (1:300) in a moist chamber at 4 °C in the dark and then incubated for 2 h with streptavidin-rhodamine (1:500) and ConA-FITC (1:50). DBA-ConA: Samples were incubated for 16 h with DBA (1:100) in a moist chamber at 4 °C and in the dark. Afterwards, sections were incubated for 2 h with streptavidin-rhodamine (1:500) and ConA-FITC (1:50). Every preparation was mounted with glycerol-PBS (50:50) and was observed and photographed in an Olympus Fluoview confocal microscope, with Fluoview software V 5.0. The images obtained were qualitatively and quantitatively analyzed by three observers who did not know the origin of the images. This analysis was applied to mucous layer, goblet cells and enterocytes (distinguishing between the apical-subapical region, which includes the glycocalyx, and the cell body). Additionally, we recorded the number of goblet cells, which reacted with each lectin in each preparation.

2.4. Abcc-like transport

We studied the effects of MCLR, as a possible competitive inhibitor of the luminal and serosal transport of two specific Abcc substrates, DNP-SG (Evers et al., 2000; Gotoh et al., 2000; Mottino et al., 2001) and calcein (Fischer et al., 2011; Gekeler et al., 1995). We incubated intestine preparations with CDNB, which is assumed to freely enter the cells due to its lipophilic nature and is subsequently conjugated with GSH to form DNP-SG in the cytoplasm in a reaction catalyzed by GST (Gotoh et al., 2000). Alternatively, we applied the non-fluorescent compound calcein-AM, which enters the cell and is hydrolyzed by cytosolic esterases to calcein, a fluorescent substrate of Abcc (Evers et al., 2000; Takano et al., 2006). The specific Abcc transport inhibitor, MK571 (Fischer et al., 2011; Gekeler et al., 1995) was used as a specificity control. In all the experiments, CDNB and calcein-AM were previously dissolved in DMSO and MK571 was dissolved in distilled water. Final solvent concentrations were always below 0.1%.

Fish were fasted for 24 h before each experiment and then sacrificed by decapitation. The entire digestive tube was immediately removed and rinsed in ice-cold Cortland solution (with 5 mmol L⁻¹ HCO₃Na and 5.55 mmol L⁻¹ glucose, pH 7.4). The digestive tube was divided into three segments as is described in Section 2.3. Pieces of 15 mm length from each segment were thoroughly washed with saline solution to eliminate gut content and then intestinal sacs or strips were prepared as described below. Preparations were kept at 20 °C with constant aeration throughout the duration of the experiments.

Intestinal sacs were prepared according to Bieczynski et al. (2014) in order to analyze intracellular to luminal transport (everted sacs) and intracellular to serosal transport (non-everted sacs). Everted and non-everted preparations were weighed, ligated at both ends and filled with saline solution ($50 \pm 3 \mu\text{L}$) through a Teflon cannula. Intestinal strips were prepared by cutting the middle intestine into small longitudinal strips, which were weighed and randomly placed in saline solution with the corresponding substrate and treatment.

2.4.1. DNP-SG efflux experiments

Portions ($0.05 \pm 0.012 \text{ g}$) from each intestinal segment were separated and cut into halves for preparing paired (control vs. treatment) everted ($n=5$) and non-everted ($n=5$) sacs. Control sacs were incubated in 5 mL Cortland saline (bath) with 200 $\mu\text{mol L}^{-1}$ CDNB under constant aeration for 1 h. Treated everted sacs were incubated in 5 mL saline with 200 $\mu\text{mol L}^{-1}$ CDNB + 1.14 $\mu\text{mol L}^{-1}$ MCLR. In non-everted sacs, the same quantity of MCLR (5.65 μg) dissolved in saline solution, was added into the sac lumen. These MCLR concentrations were chosen in order to reach a MCLR mass per g of intestine similar to those applied in previous in vivo (Williams et al., 1997; Jos et al., 2005; Bieczynski et al., 2013) and ex vivo (Bieczynski et al., 2014) experiments.

Every ten min, 2 mL aliquots were collected from the bath solution and DNP-SG was determined by reading absorbance at 340 nm with a UV-vis spectrophotometer. After measurement, aliquots were returned to the bath for keeping a constant volume. The DNP-SG transport rate was calculated from the slope of cumulative absorbance vs. time, an extinction coefficient of 9.6 mM⁻¹ cm⁻¹, 1 cm path length and referred to wet tissue mass. Results were expressed as nmol DNP-SG g⁻¹ min⁻¹ and presented as percentage of the corresponding control.

To confirm that the detected product was DNP-SG and to check for the presence of any subsequent metabolite, bath and tissue samples from 9 intestinal sacs were prepared for HPLC analysis as described by Bieczynski et al. (2014). DNP-SG was detected at 365 nm and was quantified by the external standard method by the height of the peak.

2.4.2. Concentration dependent effects of MK571 and MCLR on DNP-SG efflux

Based on the results obtained in Section 2.4.1 and in previous studies (Bieczynski et al., 2014), the middle segment of *O. hatcheri* intestine was selected for studying concentration-dependent effects of MK571 and MCLR on DNP-SG transport. Intestinal strips (repeated measurements) from four individuals ($0.021 \pm 0.006 \text{ g}$) were incubated for 10 min with different MK571 concentrations: 0 (two control strips), 0.6, 1.14, 2.3, 3.4 or 6.8 $\mu\text{mol L}^{-1}$ (final concentration) before being transferred to 200 $\mu\text{mol L}^{-1}$ CDNB solution with the same concentration of inhibitor. The same protocol was used for MCLR, treating intestinal strips ($0.019 \pm 0.006 \text{ g}$, $n=4$) with the following MCLR final concentrations: 0 (two control strips), 0.6, 1.14, 2.3, 3.4, 5.7 or 8.5 $\mu\text{mol L}^{-1}$.

DNP-SG concentration in the bath was measured during 1 h as described in Section 2.4.1. Results from treated strips were calculated as mass specific DNP-SG transport rate and expressed as percentage respect to control strips' transport rate. Nonlinear regression curves, log (inhibitor concentration) vs. response, were fitted for MK571 and MCLR and the IC₅₀ values were calculated.

2.4.3. Calcein efflux

Middle intestine strips ($0.045 \pm 0.003 \text{ g}$) from seven individuals were weighed and randomly placed in microcentrifuge tubes (two strips/tube) with 300 μL of saline solution containing 0.25 $\mu\text{mol L}^{-1}$ calcein-AM (control strips) (Zaja et al., 2008). Treated strips were placed in the same bath solution with MCLR or MK571 at 2.3 $\mu\text{mol L}^{-1}$ and 3 $\mu\text{mol L}^{-1}$ final concentrations added, respectively. During one hour, 200 μL aliquots were taken every 10 min and calcein fluorescence was read with a Qubit fluorometer (Invitrogen-Molecular Probes, Oregon) at excitation/emission wavelengths of 485/530 nm. Immediately after each measurement, the aliquots were returned to each tube. During the experiment all the tubes were protected from light. Calcein transport rate was calculated as fluorescent units per min and referred to tissue mass.

Table 1
Carbohydrate binding specificity and emission wave length for the lectins employed in this study.

Lectin	Specificity	Emission wavelength
Biotinylated- <i>Triticum vulgaris</i> agglutinin (WGA)	β -N-acetyl-D-glucosamine, sialic acid	570 nm
Fluorescein labeled- <i>Canavalia ensiformis</i> agglutinin, concanavalin A (ConA-FITC)	α -D-mannose, α -D-galactose	520 nm
Biotinylated- <i>Dolichos biflorus</i> agglutinin (DBA)	α -N-acetyl-D-galactosamine	570 nm

2.4.4. Toxic effects of MCLR in middle intestine preparations

The middle intestine segment (0.05 ± 0.004 g) from 50 individuals was separated, washed with Cortland solution and everted, keeping both extremes open to allow the contact of the bathing solution with both sides of the epithelium. These preparations were placed in 5 mL of Cortland solution alone (controls) or Cortland solution with $1.5 \mu\text{mol L}^{-1}$ MCLR (MC1.5), $1.5 \mu\text{mol L}^{-1}$ MCLR + $3 \mu\text{mol L}^{-1}$ MK571 (MC1.5/MK), $2.5 \mu\text{mol L}^{-1}$ MCLR (MC2.5), $2.5 \mu\text{mol L}^{-1}$ MCLR + $3 \mu\text{mol L}^{-1}$ MK571 (MC2.5/MK), $5 \mu\text{mol L}^{-1}$ MCLR (MC5) and $3 \mu\text{mol L}^{-1}$ MK571 (MK). Treatments with MK571 were incubated for 10 min with the inhibitor before adding MCLR. After 1 h of incubation in the corresponding solutions, intestinal segments were washed with ice-cold homogenization buffer and processed as described below.

2.4.4.1. Protein phosphatase activity and protein quantitation. According to Carmichael and An (1999), intestinal segments were homogenized in 40 mmol L^{-1} Tris-HCl, 20 mmol L^{-1} KCl, 20 mmol L^{-1} MgCl_2 buffer, pH 8.6, with a Teflon-glass homogenizer, 30 strokes (1:4 m/v). Homogenates were centrifuged at $11,000 \times g$ for 15 min and the supernatants were used for PP1 activity measurements and protein quantitation. PP1 activity was measured in a microtiter plate reader with pNPP as substrate. Absorbance at 405 nm was read in triplicate, every 5 min during 40 min at 25°C . Enzyme activity was expressed as nkatal mg^{-1} protein. Total soluble protein content was measured by the method of Bradford (1976), using BSA as standard. Results were expressed as $\text{mg protein mL}^{-1}$.

2.5. Statistical analysis

Results are presented as mean \pm SEM. Comparisons among groups were performed by paired Student *t*-tests or one-way analysis of variance (ANOVA) for independent or repeated measures as appropriate. Values of $p < 0.05$ were considered as statistically significant (Zar, 1999). The assumptions of homogeneity of variance and normality were tested by Bartlett test and Kolmogorov-Smirnov test, respectively. *Post hoc* Fischer LSD comparisons were used when ANOVA was significant.

3. Results

3.1. Lectin-histochemistry

WGA-ConA: Control and MCLR-treated fish showed differences in the reaction to WGA in the mucous layer and in goblet cells (Table 2). The mucous layer of the anterior and middle intestine of control fish presented areas with moderate reaction to WGA (red fluorescence) and areas with reaction to both lectins (colocalization, yellow-orange fluorescence) whereas in treated fish the reaction to WGA in the mucous layer was intense and predominant, especially in the anterior intestine. The posterior intestine of control fish showed a continuous mucous layer with intense reaction to WGA, while in treated fish this part of the intestine presented only scattered accumulations of mucus with intense reaction to WGA (Fig. 1a–f). In general, enterocytes showed positive reaction to ConA (green fluorescence), mostly in the apical region. Goblet cells showed moderate to intense reaction to both lectins with the exception of those in the posterior intestine of treated fish, which

showed weak to no reactivity to WGA. In the anterior intestine of control fish, goblet cells were 10% ConA-positive, 5% WGA-positive, 82% with colocalization of both lectins and 3% with no visible reaction, while in treated fish goblet cells were 21% ConA-positive, 20% WGA-positive, 24% with colocalization and 35% with no visible reaction. In the middle intestine of control fish goblet cells were 11% ConA-positive, 57% WGA-positive, 24% with colocalization of both lectins and 8% with no visible reaction. In contrast, MCLR-treated fish showed goblet cells 38% ConA-positive, 15% WGA-positive and the proportion of goblet cells with colocalization of both lectins increased to 47%. In the posterior intestine of control fish, most goblet cells reacted to WGA (78%), 16% of goblet cells were ConA-positive and there were few cells with colocalization (6%); while in MCLR-treated fish, most goblet cells were ConA-positive (49%), a small proportion of cells reacted to WGA (19%) and the rest had no reaction (32%).

3.2. Effects of MCLR on Abcc-like transport

3.2.1. Effects on DNP-SG transport

HPLC analysis showed a single significant peak for DNP-SG at a retention time of 6.8 min. No metabolites from DNP-SG degradation were detected in significant quantity, indicating that the conversion of DNP-SG to other metabolites, e.g. those mediated by γ -glutamyl transferase, was minimal (data not shown).

Both in everted and in non-everted intestinal sacs, transport of DNP-SG was inhibited by MCLR (Fig. 3a). The transport rate was reduced from 7.7 ± 3.9 to $5.5 \pm 2.8 \text{ nmol DNP-SG g}^{-1} \text{ min}^{-1}$ and from 10 ± 4.2 to $5.3 \pm 1.2 \text{ nmol DNP-SG g}^{-1} \text{ min}^{-1}$ in anterior and middle intestine everted sacs, respectively (paired Student's *T* test, $p < 0.05$ for both sections). No significant differences were detected in posterior intestine. In non-everted sacs, DNP-SG transport was significantly inhibited by MCLR only in the middle segment, from 8.3 ± 1.4 to $6.3 \pm 0.8 \text{ nmol DNP-SG g}^{-1} \text{ min}^{-1}$ ($p < 0.05$). We could not obtain consistent DNP-SG transport rates in anterior intestine non-everted sacs, possibly because the anterior part of some of these preparations could have included a portion of esophagus, which is difficult to distinguish from the intestine in small individuals. The lower DNP-SG transport capacity of the esophagus could have artificially reduced the DNP-SG transport rate of some preparations increasing data variability.

Results from DNP-SG transport experiments in middle intestine strips are shown in Fig. 3b. Both, MCLR and the specific ABCC inhibitor MK571 inhibited DNP-SG transport in a concentration-dependent fashion. The non-linear regression analysis of log inhibitor concentration vs. percentage transport rate yielded IC_{50} values of $3.3 \mu\text{mol L}^{-1}$ ($R^2 = 0.75$) for MCLR and $0.6 \mu\text{mol L}^{-1}$ ($R^2 = 0.6$) for MK571.

3.3. Calcein efflux

Middle intestine strips incubated with $0.25 \mu\text{mol L}^{-1}$ calcein-AM plus $2.3 \mu\text{mol L}^{-1}$ MCLR or $3 \mu\text{mol L}^{-1}$ MK571 showed a significant reduction on calcein efflux rate respect to control strips incubated only with calcein-AM (repeated measures ANOVA $p < 0.05$). Percentage inhibition with respect to the control was 38% and 27% for MCLR and MK571, respectively. There were no significant differences between treatments (Fig. 4).

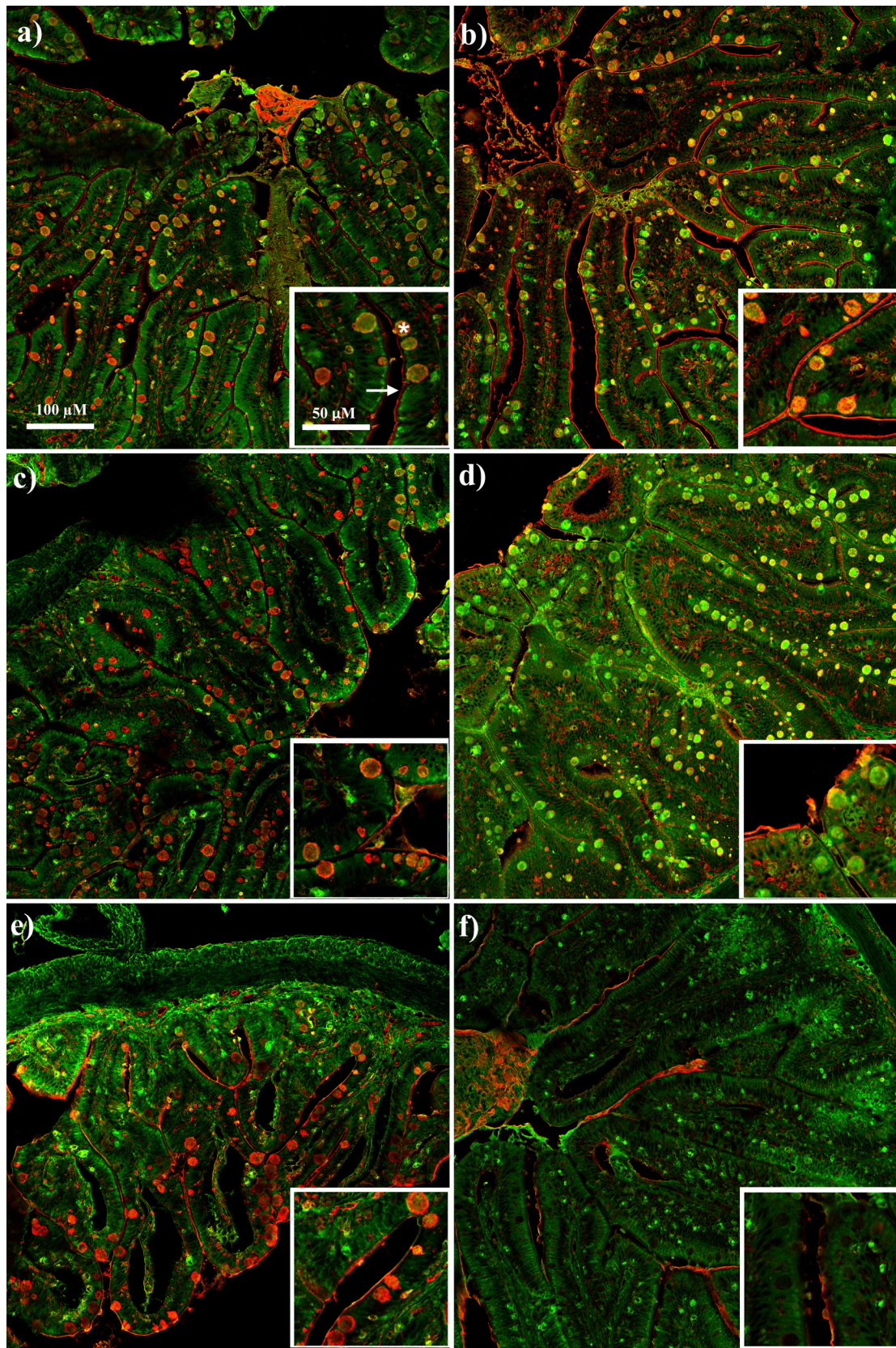


Fig. 1. Histological preparations from *Odontesthes hatcheri* intestine treated with concanavalin A (ConA, green fluorescence) and *Triticum vulgaris* agglutinin (WGA, red fluorescence). a, c, e, anterior, middle and posterior intestine preparations from control fish. b, d, e, anterior, middle and posterior intestine preparations from control fish. 200×. Goblet cell (asterisk), mucous layer (arrow).

Table 2
Semi-quantitative analysis of concanavalin A (ConA) and *Triticum vulgaris* agglutinin (WGA) reactivity in the three intestine segments of *Odontesthes hatcheri*, control and treated with 5 μg MCLR g^{-1} . References: 0 (no reaction), 1 (weak reaction), 2 (moderate reaction), 3 (intense reaction); n = 2.

	Anterior		Middle		Posterior	
	Control	MCLR	Control	MCLR	Control	MCLR
Mucous layer	ConA:1-2 WGA:2	ConA:1 WGA:3	ConA:1 WGA:2	ConA:1 WGA:3	ConA:1 WGA:3	ConA:1 WGA:3
Goblet cells	ConA:2 WGA:2	ConA:2-3 WGA:2-3	ConA:2 WGA:2	ConA:2-3 WGA:2	ConA:2 WGA:2-3	ConA:2 WGA:0-1
EnterocytesApical-subapicalBody	ConA:2 WGA:0 ConA:1 WGA:0	ConA:2 WGA:0 ConA:1 WGA:0	ConA:1-2 WGA:0 ConA:1 WGA:0	ConA:1-2 WGA:0 ConA:1 WGA:0	ConA:1-2 WGA:0 ConA:1 WGA:0	ConA:1-2 WGA:0 ConA:1 WGA:0

DBA-ConA: Differences between treatments were only observed in the proportion of goblet cells that reacted with each lectin in middle intestine. MCLR treatment increased goblet cells with colocalization of both lectins from 20% to 47%; and ConA-positive goblet cells from 36% to 43%, while DBA-positive goblet cells decreased from 33% to 10% upon MCLR treatment. Non-reactive goblet cells (11%) were seen only in control fish. The middle intestine of control and treated fish showed only scattered accumulations of mucus with intense reaction to DBA (Table 3, Fig. 2).

Table 3
Semi-quantitative analysis of concanavalin A (ConA) and Dolichos biflorus agglutinin (DBA) reactivity in the intestine segments of *Odontesthes hatcheri*, control fish and treated fish (5 μg MCLR g^{-1} fish). References: 0 (no reaction), 1 (weak reaction), 2 (moderate reaction), 3 (intense reaction); n = 2.

	Anterior		Middle		Posterior	
	Control	MCLR	Control	MCLR	Control	MCLR
Mucous layer	ConA:1-2 DBA: 0	ConA:1-2 DBA: 0	ConA:1-2 DBA:3	ConA:1-2 DBA:3	ConA:1 DBA: 0	ConA:1 DBA: 0
Goblet cells	ConA:3 DBA:3	ConA:2-3 DBA:3	ConA:3 DBA:3	ConA:3 DBA:3	ConA:3 DBA:3	ConA:3 DBA:3
EnterocytesApical-Subapicalbody	ConA:3 DBA:0 ConA:1 DBA:0	ConA:3 DBA:0 ConA:1 DBA:0	ConA:3 DBA:0 ConA:1 DBA:0	ConA:3 DBA:0 ConA:1 DBA:0	ConA:2 DBA:0 ConA:1 DBA:0	ConA:2 DBA:0 ConA:1 DBA:0

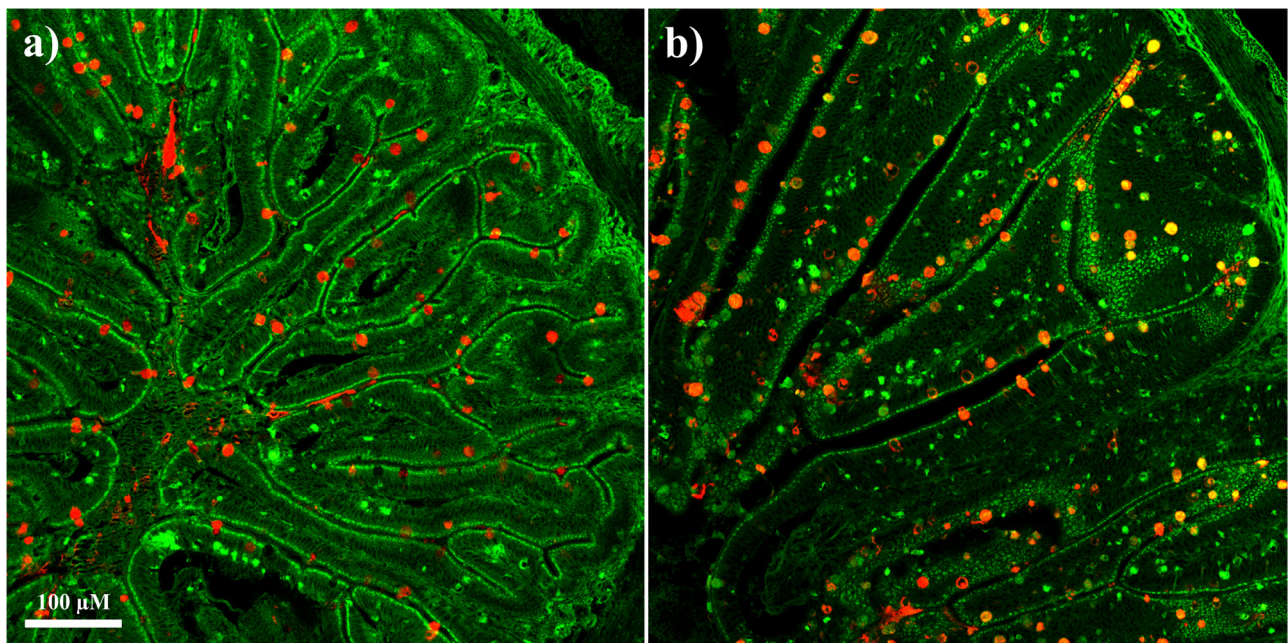


Fig. 2. Histological preparations from *Odontesthes hatcheri* intestine treated with concanavalin A (ConA, green fluorescence) and Dolichos biflorus agglutinin (DBA, red fluorescence). a, middle intestine preparations from control fish. b, middle intestine preparations from control fish. 200 \times .

3.4. Intracellular effects of MCLR in middle intestine segments

Middle intestine segments incubated with saline solution (controls) or with different concentrations of MCLR, MK571 or combination of both showed significant differences among treatments in PP1 activity (ANOVA, $p < 0.05$; Fig. 5). Intestine segments incubated with MC2.5/MK showed 42.7% lower PP1 activity than

control segments ($p < 0.05$); while the same concentration of MCLR applied alone (MC2.5) did not cause significant inhibition. The effect of MC2.5/MK was similar to that of the higher dose of MCLR applied, MC5 (46.5% of inhibition respect to the control). The lowest MCLR dose tested (MC1.5) did not cause significant effects in PP1 activity with respect to control segments, even when MC1.5 was applied in combination with 3 $\mu\text{mol L}^{-1}$ MK571 (MC1.5/MK).

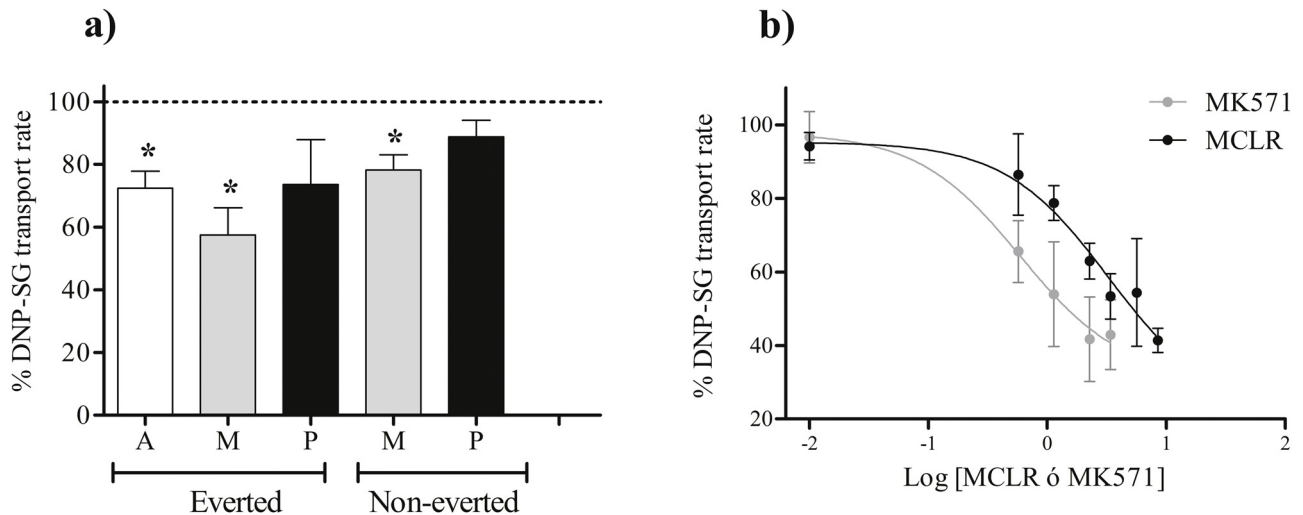


Fig. 3. a) Effects of MCLR on DNP-SG transport as percentage of respective controls in everted and non-everted sacs from *Odontesthes hatcheri* anterior, middle and posterior intestine. Values are means \pm SEM (n = 4–5). Asterisks indicate significant differences between each group and its respective control at $p < 0.05$. b) Non-linear regression curve for log inhibitor (MCLR and MK571) concentration vs. DNP-SG transport rate (as percentage of control), in *Odontesthes hatcheri* middle intestine strips. Values are means \pm SEM (n = 5 for each MCLR/MK571 concentration).

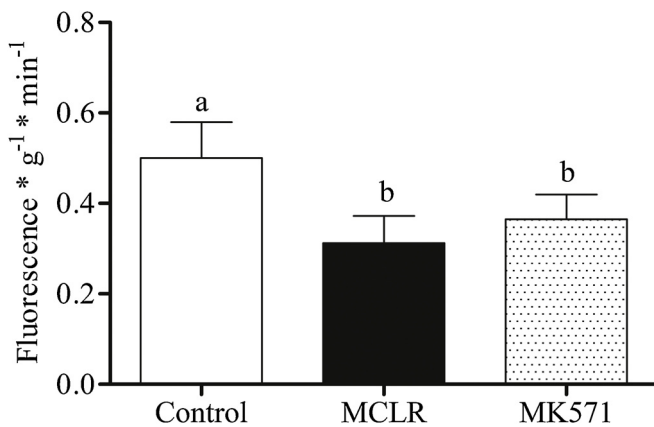


Fig. 4. Effects of $2.3 \mu\text{mol L}^{-1}$ MCLR and $3 \mu\text{mol L}^{-1}$ MK571 on calcein efflux measured in *Odontesthes hatcheri* middle intestine strips. Values are means \pm SEM (n = 7). Different letters indicate significant differences between treatments at $p < 0.05$.

4. Discussion

This study provides original information on the effects of orally applied MCLR on the glycosylation pattern of the gastrointestinal epithelium, and on the role of Abcc proteins in the protection against this toxin in the freshwater fish *Odontesthes hatcheri*. The relative abundance of different monosaccharide residues along the gastrointestinal tract was obtained through lectin-histochemistry analysis after *in vivo* exposure to dietary MCLR for 24 h. Abcc-like transport and intracellular effects of MCLR were studied in *ex vivo* intestinal preparations.

Lectin-histochemistry shows that reaction to ConA, which binds to α -D-mannose and/or α -D-galactose, is intense in goblet cells, in the apical region of the enterocytes and in the mucous layer along the intestine. There is little or no effect of MCLR in the intensity of the reaction to this lectin. Reactivity to WGA, which binds to β -N-acetyl-D-glucosamine and sialic acids, is present only in the mucous layer and in goblet cells (Fig. 1). Control fish preparations (Fig. 1a,c,e) show abundant WGA-positive goblet cells (except for the anterior intestine) while all the intestine segments of MCLR-treated fish show predominant ConA-positive goblet cells; together with intense reaction to WGA in the mucous layer, alternated with

zones reactive to ConA or to both lectins (Fig. 1b,d,f). This suggests a defensive response based on increased secretion of β -N-acetyl-D-glucosamine and/or sialic acid-containing mucins to the mucous layer and increased production and secretion of α -D-mannose and/or α -D-galactose-containing mucins in goblet cells. Mannose residues have been related with a protective role against bacterial infections in fish (Burkhardt-Holm, 1997; Díaz et al., 2005; Lemaitre et al., 1996), while β -N-acetyl-D-glucosamine is associated with epithelial protection against digestive enzymes and other harmful compounds present in the intestinal content (Pavlova et al., 2013). Sialic acid residues have been detected in fish digestive tract and gills (Díaz et al., 2008, 2010, 2012) and are associated with regulation of mucus viscosity, stabilization of molecules and membranes, protection against bacterial and viral invasion and protection of molecules and cells from the attack of proteases or glycosidases (Domeneghini et al., 1998; Meyer et al., 2009; Suprasert et al., 1987; Varki and Schauer, 2009). Thus, the secretion of ConA and WGA-positive mucins in the presence of MCLR could increase the defensive capacity of the mucous layer but also could change the normal mucus viscosity, reducing and affecting digestive processes. Particularly, the loss of WGA reactivity in posterior intestine goblet cells (Fig. 1f) seems to reflect deleterious effects of MCLR on the mucins production functions. The 32% increase in cells with no reaction to these lectins in anterior and posterior segments of treated fish suggests that part of the secreted mucins are not replaced inside goblet cells, at least at this time exposure (Fig. 1b,f). In contrast, not reacting cells decrease from 8 to 0% in the middle intestine of treated fish (Fig. 1d), indicating stimulated mucins production, especially for those reactive to ConA.

The lectin DBA binds to α -N-acetyl-D-galactosamine residues, which have been related to lubrication, protection and molecule transport (Díaz et al., 2012). This lectin shows positive reaction in goblet cells and in few localized zones of the mucous layer (Fig. 2). There is an increment in the proportion of goblet cells with colocalization of DBA and ConA at the expense of DBA-positive cells, an increase of ConA-positive cells and a decrease in not reacting cells in treated fish middle intestine with respect to control fish (Fig. 2a,b). This coincides with the increased production of ConA targets described above and suggests that some goblet cells, which predominantly produce α -N-acetyl-D-galactosamine, are stimulated to also produce α -D-mannose/ α -D-galactose in the presence of MCLR. In such conditions, the protective functions of β -N-acetyl-

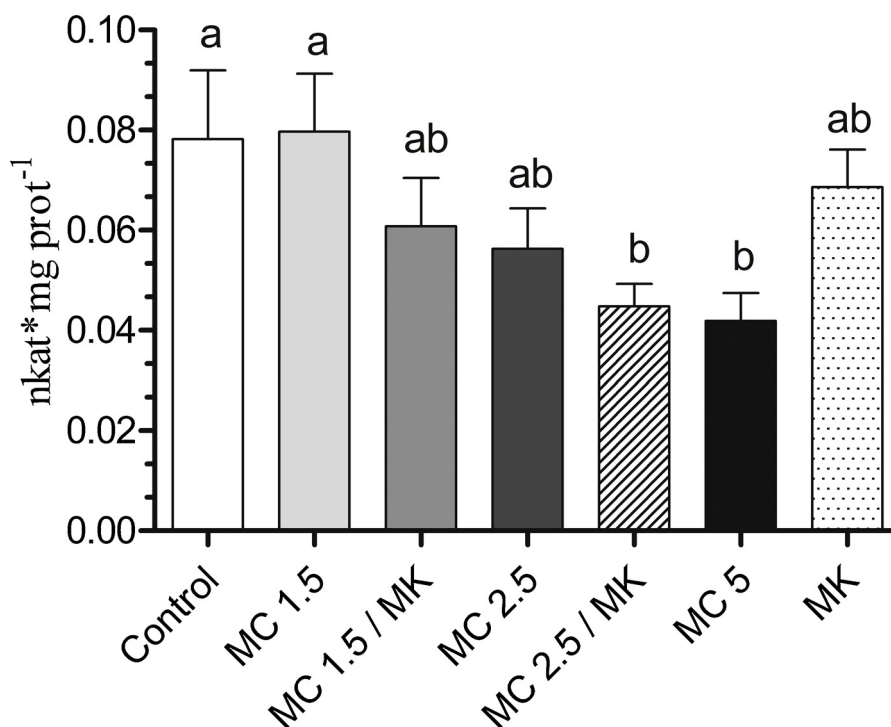


Fig. 5. Toxic effects of MCLR on PP1 activity in *Odontesthes hatcheri* middle intestine segments. Treatments: Control, $1.5 \mu\text{mol L}^{-1}$ MCLR (MC1.5), $1.5 \mu\text{mol L}^{-1}$ MCLR + $3 \mu\text{mol L}^{-1}$ MK571 (MC1.5/MK), $2.5 \mu\text{mol L}^{-1}$ MCLR (MC2.5), $2.5 \mu\text{mol L}^{-1}$ MCLR + $3 \mu\text{mol L}^{-1}$ MK571 (MC2.5/MK), $5 \mu\text{mol L}^{-1}$ MCLR (MC5) and $3 \mu\text{mol L}^{-1}$ MK571 (MK). Values are expressed as means \pm SEM, $n = 5$ –9. Letters indicate significant differences between treatments, $p < 0.05$.

D-galactosamine would be conserved while the defensive effects of α -D-mannose/ α -D-galactose mucins would be increased. Besides, the conserved ConA-positive reaction of the apical zone of enterocytes indicates that the glycocalyx composition is not altered by MCLR under our experimental conditions.

Nonetheless, an important mechanism in MCLR toxicity is the inhibition of protein phosphatases, particularly PP1 and PP2A (MacKintosh et al., 1995; Runnegar et al., 1995). These enzymes are principal components in the regulatory pathways of many cell processes and their inhibition probably affects glycoprotein synthesis and/or secretion in goblet cells, as it has been reported for mammalian goblet cells (Kim and Khan, 2013; Li et al., 2013). In this sense, Ito et al. (2000, 2002) have found MCLR and MCRR accumulated mostly in goblet cells in both the small and large intestine in mice intoxicated orally or intratracheally. However, our conclusions on MCLR effects on the intestinal mucosa glycoconjugate composition are limited by the fact that we used a toxin extract from a cyanobacterial culture which could have contained lipopolysaccharides (LPS). Although we did not find any peak suggesting the presence of LPS in the HPLC-UV scan, small quantities of these compounds could have been present in the extract contributing to the observed effects.

Besides the protection of the mucous layer, fish intestinal epithelium limits MCLR intake, toxicity and accumulation through detoxification and elimination processes (Bieczynski et al., 2013, 2014; Soares et al., 2004; Xie et al., 2004). MC biotransformation has been reported to occur by a phase II reaction catalyzed by GST (Kondo et al., 1996; Pflugmacher et al., 1998) resulting in a product less harmful and more susceptible to be excreted by phase III transporters such as Abcc proteins, which typically export glutathione and glucuronic acid conjugates (Luckenbach et al., 2014).

In *O. hatcheri*'s intestine, MCLR inhibits the cellular transport of the Abcc substrate, DNP-SG both, in everted (anterior and middle intestine) and non-everted (middle) intestinal sacs (Fig. 3a), sug-

gesting that MCLR is exported from epithelial cells by Abcc-like proteins to the luminal and to the serosal side, respectively.

Our experiments with middle intestine strips show that inhibition of DNP-SG efflux by MCLR is concentration-dependent and similar to the obtained with the specific Abcc inhibitor, MK571, although MCLR has lower affinity than MK571 (IC_{50} $3.3 \mu\text{mol L}^{-1}$ and $0.6 \mu\text{mol L}^{-1}$ for MCLR and MK571, respectively, Fig. 3b). In previous works of our laboratory (Bieczynski et al., 2014; Bieczynski, 2015), MCLR and MK571 inhibited DNP-SG efflux in *Oncorhynchus mykiss* middle intestine strips in a similar fashion but with higher affinity (IC_{50} of $1.4 \mu\text{M}$ and $0.2 \mu\text{M}$ for MCLR and MK571, respectively). Interestingly, the ratio MCLR IC_{50} /MK571 IC_{50} is similar in both species, which suggests that the same kind of transporters are involved in MCLR transport in the middle intestine epithelium of both species.

Since MCLR and CDNB are both conjugated with GSH in a reaction catalyzed by GST (Gotoh et al., 2000; Pflugmacher et al., 1998), MCLR could competitively inhibit DNP-SG formation and thus DNP-SG efflux. To discard this possibility, we also evaluated the effect of MCLR and MK571 on the efflux of the fluorescent Abcc substrate calcein (as calcein-AM), which does not depend on GST (Fig. 4). As has previously been reported by Bieczynski et al. (2014) for *O. mykiss*, calcein efflux is inhibited by both MCLR and MK571, indicating that the results obtained in the transport experiments are more likely due to direct inhibition of Abcc-like proteins by MCLR than to inhibition of GST activity.

In order to further assess whether MCLR is exported by Abcc-like proteins, we have studied PP1 activity as a characteristic intracellular effect of MCLR in middle intestine segments incubated with MCLR alone or combined with MK571 (Fig. 5). The results obtained indicate that $1.5 \mu\text{mol L}^{-1}$ and $2.5 \mu\text{mol L}^{-1}$ MCLR do not affect PP1 activity although these MCLR concentrations inhibit Abcc-like transport in the DNP-SG and calcein efflux experiments. MK571 is expected to block MCLR efflux through Abcc proteins increasing intracellular MCLR accumulation which in turn

enhances PP1 inhibition. However, this effect is not evident when MK571 is applied along with $1.5 \mu\text{mol L}^{-1}$ MCLR. PP1 activity is significantly inhibited only with $2.5 \mu\text{mol L}^{-1}$ MCLR plus MK571 or with $5 \mu\text{mol L}^{-1}$ MCLR applied alone. A similar response has been observed in *O. mykiss* under the same experimental conditions but at lower MCLR concentrations (Bieczynski et al., 2014). The lower sensitivity of *O. hatcheri* intestinal PP1 compared with that of *O. mykiss* could be related to differences in feeding habits. While *O. mykiss* is a carnivorous fish which rarely consumes cyanobacteria, *O. hatcheri* is an omnivorous-planktivorous fish that habitually consumes cyanobacteria and is able to digest cyanobacterial cells (Bieczynski et al., 2013).

Until now, Abccs have not been considered as MC transporters in the literature although Ito et al. (2002) and Malbrouck and Kestemont (2006) have suggested this possibility. The present work on *O. hatcheri*, together with our previous work on *O. mykiss* (Bieczynski et al., 2014), supplies experimental evidence on the involvement of Abcc proteins in the elimination of MCLR from fish enterocytes. In contrast, in the literature, Abcb1 is considered as the principal candidate for mediating MCLR efflux (Amé et al., 2009; Contardo-Jara et al., 2008; Lu et al., 2015). However, this assumption is based on only three experimental works, Amé et al. (2009), who detected increased expression of Abcb1 mRNA in gills and brain of the fish *Jenynsia multidentata* exposed to MCLR; Contardo-Jara et al. (2008), who studied the effect of MCLR on the transport of an Abcb1 fluorescent substrate through the gills of the mussel *Dreissena polymorpha* and Lu et al. (2015), who studied the effect of MCLR on the expression of Abcb4 in zebrafish and LLC-PK1. Although we cannot discard the involvement of ABC proteins, such as Abcb1 or Abcg2, in cellular transport of MCLR in *O. hatcheri*'s intestine, the principal substrate used in our experiments, DNP-SG, has not been reported to act as a substrate of Abcb1 or Abcg2. Additionally, Abcb1 exports the non-fluorescent calcein-AM rather than calcein (Takano et al., 2006). This activity tends to reduce the intracellular concentration of the fluorescent product calcein. Thus, inhibition of Abcb1 by MCLR would favor the accumulation of calcein-AM, increasing the intracellular concentration of fluorescent calcein, which would favor calcein efflux by Abcc. However, we have observed inhibition of calcein efflux. This indicates interaction of MCLR with Abcc transporters rather than with Abcb1 in fish intestines.

Bieczynski et al. (2013) have shown that, in *O. hatcheri* treated with a single oral dose of $1.3 \mu\text{g MCLR g}^{-1} \text{bm}$ MCLR, 34% of the initial dose was accumulated after 3 h, mainly in intestine (ca. 32%). After 6 h only 6.5% of the initial dose remained in the intestine while 11% was found in liver, after 48 h MCLR was almost totally removed from both organs. The only toxic effect detected in this experiment was a transient increase in lipid peroxidation in liver. Our present results suggest that the intestinal epithelium avoids MCLR toxicity by rapidly excreting most of the absorbed toxin to the gut lumen through apical (likely Abcc2 proteins), while a smaller part is transported to the blood and then to the liver by basolateral Abcc proteins. From an ecotoxicological point of view, the elimination of MCLR through the transport mechanism reported herein for *O. hatcheri* and by Bieczynski et al. (2014) for *O. mykiss*, reduces the amount of toxin available for trophic transference, which could explain the observed trend to biodilution of MCLR along diverse food chains (Ferrão-Filho and Kozłowski-Suzuki, 2011).

5. Conclusions

MCLR alters the glycosylation pattern of *O. hatcheri*'s intestinal epithelium. These changes could be related to a defensive response activated in the presence of the toxin and/or to dysregulation of glycoprotein synthesis and secretion.

In *O. hatcheri* enterocytes, MCLR would be metabolized by conjugation with GSH, followed by luminal excretion through an Abcc-like apical transporter, or by transport to the serosal side through Abcc-like basolateral transporters.

The presence of MCLR diminishes the capacity of enterocytes to eliminate other toxicants, which are eliminated through Abcc proteins.

Acknowledgements

This work was supported by grants: CONICET PIP 2014-2016-11220130100529CO and ANPCYT-PICT 2013-1415 to CML; PICT 2014-1121 to S.S.M.V. We would like to thank Mariela Demicheli, Pablo Hualde, Leonardo Molinari, and Pablo Moreno (CEAN) for their kind help. Calcein-AM was a generous gift from Dr. Amro M. Hamdoun. We acknowledge material support from Cooperativa de Agua y Saneamiento de San Martín de los Andes and from the Junín de los Andes Hospital laboratory. We are also grateful to Dr. Aldo Mottino and Marcelo G. Luquita, for technical advice and help, respectively, and María Silvina Villemur, Laboratorio Experimental de Calidad de Aguas – Instituto Nacional del Agua, Argentina for chromatographic analyses. We acknowledge the helpful suggestions of two anonymous reviewers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2016.07.016>.

References

- Amado, L.L., Monserrat, J.M., 2010. Oxidative stress generation by microcystins in aquatic animals: why and how. *Environ. Int.* 36, 226–235, <http://dx.doi.org/10.1016/j.envint.2009.10.010>.
- Amé, M.V., Baroni, M.V., Galanti, L.N., Bocco, J.L., Wunderlin, D.A., 2009. Effects of microcystin-LR on the expression of P-glycoprotein in *Jenynsia multidentata*. *Chemosphere* 74, 1179–1186, <http://dx.doi.org/10.1016/j.chemosphere.2008.11.068>.
- Atencio, L., Moreno, I., Jos, A., Pichardo, S., Moyano, R., Blanco, A., Cameán, A.M., 2008. Dose-dependent antioxidant responses and pathological changes in *Tinca tinca* after acute oral exposure to *Microcystis* under laboratory conditions. *Toxicol.* 52 (1), 1–12, <http://dx.doi.org/10.1016/j.toxicol.2008.05.009>.
- Azevedo, S.M.F.O., Evans, W.R., Carmichael, W.W., Namikoshi, M., 1994. First report of microcystins from a Brazilian isolate of the cyanobacterium *Microcystis aeruginosa*. *J. Appl. Phycol.* 6, 261–264, <http://dx.doi.org/10.1007/BF02181936>.
- Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* 48, 357–389, [http://dx.doi.org/10.1016/S0166-445X\(00\)00088-6](http://dx.doi.org/10.1016/S0166-445X(00)00088-6).
- Barltrop, D., Brueton, M.J., 1990. Chapter 8: the gastrointestinal tract and short-term toxicity tests. In: Bourdeau, P.J. (Ed.), *Short-Term Toxicity Tests for Non-genotoxic Effects*. John Wiley and Sons Ltd, 99–110 pp.
- Bieczynski, F., Bianchi, V.A., Luquet, C.M., 2013. Accumulation and biochemicaleffects of microcystin-LR on the Patagonian pejerrey (*Odontesthes hatcheri*) fed with the toxic cyanobacteria *Microcystis aeruginosa*. *Fish. Physiol. Biochem.* 39 (5), 1309–1321, <http://dx.doi.org/10.1007/s10695-013-9785-7>.
- Bieczynski, F., De Anna, J.S., Pirez, M., Brena, B.M., Villanueva, S.S.M., Luquet, C.M., 2014. Cellular transport of microcystin-LR in rainbow trout (*Oncorhynchus mykiss*) across the intestinal wall: possible involvement of multidrug resistance-associated proteins. *Aquat. Toxicol.* 154, 97–106, <http://dx.doi.org/10.1016/j.aquatox.2014.05.003>.
- Bieczynski, F., 2015. Cellular transport mechanisms, accumulation kinetics and toxic effects of microcystin-LR in Patagonian freshwater fish. In: PhD Thesis. National University of Comahue, Argentina.
- Boaru, D.A., Dragoș, N., Schirmer, K., 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures and cell lines: a comparative study. *Toxicology* 218, 134–148, <http://dx.doi.org/10.1016/j.tox.2005.10.005>.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248–254.
- Burkhardt-Holm, P., 1997. Lectin histochemistry of rainbow trout (*Oncorhynchus mykiss*) gill and skin. *Histochem. J.* 29 (11–12), 893–899.

- Bury, N.R., Newlands, A.D., Eddy, F.B., Codd, G.A., 1998. *In vivo* and *in vitro* intestinal transport of 3H-microcystin-LR, a cyanobacterial toxin, in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 42, 139–148.
- Campos, A., Vasconcelos, V., 2010. Molecular mechanisms of microcystin ioxicity in animal cells. *Int. J. Mol. Sci.* 11, 268–287, <http://dx.doi.org/10.3390/ijms11010268>.
- Carmichael, W.W., An, J., 1999. Using an enzyme linked immunosorbent assay (ELISA) and a protein phosphatase inhibition assay (PPiA) for the detection of microcystins and nodularins. *Nat. Toxins* 7, 377–385.
- Carmichael, W.W., 1992. Cyanobacteria secondary metabolites- the cyanotoxins. *J. Appl. Bacteriol.* 72, 445–459.
- Chan, L.M.S., Lowes, S., Hirst, B.H., 2004. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *Eur. J. Pharm. Sci.* 21, 25–51, <http://dx.doi.org/10.1016/j.ejps.2003.07.003>.
- Codd, G.A., Morrison, L.F., Metcalfe, J.S., 2005. Cyanobacterial toxins: risk management for health protection. *Toxicol. Appl. Pharmacol.* 203, 264–272, <http://dx.doi.org/10.1080/09670269910001736462>.
- Contardo-Jara, V., Pflugmacher, S., Wiegand, C., 2008. Multi-xenobiotic-resistance a possible explanation for the insensitivity of bivalves towards cyanobacterial toxins. *Toxicol.* 52, 936–943, <http://dx.doi.org/10.1016/j.toxicol.2008.09.005>.
- Díaz, A.O., García, A.M., Devincenzi, C.V., Goldemberg, A.L., 2003. Morphological and histochemical characterization of the mucosa of the digestive tract in *Engraulis anchoita* (Hubbs and Marini, 1935). *Anat. Histol. Embryol.* 32, 341–346.
- Díaz, A.O., García, A.M., Goldemberg, A.L., 2005. Glycoconjugates in the branchial mucous cells of *Cynoscion guatucupa* (cuvier, 1830) (Pisces: Sciaenidae). *Scientia Marina* 69, 545–553.
- Díaz, A.O., García, A.M., Goldemberg, A.L., 2008. Glycoconjugates in the mucosa of the digestive tract of *Cynoscion guatucupa*: a histochemical study. *Acta Histochem.* 110, 76–85.
- Díaz, A.O., García, A.M., Escalante, A.H., Goldemberg, A.L., 2010. Glycoproteins histochemistry of the gills of *Odontesthes bonariensis* (Teleostei, Atherinopsidae). *J. Fish Biol.* 77, 1665–1673.
- Díaz, A.O., Tano de la Hoz, M.F., García, A.M., Escalante, A.H., Goldemberg, A.L., 2012. Characterization of glycoconjugates in the pharyngeal cavity and the oesophagus of *Odontesthes bonariensis* by lectins. *Biotemas* 25 (4), 157–163.
- Dörr, F.A., Pinto, E., Soares, R.M., Azevedo, S.M.F.O., 2010. Microcystins in South American aquatic ecosystems: occurrence, toxicity and toxicological assays. *Toxicol.* 56, 1247–1256, <http://dx.doi.org/10.1016/j.toxicol.2010.03.018>.
- Dietrich, D., Hoeger, S., 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol. Appl. Pharmacol.* 203, 273–289, <http://dx.doi.org/10.1016/j.taap.2004.09.005>.
- Domeneghini, C., Pannelli Straini, R., Veggetti, A., 1998. Gut glycoconjugates in *Sparus aurata* L. (Pisces: Teleostei). A comparative histochemical study in larval and adult ages. *Histol. Histopathol.* 13, 359–372.
- Echenique, R., Aguilera, A., 2009. Chapter III: Floraciones de Cyanobacteria toxigenas en la República Argentina: antecedentes. In: *Cianobacterias y Cianotoxinas: Identificación, Toxicología, Monitoreo y Evaluación de riesgo*. Giannuzzi, L., (Ed.), Moglia editors, Corrientes, 1st ed. 238 pp. ISBN 978-987-05-5749-4.
- Eriksson, J.E., Toivola, D., Meriluoto, J.A., Karaki, H., Han, Y.G., Hartshorne, D., 1990. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. *Biochem. Biophys. Res. Commun.* 173, 1347–1353, [http://dx.doi.org/10.1016/S0006-291X\(05\)80936-2](http://dx.doi.org/10.1016/S0006-291X(05)80936-2).
- Evers, R., Kool, M., Smith, A.J., van Deemter, L., de Haas, M., Borst, P., 2000. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-MRP1- and MRP2-mediated transport. *Cancer Res. Campaign* 83 (3), 366–374, <http://dx.doi.org/10.1054/bjoc.2000.1260>.
- Falconer, I.R., Yeung, D.S., 1992. Cytoskeletal changes in hepatocytes induced by Microcystis toxins and their relation to hyperphosphorylation of cell proteins. *Chem. Biol. Interact.* 81, 181–196.
- Fardel, O., Jigorel, E., Le Vee, M., Payen, L., 2005. Physiological, pharmacological and clinical features of the multidrug resistance protein 2. *Biomed. Pharmacother.* 59, 104–114, <http://dx.doi.org/10.1016/j.biopha.2005.01.005>.
- Ferrão-Filho, A.S., Kozłowsky-Suzuki, B., 2011. Cyanotoxins: bioaccumulation and effects on aquatic animals. *Mar. Drugs* 9, 2729–2772, <http://dx.doi.org/10.3390/md9122729>.
- Ferreira, M., Costa, J., Reis-Henriques, M.A., 2014. ABC transporters in fish species: a review. *Frontiers Physiol.* 5 (266), 1–12, <http://dx.doi.org/10.3389/fphys.2014.00266>.
- Fischer, W.J., Dietrich, D.R., 2000. Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* 164, 73–81, <http://dx.doi.org/10.1006/taap.1999.8861>.
- Fischer, W.J., Althheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol. Appl. Pharmacol.* 203, 257–263, <http://dx.doi.org/10.1016/j.taap.2004.08.012>.
- Fischer, S., Lončar, J., Zaja, R., Schnell, S., Schirmer, K., Smital, T., Luckenbach, T., 2011. Constitutive mRNA expression and protein activity levels of nine ABC efflux transporters in seven permanent cell lines derived from different tissues of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 101, 438–446, <http://dx.doi.org/10.1016/j.aquatox.2010.11.010>.
- Gekeler, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The leukotriene LTD4 receptor antagonist MK571 specifically modulates mrp associated multidrug resistance. *Biochem. Biophys. Res. Commun.* 208 (1), 345–352, <http://dx.doi.org/10.1006/bbrc.1995.1344>.
- Gotoh, Y., Suzuki, H., Kinoshita, S., Hirohashi, T., Kato, Y., Sugiyama, Y., 2000. Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J. Pharmacol. Exp. Ther.* 292, 433–439.
- Gottesman, M.M., Pastan, I., 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62, 385–427, <http://dx.doi.org/10.1146/annurev.bi.62.070193.002125>.
- Honkanen, R.E., Zwiller, J., Moore, R.E., Daily, S.L., Khatra, B.S., Dukelow, M., Boynton, A.L., 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* 265, 19401–19404.
- Hooser, S.B., Beasley, V.R., Lovell, R.A., Carmichael, W.W., Haschek, W.M., 1989. Toxicity of microcystin-LR, a cyclic heptapeptide hepatotoxin from *Microcystis aeruginosa*, to rats and mice. *Vet. Pathol.* 26, 246–252.
- Horn, M.H., Gawlicka, A.K., German, D.P., Logothetis, E.A., Cavanagh, J.W., Boyle, K.S., 2006. Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory. *Mar. Biol.* 149, 1237–1245, <http://dx.doi.org/10.1007/s00227-006-0281-9>.
- Hualde, J.P., Torres, W.D.C., Moreno, P., Ferrada, M., Demicheli, M.A., Molinari, L.J., Luquet, C.M., 2011. Growth and feeding of patagonian pejerrey *Odontesthes hatcheri* reared in net cages. *Aquacult. Res.* 42, 754–763, <http://dx.doi.org/10.1111/j.1365-2109.2011.02827>.
- Ito, E., Kondo, F., Harada, K., 2000. First report on the distribution of orally administered microcystin-LR in mouse tissue using an immunostaining method. *Toxicol.* 38, 37–48.
- Ito, E., Takai, A., Kondo, F., Masui, H., Imanishi, S., Harada, K., 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. *Toxicol.* 40 (7), 1017–1025, [http://dx.doi.org/10.1016/S0041-0101\(02\)00099-5](http://dx.doi.org/10.1016/S0041-0101(02)00099-5).
- Jos, A., Pichardo, S., Prieto, A.I., Repetto, G., Vázquez, C.M., Moreno, I., Cameán, A.M., 2005. Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis sp.*) under laboratory conditions. *Aquat. Toxicol.* 72, 261–271, <http://dx.doi.org/10.1016/j.aquatox.2005.01.003>.
- Kim, J.J., Khan, W.I., 2013. Goblet cells and mucins: role in innate defense in enteric infections. *Pathogens* 2 (1), 55–70, <http://dx.doi.org/10.3390/pathogens2010055>.
- Klaassen, C.D., Aleksunes, L.M., 2010. Xenobiotic, bile acid, and cholesterol transporters: function and regulation. *Pharmacol. Rev.* 62, 1–96, <http://dx.doi.org/10.1124/pr.109.002014>.
- Kondo, F., Matsumoto, H., Yamada, S., Ishikawa, N., Ito, E., Nagata, S., Ueno, Y., Suzuki, M., Harada, K., 1996. Detection and identification of metabolites of microcystins formed in vivo in mouse and rat livers. *Chem. Res. Toxicol.* 9, 1355–1359.
- Kuiper-Goodman, T., Falconer, I., Fitzgerald, J., 1999. Human health aspects. In: Chorus, I., Bartram, J. (Eds.), *Toxic Cyanobacteria in Water: a Guide to Their Public Health Consequences, Monitoring and Management*, first ed. WHO, New York, 400 pp.
- Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. *Crit. Rev. Toxicol.* 22, 23–43.
- Lemaitre, C., Orange, N., Saglio, P., Saint, N., Gagnon, J., Molle, G., 1996. Characterization and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*). *Eur. J. Biochem.* 240, 143–149.
- Leslie, E.M., Deeley, R.G., Cole, S.P.C., 2005. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* 204, 216–237, <http://dx.doi.org/10.1016/j.taap.2004.10.012>.
- Li, D., Jiao, J., Shatos, M.A., Hodges, R.R., Dartt, D.A., 2013. Effect of VIP on intracellular [Ca²⁺], extracellular regulated kinase 1/2, and secretion in cultured rat conjunctival goblet cells. *Invest. Ophthalmol. Visual Sci.* 54 (4), 2872–2884, <http://dx.doi.org/10.1167/jovs.12-11264>.
- Linden, S.K., Sutton, P., Karlsson, N.G., Korolik, V., McGuckin, M.A., 2008. Mucins in the mucosal barrier to infection. *Mucosal Immunol.* 1 (3), 183–197, <http://dx.doi.org/10.1038/mi.2008.5>.
- Loretz, C.A., 1995. Electrophysiology of ion transport in teleost intestinal cells. In: Wood, C.H., Shuttleworth, T.J. (Eds.), *Cellular and Molecular Approaches to Fish Ionic Regulation*. Academic Press, London, UK, 1–352 pp.
- Lu, H., Choudhuri, S., Ogura, K., Csanaky, I.L., Lei, X., Cheng, X., Song, P.Z., Klaassen, C.D., 2008. Characterization of organic anion transporting polypeptide 1b2-null mice: essential role in hepatic uptake/toxicity of phalloidin and microcystin-LR. *Toxicol. Sci.* 103, 35–45, <http://dx.doi.org/10.1093/toxsci/kfn038>.
- Lu, X., Long, Y., Sun, R., Zhou, B., Lin, L., Zhong, S., Cui, Z., 2015. Zebrafish Abcb4 is a potential efflux transporter of microcystin-LR. *Comp. Biochem. Physiol. C* 167, 35–42, <http://dx.doi.org/10.1016/j.cbpc.2014.08.005>.
- Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. *Comp. Biochem. Physiol. C* 165, 28–52, <http://dx.doi.org/10.1016/j.cbpc.2014.05.002>.
- MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P., MacKintosh, C., 1995. The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. *FEBS Lett.* 371, 236–240.
- Malbrouck, C., Kestemont, P., 2006. Effects of microcystins on fish. *Environ. Toxicol. Chem.* 25 (1), 72–86.
- Meier-Abt, F., Hammann-Hanni, A., Stieger, B., Ballatori, N., Boyer, J.L., 2007. The organic anion transport polypeptide 1d1 (Oatp1d1) mediates hepatocellular

- uptake of phalloidin and microcystin into skate liver. *Toxicol. Appl. Pharmacol.* 218, 274–279, <http://dx.doi.org/10.1016/j.taap.2006.11.015>.
- Meyer, W., Luz, S., Schnapper, A., 2009. Lectin histochemical aspects of mucus function in the oesophagus of the reticulated Python (*Python reticulatus*). *Anat. Histol. Embryol.* 38, 316–318.
- Mottino, A.D., Hoffman, T., Jennes, L., Cao, J., Vore, M., 2001. Expression of multidrug resistance-associated protein 2 in small intestine from pregnant and postpartum rats. *American Journal of Physiology. Gastrointest. Liver Physiol.* 280, G1261–G.
- Othaz Brida, M.A., Agúndez, J.P., Gil, M.I., Labollita, H.A., 2010. Control de floraciones algales en los embalses de la Cuenca del Río Negro. In: VI Congreso Argentino De Presas Y Aprovechamientos Hidroeléctricos, Neuquén, 3–6 noviembre, p. 2010.
- Pavagadhi, S., Balasubramanian, R., 2013. Toxicological evaluation of microcystins in aquatic fish species: current knowledge and future directions. *Aquat. Toxicol.* 142–143, 1–16, <http://dx.doi.org/10.1016/j.aquatox.2013.07.010>.
- Pavlova, V., Georgieva, L., Paunova, T., Stoitsova, S., Nikolova, E., 2013. Carbohydrate localization in intestinal glycocalyx. *Sci. Technol.* 3 (1), 17–21.
- Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd, G.A., 1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochim. Biophys. Acta* 1425, 527–533, [http://dx.doi.org/10.1016/S0304-4165\(98\)00107-X](http://dx.doi.org/10.1016/S0304-4165(98)00107-X).
- Puig, A., 1992. Estructura espacial y temporal de la taxocenosis de entomostracos (Crustacea) limnéticos en el embalse Ramos Mexía (provincia del Neuquén y de Río Negro, Argentina). In: PhD Thesis. University of Buenos Aires.
- Runnegar, M., Berndt, N., Kong, S.-M., Lee, E.Y.C., Zhang, L., 1995. In vivo and in vitro binding of microcystin to protein phosphatases 1 and 2A. *Biochem. Biophys. Res. Commun.* 216 (1), 162–169.
- Sahin, A., Tencalla, F.G., Dietrich, D.R., Naegeli, H., 1996. Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology* 106, 123–130.
- Sivonen, K., Jones, G., 1999. Chapter 3: cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, first ed. WHO, New York, NY, 12–23pp.
- Soares, R.M., Magalhães, V.F., Azevedo, S.M., 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. *Aquat. Toxicol.* 70, 1–10, <http://dx.doi.org/10.1016/j.aquatox.2004.06.013>.
- Suprasert, A.T., Fujioka, T., Yamada, K., 1987. The histochemistry of glycoproteins in the colonic epithelium of the chicken. *Histochemistry* 86, 491–497.
- Takano, M., Yumoto, R., Murakami, T., 2006. Expression and function of efflux drug transporters in the intestine. *Pharmacol. Ther.* 109, 137–161, <http://dx.doi.org/10.1016/j.pharmthera.2005.06.005>.
- Tencalla, F., Dietrich, D., 1997. Biochemical characterization of microcystin toxicity in rainbow trout (*Oncorhynchus mykiss*). *Toxicon* 35, 583–595.
- Varki, A., Schauer, R., 2009. Chapter 14: sialic acids. In: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E. (Eds.), *Essentials of Glycobiology*, 2nd ed. Cold Spring Harbor Laboratory Press, New York.
- Vila, I., Soto, D., 1981. Atherinidae (Pisces) of rapel reservoir, Chile. *V erh. Internat. Verein. Limnology* 21, 1334–1338.
- Wiegand, C., Pflugmacher, S., 2005. Ecotoxicological effects of selected cyanobacterial secondary metabolites a short review. *Toxicol. Appl. Pharmacol.* 203, 201–218, <http://dx.doi.org/10.1016/j.taap.2004.11.002>.
- Williams, D.E., Craig, M., Dawe, S.C., Kent, M.L., Holmes, C.F.B., Andersen, R.J., 1997. Evidence for a covalently bound form of microcystin-LR in salmon liver and dungeness crab larvae. *Chem. Res. Toxicol.* 10, 463–469.
- Xie, L., Xie, P., Ozawa, K., Honma, T., Yokoyama, A., Park, H.D., 2004. Dynamics of microcystins-LR and -RR in the phytoplanktivorous silver carp in a sub-chronic toxicity experiment. *Environ. Pollut.* 127, 431–439, <http://dx.doi.org/10.1016/j.envpol.2003.08.011>.
- Zaja, R., Munić, V., Sauerborn Klobučar, R., Ambriović-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1 ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology* 90, 322–332, <http://dx.doi.org/10.1016/j.aquatox.2008.09.012>.
- Zar, H.J., 1999. *Biostatistical Analysis*, 4th ed. Prentice Hall, New Jersey.