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ARTICLE

Frequency of fish micronuclei to diagnose aquatic environmental conditions from Brazilian megacities: a case study of Iguaçu river, Southern Brazil

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ABSTRACT: (Frequency of fish micronuclei to diagnose aquatic environmental conditions from Brazilian megacities: a case study of Iguaçu river, Southern Brazil). More sensitive methodologies for detecting alterations in organisms due to the action of pollutants have been largely used. Among them, studies of genotoxicity with analyses of genomic, such as the frequency of micronuclei, damaging have been very useful. The present research aimed on using the frequency of micronuclei (MN) for diagnosing and predicting the environmental conditions from upper Iguaçu river with influences from Southern Brazil megacity (Curitiba, PR). Peripheral blood smear specimens of the native *Rhamdia quelen* from 10 sites were analyzed and it was computed their frequency of MN cells. The results were compared with a control group with the (zero MNs) in the absence of any altered circumstances. One-way ANOVA and Tukey test ($p < 5\%$, in both tests) was computed in order to verify the statistical robustness of the variation observed. Three of the 10 evaluated localities showed a high average number of MN cells, as well as, a high frequency of MN cells. Tukey test ($p < 5\%$), revealed the existence of statistically significant differences in 18 apart 55 possible pairwise comparisons. The results suggest that the species is undergone to genotoxic impact with a large variation in terms of intensity of the impact depending upon the inhabiting site. Considering that drastic changes in genetic material reduces the biotic evolutionary potential the results obtained herein suggest that *Rhamdia quelen* requires differential management programs in order to preserve the species and their ecosystems.
Keywords: Genotoxicity, Environmental Risk, *Rhamdia quelen.*

RESUMO: (Frequência de micronúcleos em peixes para diagnosticar as condições ambientais aquáticas de megacidades brasileiras: um caso de estudo do rio Iguaçu, sul do Brasil). Metodologias mais sensíveis para detectar alterações nos organismos devido à ação de poluentes têm sido amplamente utilizadas. Entre elas, os estudos de genotoxicidade com análises de danos genômicos, tal como a frequência de micronúcleos, têm sido muito eficazes. O presente trabalho objetivou através do uso na frequência de micronúcleos (MN), diagnosticar e prever as condições ambientais do rio Iguaçu superior, com influências no sul do Brasil na megacidade (Curitiba, PR). Esfregaços de sangue periférico de espécimes do nativo jundiá (*Rhamdia quelen)* de 10 localidades, foram analisados e quantificadas as células MN. Os resultados foram comparados com um grupo controle (zero MNs) na ausência de quaisquer circunstâncias alteradas. ANOVA one-way e teste de Tukey ($p < 5\%$, em ambos os testes) foram calculados de forma a verificar a robustez estatística da variação observada. Três das 10 localidades avaliadas mostraram um elevado número médio de células MN, assim como, uma alta frequência de células MN. O Teste de Tukey ($p < 5\%$) revelou a existência de 18 diferenças estatisticamente significativa de 55 possíveis comparações pareadas. Os resultados sugerem que esta espécie está sendo submetida a um impacto genotóxico com uma grande variação de intensidade, dependendo do local habitado. Considerando-se que as mudanças drásticas no material genético reduz o potencial evolutivo biótico, os resultados obtidos sugerem que o jundiá requer programas de gestões diferenciais, a fim de preservar a espécie e os seus ecossistemas.
Palavras chave: Genotoxicidade, Risco Ambiental, *Rhamdia quelen.*

INTRODUCTION

Loss of biological diversity might be a reflex of environmental degradation. Therefore, the maintenance of native habitat constitutions is important in order to preserve the biodiversity. However, the human activities have contributed for degradation of ecosystems, enhancing the causes of environmental impacts. Such causes, including environmental contaminants, might also contribute, even slowly, for species extinctions (Gavrilescu *et al*. 2015). Environmental contaminants promote intense degradation, resulting in both ecosystem alterations and harmful development of organisms due to the presence of prejudicial substances (Cardellicchio *et al*. 2015). Pesticides, chemical compounds, and sewer released by industries and human communities are frequently the causes of environmental contamination. Their effects comprise true threats of impacted ecosystems on biological diversity as well as for human health (Rose & Ruppel 2015, Gavrilescu *et al*. 2015).

In some cases, the environmental contamination is quite visible and dramatic. In the year 2000 an oil overflow at one of the tributaries of Iguaçu river (Southern Brazil) caused a huge impact in the river, besides the aquatic pollution raised from Curitiba (statewide principal me-

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gacity). A genotoxic evaluation in a native fish species revealed an increased genomic damaging associated to highest concentrations of aromatic polycyclic hydrocarbon derived from that oil overflowing (Filho 2006).

In the last decades the evaluations of altered ecosystems were improved given the increasing development of most sensible methods based upon genomic alterations. Such methods based upon the damage on the genetic material have provided protocols of proven accuracy (Bolognesi & Cirillo 2014; Lacroix *et al*. 2014).

Genomic damaging might be caused genotoxic agents that interact with DNA and RNA resulting in chemical--structural alterations in these molecules. Depending upon the rigor of such interactions, the alterations might reduce remarkably the maintenance of evolutionary potential of the biota (Angeletti & Carere 2014; Brandão 2014). Genotoxic effects of those agents are easily detected by genetic biomarkers in which allow monitoring native populations by estimating their putative genomic abnormalities (Bolognesi& Cirillo 2014).

Some of those abnormalities are named micronuclei that can be entire chromosomes or fragments of chromatin freely lost in the citosol in which their elevated frequency is normally raised by a global genetic instability. The occurence of micronucleated cells can be from spontaneous nature or induced by genotoxic compounds. Thus, MN test (micronuclei test) is an important instrument for the genotoxical research, especially in pharmacological therapy, ecotoxicological studies, and biomonitoring research (Stopper *et al*. 1997). In terms of global genotoxicity, MN test at peripheral blood comprises one of the best *in vivo* test focused on indicating genomic damaging by means of its simplicity, celerity, and low cost at chromosomal and interphase levels (Benincá 2006).

The evaluation genotoxic pollution of water (Adam *et al.* 2010), the genotoxicity induction by agrochemical compounds (Botelho *et al.* 2015), by heavy metals (Martins *et al*. 2015), and by diesel (Martinović *et al*. 2015) are true examples of genotoxic studies. In general, problems caused by pollutants have motivated the using of biomarkers in order to describe the real impact over the biodiversity from a given environment (Obiakor *et al.* 2012).

Several authors have evaluated genotoxicity in animals as manner for analysing the cyto-genotoxic effects of medicines in animals, and especially those effects caused by the exposure of native and exotic fish species to drastically altered environmental conditions (Adam *et al*. 2005, Adam *et al*. 2010, Ragugnetti *et al*. 2011, Sponchiado *et al.* 2011, Floehr *et al.* 2015, Baršienė *et al*. 2015),

The present research aimed on obtaining an environmental diagnosis in the upper Iguaçu river at a metropolitan region from a megacity in Southern Brazil (Curitiba, state of Paraná), by evaluating the frequency of micronucleated (MN) cells from a native fish species (*Rhamdia quelen* - Siluriformes (Quoy & Gaimard 1824). Furthermore, we focused on providing a fast, low cost, and accurate protocol in terms of diagnosing and predicting environmental quality in brazilian megacities. Indeed, the research aimed on responding the following questions: (a) *R. quelen* is suffering any genotoxic impact? (b) There are any significant differences of MN cells among several places in the upper Iguaçu river? (c) What is the environmental diagnosis for the upper Iguaçu river? (d) Would be the aquatic assemblage subjected to a serious threat? and (e) What is the observed environmental diagnosis for megacity similar to Curitiba?

MATERIAL AND METHODS

Species and sampling sites

Fourty-six specimens of *R. quelen* (Siluriformes) were sampled from 10 sites in the upper Iguaçu river, including a fish-farming supplied by water from the river. Table 1 provides the site names and a map with the accessed locations in the Iguaçu river.

Slide preparations and analysis

Smears with peripheral blood were prepared from the specimens sampled. Slides were stained with Giemsa (7.5%) for around six minutes at room temperature and the erythrocytes were analyzed at immersion microscopy (100X). It was used several criteria on recognizing a typical micronucleus such as morphology and staining equal to the main nuclei. Indeed, typical micronuclei cannot show bright and refractive light as well as no superposition with the main nuclei.

Statistical analyses

Total number of erythrocytes analyzed was standardized into 3000 cells for each analyzed specimen. Thus, the MN cells observed represent the total number of micronuclei for each 3000 analyzed cells. Based on that, we determine the frequency of MN cells. The amount of observed micronuclei was Log transformed [Ln(X+1)] and variance homoscedasticity was verified with the Bartlet test. Observed differences among the occurrence of MN cells through sampled sites were tested by one-way ANOVA and Tukey test. Comparisons were carried out by using a *R. quelen* control group with an occurrence of

Table 1. Locations, sampled sites and total number of specimens analyzed at each sampled site.

Location	Sampled site	Number of specimens
2	Zoológico lowland	6
3	Engenheiro Bleystream	4
4	PUCPR*	4
5	Guajuvira river	3
6	Zaniolo river	2
7	Cotia river	4
8	Irai river	5
9	Água azul river	6
10	Piraquara river	6
11	Passaunariver	6

* Location of the fish farming.

zero MN cells in the absence of any altered environmental conditions, according to the results obtained by Correa *et al*. (2008). The use of a control group from the literature was due to the difficulty on finding a suitable location (not impacted and with the same environmental conditions) to obtain these fish. All statistical analyses were performed in the software Statistica v. 6.0 (STATSOFT, INC.).

RESULTS

There was a large variation in the proportion of MN cells from the erythrocytes of *R. quelen* native from the 10 evaluated sites. The number of MN cells per specimen varied from 4.96 (Zaniolo; Table 1) to 339.85 (Passaúna; Table 1). Yet, the average number of MN cells (ANM) varied from 15 (Água Azul) to 170.1 (Passaúna). The frequencies of MN cells (FMN) varied from 0.5% (Água Azul) to 5.67% (Passaúna) (Table 2).

One-way ANOVA showed that the number of MN cells at *R. quelen* is high with reference to superior limit of confidence interval of control group in all evaluated sites (Fig. 1). Such variation among the sites analyzed was significantly different (p= 0,00002, significance level p <5%). The FMN in the site Passaúna was significantly higher than in the majority of other analyzed sites (Table 2; Fig. 1), with exception of the sites Zoológico and Engenheiro Bley that showed similar values to those observed in Passaúna.

Tukey test (Tab.3) revealed a differential statistically significant ($p \leq 5\%$) genotoxic impact occurring in *R. quelen* in 18 apart of the 55 possible comparisons (Table 3 background in gray). Such differential impacts comprise the totality of evaluated sites compared with control group. In addition, similar situation was also observed by comparing the sites Zoológico and Engenheiro Bley with the site Água Azul. Furthermore, other similar comparisons revealed equally like differential genotoxic impact involving the sites Guajuvira, Zaniolo, Rio Cotia, Irai, Água Azul, and Piraquara by comparing with Passaúna (Tab. 3)

DISCUSSION

Several authors have used the MN test in order to analyze the resulting genotoxic effects over a native species from a freshwater ecosystem (Furnus *et al.* 2014; Obiakor *et al.* 2014; Vincze *et al*. 2015). Given the global results obtained herein the effectiveness of the method is reinforced, especially in terms of gathering an environmental diagnosis from an aquatic ecosystem from a megacity in Southern Brazil.

Analyses of the *R. quelen* specimens from the sites of the upper Iguaçu river highlighted our results obtained in Zoológico, Engenheiro Bley, and Passaúna, showing the highest FMNs (Tab. 2). According to Bühler *et al*. (2014), Obiakor *et al*. (2014), Gutiérrez *et al*. (2015), Colin *et al*. (2016), fishes inhabiting polluted regions have shown elevated FMNs. Thus, the results observed here suggest that this species might be undergoing to hexogen aquatic compounds, indicating a sharp degree of pollution at these sites.

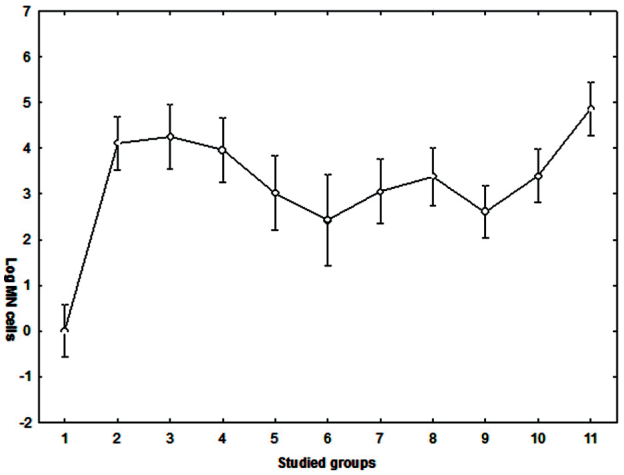

Figure. 1 Graph showing the result from one-way Anova. The vertical bars correspond to the confidence intervals (95%). Note the striking genotoxicity occurring on *Rhamdia quelen* at the whole sampled sites. For the sites, please consult the Table 1.

Table 2. Summary of the results obtained for the frequency of MN erythrocytes in *Rhamdia quelen* at the 10 sampled sites from upper Iguaçu river. The values with grayed background show the 3 sites with the highest FMNs. The values with black background correspond to the lowest FMNs.

	Control group		Zoológico lowland		Eng. Bley stream		PUCPR		Guajuvira river		Zaniolo river		Cotia river		Irai river		Água Azul river		Piraquara river		Passauna river	
Sp	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**	**T**	**MN**
I	3000	0	3000	80,7	3000	71,4	3000	40,3	3000	31,4	3000	4,9	3000	176	3000	59,4	3000	5,8	3000	26,5	3000	61,6
II	3000	0	3000	74,2	3000	82,4	3000	45,6	3000	23,7	3000	25,8	3000	21,5	3000	16,7	3000	18,8	3000	27,9	3000	102
III	3000	0	3000	82,4	3000	63,4	3000	87,4	3000	11,6	-	-	3000	7,9	3000	18,9	3000	11,8	3000	13,8	3000	311
IV	3000	0	3000	13,5	3000	65,7	3000	47,4	-	-	-	-	3000	6,8	3000	21,5	3000	23,6	3000	77,5	3000	42,3
V	3000	0	3000	89,9	-	-	-	-	-	-	-	-	-	-	3000	54,9	3000	20,5	3000	41,6	3000	340
VI	3000	0	3000	83,8	-	-	-	-	-	-	-	-	-	-	-	-	3000	9,7	3000	21,4	3000	164
Total	18000	0	18000	424	12000	283	12000	221	9000	66,7	6000	30,7	12000	212	15000	171	18000	90,2	18000	209	18000	1021
ANM	-	0	-	70.6	-	70.7	-	55.2	-	22.2	-	15.3	-	53	-	34.2	-	15	-	34.8	-	170.1
FMN	0		0,0235		0,0236		0,0184		**0,0074**		**0,0051**		0,0177		0,0114		**0,005**		0,0116		**0,0567**	

Abbreviations: Sp, specimen; MN, number of micronucleated cells; T, total number of cells; ANM, average number of MN cells; FMN, frequency of micronucleated cells; I-VI, specimens analyzed.

Table 3. Pairwise results from Tukey test among the Log of MN cells observed in *Rhamdia quelen* in the studied sites from upper Iguaçu river (2-11). (1) represents the control group. The values with grayed backgrounds show the statistical bases for a differential genotoxic impact. For the sites, please consult the Table 1.

	1	2	3	4	5	6	7	8	9	10	11
1	-										
2	0,0001	-									
3	0,0001	1,0000	-								
4	0,0001	1,0000	0,9999	-							
5	0,0001	0,5208	0,4477	0,7948	-						
6	0,0052	0,1450	0,1235	0,3147	0,9969	-					
7	0,0001	0,4346	0,3769	0,7538	1,0000	0,9928	-				
8	0,0001	0,8224	0,7400	0,9744	0,9997	0,8577	0,9997	-			
9	0,0001	0,0224	0,0267	0,1271	0,9987	1,0000	0,9948	0,7522	-		
10	0,0001	0,7965	0,7142	0,9718	0,9995	0,8269	0,9995	1,0000	0,6757	-	
11	0,0001	0,7345	0,9551	0,6566	0,0224	0,0049	0,0104	0,0407	0,0002	0,0287	-

Among sampled sites, the results obtained in the specimens from Passaúna were highlighted, given it was observed both the highest FMNs and average number of MN cells (Tab. 2). Such evidences indicate this site with probably the worst environmental quality among those worst, given the degree of genomic damaging observed in the specimens. Furthermore, it allows suggesting the priority on the recovering of the Passaúna river. This suggestion is reinforced by the results obtained on the water quality from Passaúna river, indicating its polluted situation (Bollmann *et al*. 2005).

One-way ANOVA confirmed that *R. quelen* is undergone to a genotoxic impact in the upper Iguaçu river (p = 0,00002 (p <5%); Fig. 1). The sites Zoológico, Engenheiro Bley, and Passaúna showed the highest statistical differences compared with control group. The results from table II are additional supports for such observations. A similar study with the species *Oreochromis niloticus* from Paraíba do Sul river that suffers similar influences from another megacity (Rio de Janeiro, Southeastern Brazil), has revealed by one-way ANOVA equally like impact at the species from that river (Linde-Arias *et al*. 2008). One-way ANOVA has also detected genotoxic impact over *Astyanax jacuhienses* from the stream Bom Jardim (Southern Brazil) that flows throughout a region under influences of a petrochemical complex (Lemos *et al*. 2008). Therefore, it is possible to reinforce that the sites Zoológico, Engenheiro Bley, and Passaúna show the worst environmental situations, given the degree of genomic damaging observed in the examined *R. quelen*.

In contrast, the results from ANOVA also indicated that fishes from the sites Zaniolo and Água Azul show both the lowest average number of MN cells and FMN cells. Conclusions about Zaniolo site must be careful and the region should be re-evaluated in order to organize a most consistent sampling for most conclusive results. Therefore, the evidences might suggest that Água Azul site could host the best environmental conditions, given the sites evaluated in the studied ecosystem. As aforementioned the loss of biodiversity might be a reflex of habitat losses in which critical alterations (with pollutants, p. ex.) along the ecosystems represent a large part of population declines in several species (SIH *et al*. 2000; Obiakor *et al.* 2012; Anil *et al.* 2014). Restocking programs could comprise a viable alternative of environmental rescue.

Hence, the results obtained herein also seem to answer for an essential requesting to environmental rescue, i.e., the identification of areas with suitable conditions for the effectiveness of biotic restocking. Therefore, the genotoxicity profiles observed in *R. quelen* from Água Azul arguments in favor to this site as the possible main target for future native biotic restocking in Iguaçu river. This argument is supported by the idea that critical alterations along the ecosystems might interfere in the genetic structure of populations, causing genetic depletion and reducing their capability on confronting with historical environmental changes (Oziolor & Matson 2015). Indeed, based on the results observed in the site Água Azul we suppose that low genotoxicity impact over *R. quelen* could be resulted from the possible minor influence of a megacity (Curitiba) over the species. Such hypothesis is supported by the largest distance between the site Água Azul and Curitiba rather that the other sites (Fig. 1).

In the Tukey test (Tab. 3) the sites were compared and 18, apart 55 possible comparisons, were statistically significant (p < 0,05). Linde-Arias *et al*. (2008), also used Tukey test in order to compare different localities from Paraíba do Sul river (Rio de Janeiro). However, no statistically significant differences were observed among localities. In contrast, results obtained herein allow recognizing some sites from Iguaçu river imposing different genotoxic circumstances for *R. quelen*. This hypothesis gathers aid given those differential statistical supports observed among the sites (Tab. 3). Furthermore, such evidences corroborate the results provided by tab.2 and fig.1, given *R. quelen* from Zoológico, Engenheiro Bley, and Passaúna comprises equally like a part of those 18 comparisons showing statistically significant support for Tukey test (Tab.3). Thus, such evidences reinforce the serious threats of those 3 sites over not only to *R. quelen*, but possibly to the whole aquatic assemblage.

CONCLUSIONS AND RECOMENDATIONS

Fishes are considered sentinel organisms for exposures of genotoxic substances to drinking waters, and vectors of pollutant transference to humans (López-López &

Sedeño-Díaz 2015). Thus, we can consider that besides the impact over aquatic assemblages, human populations might be equally like undergone to ineffective sanitation and/or environment management in the region.

The results obtained herein for *R. quelen* allow us to conclude that the sites Zoológico, Engenheiro Bley, and Passaúna might dock a largest bad managed amount of pollutants rather than the other sites. In addition, *R. quelen* and its environments request different priorities with reference of preservation managements. Thus, the sites Zoológico, Engenheiro Bley, and Passaúna should have the priority focus in terms of ecological management.

As important as to diagnose and to predict an imminent environmental risk, this approach allows detecting the degree of commitment in animal and human populations in terms of water dependency. Despite the present research be related to a study case, it reinforces the powerful of the MN test as a fast and accurate protocol to evaluate other rivers suffering similar influences of a megacity around the world.

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