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Planarians as invertebrate bioindicators in freshwater environmental quality: the biomarkers approach

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Abstract

Environmental contamination has become an increasing global problem. Different scientific strategies have been developed in order to assess the impact of pollutants on aquatic ecosystems. Planarians are simple organisms with incredible regenerative capacity due to the presence of neoblastos, which are stem cells. They are easy test organisms and inexpensive to grow in the laboratory. These characteristics make planarians suitable model-organisms for studies in various fields, including ecotoxicology. This article presents an overview of biological responses measured in planarians. Nine biological responses measured in planarians were reviewed: 1) histo-cytopathological alterations in planarians; 2) Mobility or behavioral assay; 3) regeneration assay; 4) comet assay; 5) micronucleus assay; 6) chromosome aberration assay; 7) biomarkers in molecular level; 8) sexual reproduction assay; 9) asexual reproduction assay. This review also summarizes the results of ecotoxicological evaluations performed in planarians with metals in different parts of the world. All these measurement possibilities make Planarians good bioindicators. Due to this, planarians have been used to evaluate the toxic, cytotoxic, genotoxic, mutagenic, and teratogenic effects of metals, and also to evaluate the activity of anti-oxidant enzymes. Planarians are also considered excellent model organisms for the study of developmental biology and cell differentiation process of stem cells. Therefore, we conclude that these data contributes to the future establishment of standardized methods in tropical planarians with basis on internationally agreed protocols on biomarker-based monitoring programmes.

Keywords: ecotoxicology, test organism, chromosome aberrations, mutagenicity assay, regeneration assay, micronucleus assay.

INTRODUCTION

Pollution and deforestation threaten the environmental balance of several ecosystems, affecting directly the biodiversity of the planet. Analyses suggest that the biodiversity of freshwater ecosystems is reducing very quickly in a global level, and this reduction is more accentuated than that observed in the terrestrial ecosystems impacted by anthropic actions (Sala *et al*., 2000). This way, the increase in chemical contaminants discharges into the aquatic environmental has led to an added urgency for the development of sensitive and reliable methods to assess the impact of these toxic agents on organisms that inhabit lakes, rivers and seas (Vargas *et al*., 2001; Morihama *et al*., 2012; Seiler & Berendonk, 2012). Therefore, actions are needed to control environmental pollution in order to restore impacted ecosystems, which can be achieved by the amplification of environmental monitoring activities. The use of aquatic organisms as biological sentinels has proved to be useful for environmental monitoring (Hutchinson *et al*., 1997; Bebianno *et al*., 2004; Zagatto & Bertoletti, 2006). Organisms in the classes of Turbellaria and, Cestoda, have been suggested as potential bioindicators for environmental pollutants (Gamo & Noreña-Janssem, 1998; Sures, 2004; Lau *et al*., 2007).

However, the use of new bioindicators for environmental monitoring requires previous systematic studies to firstly, establish the natural behavior of the organism in nature and in laboratory conditions (Stohler *et al*., 2004); secondly, identify biomarkers altered in response to environmental conditions (Prá *et al*., 2005; Villela *et al*., 2006; Knakievicz & Ferreira,

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2008); and thirdly establish the degree of susceptibility of the organism to specific agents (Chèvre *et al*., 2003; Knakievicz *et al*., 2008).

In this context, the proposal of the following review is to show a compilation of planarian ecotoxicological studies to assist in the standardization of this organism as a bioindicator. Hence, it will be possible to collaborate with the elaboration of experimental methodologies that will allow verifying which pollutants offer more danger for the health of aquatic ecosystems and setting a priority list for investments in recovery.

In this paper, the first section consists of a planarian description, the second section a brief review of the advantages and disadvantages of the planarians predicate for bioindicator organism. The third section basic studies on suitable biological responses and bioassays in planarians, and the fourth and concluding section will point out how this study can be practical for biomonitoring of aquatic pollution.

Planarians (Platyhelminthes, Turbellaria, Tricladida)

Recent progress in molecular phylogeny has provided trees that constitute a reference frame for discussing the still controversial evolution of body plans, resulting, for example, in the disappearance of two superphyla (acoelomates such as flatworms, pseudocoelomates such as nematodes) previously thought to represent grades of intermediate complexity between diploblasts (organisms with two germ layers) and triploblasts (organisms with three germ layers). These data analysis recognizes a clade of acoelomates, the Platyzoa (Giribet *et al*., 2000). The overall image now emerging is of a fairly simple global tree of metazoans, comprising only a small number of major branches (Adoutte, 1999). In Class Turbellaria, the most commonly studied order of planarians is Tricladida, so named because of the one ascending and two descending branches of their gastrovascular organ system, and is known to possess remarkable regenerative capacities (Baguña, 2012).

Phylogenetic relationships among major taxonomic groups within the Tricladida order postulated that the Maricola infraorder (marine planarian) constitutes the primitive sistergroup of Terricola (terrestrial planarian) and Paludicola (freshwater planarian) infraorders together and that Paludicola represents the most advanced group within the Tricladida (Sluys, 1989, 1999).The Dugesiidae (Family) belong to the Paludicola infraorder, and is a paraphyletic taxa with 19 terminal genera, which contain all freshwater planarian genera (Vries & Sluys, 1991; Riurtort *et al*., 2012).

Planarians are triploblastic acoelomate, and commonly dorsoventrally flattened soft-bodied unsegmented organisms, without circulatory, respiratory or skeletal structures. It has a pharynx and a blind gut lacking anus from the digestive system. The content of a prey body partially digested by proteolytic enzymes is sucked by the pharynx. Planarians are carnivore and catch various small invertebrates, such as protozoan, rotifers, larva insects, small crustaceans, snails and small annelids, and they can pass long periods without food (Ruppert *et al*., 2005). A solid tissue called mesenchyme fills the space between the epidermis and the gut. Flatworms lack a circulatory system and endocrine glands, but nervous system takes over an endocrine role by its production of peptidergic (neurosecretory) molecules. The peptidergic system is responsible for controlling and coordinating many aspects of the flatworm physiology, especially growth and development. Therefore, the platyhelminth nervous system takes a triple role as nervous system, endocrine system and in the regulation of stem cell proliferation (Fairweather & Skuce, 1995; Rossi *et al*., 2012).

Some of the most interesting aspect of the planarians' physiology and toxicology derive from their stem-cell system. Their stem-cell system are called neoblasts, which enable planarians to regenerate all tissue types and originate new organisms from any animal fragments. A fundamental step in planarian regeneration is the formation of a blastema by a process known as epimorphosis. A blastema is formed and grown by the continuous incorporation of neoblasts which, after proliferation, actively migrate from the stump (postblastema) to the blastema (Newmark & Sánchez-Alvarado, 2000). This structure is similar in form and organization to the embryonic limb buds produced during vertebrate embryogenesis. As in a limb bud, a regeneration blastema is made of two well-defined compartments: a superficial sheet of cells of epithelial origin covering the full extents of the bud, and an underlying mass of cells of mesenchymal origin. Interestingly, the definition of a developmental module also holds true for both limb and buds and regeneration blastemas (Sánchez-Alvarado, 2000).

In planarians, the control of regeneration, asexual and sexual reproduction is made by neurosecretions, such as melatonin that implicates in control of fission (Morita & Best, 1984) and latter, may stimulates the maturation and differentiation of gametes from neoblast cells (Fairweather & Skuce, 1995; Rossi *et al*., 2012). Besides, planarians are easy and inexpensive test organisms to culture in laboratory and they offer several responses that can be used to assess the effects of potentially harmful substances (Best & Morita, 1991; Knakievicz & Ferreira, 2008). Hence, planarians have shown suitable phylogenetical, physiological and ecological aspects for its use in ecotoxicology.

PLANARIANS AS POTENTIAL BIOINDICATORS OF FRESHWATER QUALITY

Freshwater planarians are an important component of the aquatic fauna of unpolluted streams or lakes (Indeherberg *et al*., 1999; Knakievicz *et al*., 2006). Planarians are slow-moving animals, gliding over the surface on a self-made mucous carpet and the egg containing cocoons are cemented firmly to substrate (Ball & Reynoldson, 1981), not larval stages or any resting stages and the adults are fragile (Weinzierl *et al*., 1999; Ruppert *et al*., 2005) making long distance dispersion unlikely. The salinity resistance of freshwater planarians is negligible making trans-oceanic dispersal unlikely. Known cases of trans-oceanic distribution of freshwater planarians are undoubtedly a result of introduction by man (Reynoldson

et al., 1981; Vries *et al*., 1984; Tamura *et al*., 1995).

However, other factors can determinate their distribution and abundance, such as temperatures and ecological processes. Water extreme temperatures, below 4ºC or above 25ºC, result in the disappearance of these organisms (Gamo & Noreña-Janssem, 1998). The ecological process, such as historical or zoogeographical events, physiological limitations of the species *vis-à-vis* the habitat, access to suitable energy sources, and the effects of competition can permit or prevent a species from reaching a habitat and determine both distribution and abundance of flatworms. Sympatric populations of planarians have used spatial and temporal separation methods to reduce competition which involves responses to physical aspects of their microhabitats (Reynoldson, 1981; Knakievicz *et al*., 2007).

The planarian physiology is studied, including the effects of age, size, season and reproductive activity on the assimilation of the pollutant, inability of long-term reproduction due to maximum possible levels of the pollutant in the environment (Kostelecky *et al*., 1989; Indeherberg *et al*., 1999; Knakievicz *et al*., 2006, 2007). Although planarians do not have large bodies, they provide versatility of analysis (Guecheva *et al*., 2001; Knakievicz & Ferreira, 2008; Plusquin *et al*., 2012).

However, potential accumulation of pollutants in recording with environmental pollutant content need to be evaluated in different populations, as isolated populations show divergence in sensitivity according to different localizations (Indeherberg *et al*., 1999). These differences can be explicated by natural selection and occurrence of bottlenecks (Weinzierl *et al*., 1999), which reflect evolutionary changes in the regulation of the stress response system in populations residing in long-term contaminated areas (Schill & Köhler, 2004).

Planarians have some disadvantage as bioindicator. The age of such organisms is hard to measure, but they live long, making it difficult to precisely follow up the result of pollutant integration over long periods (Indeherberg *et al*., 1999; Knakievicz *et al*., 2006). Besides, their abundance, distribution and alteration in the environment are unfamiliar. The biological character, life cycle, habitat and ecological niche of the population and specie planarians in the environment to be monitored in South America are familiar only for low localizations (Carbayo & Froehlich, 2008).

In this case, it is indispensable to obtain information on the ecological structure of regional populations to their use as sentinel organisms. Nevertheless, as described above, planarians are useful sentinel organisms as representatives of the invertebrate fauna of aquatic environments, due to their natural sensibility to environmental changes and pollutants (Prá *et al*., 2005; Knakievicz *et al*., 2007). Considering tropical environments, more ecological studies are essencial to elicit the basic characteristics of each regional population.

PLANARIANS AS TEST ORGANISMS

Most model systems used for monitoring of the environmental contamination are limited in their repertoire of responses when compared to the wide variety of toxins (Zagatto & Bertoletti, 2006). However, freshwater planarians have been used as test organisms to evaluate the toxic, cytotoxic, genotoxic, mutagenic, teratogenic effects of metals and other pollutants (Erichsen, 1940; Best & Morita, 1982; Prá *et al*., 2005, see Box 1, 2, 3, 4 and 4). Besides that, planarians permit assessment of contaminant effects through the simultaneous analysis of several different responses in distinct biological organization levels, such as molecular, cellular, morphological and behavioral (Best & Morita, 1991; Knakievicz & Ferreira, 2008; Plusquin *et al*., 2012). Thus, planarians are a promising approach to monitor the contamination of environmental because they allow complementary information about pollutants obtained from different types of biological responses at various levels of biological organization.

Planarian susceptibility

Mortality tests have been used to supplement chemical analyses. Such analyses are also useful for assessment of an organism sensitivity in comparison to different populations or species to the same substance (Milam *et al*., 2005; Zagatto & Bertoletti, 2006; Knakievicz & Ferreira, 2008). Comparable responses between standard test organisms and suitable surrogate species can validate the applicability of toxicity assessments for the protection of biodiversity. In addition, the use of regional surrogate species to assess potential ecological impacts is recommend because the toxicity endpoint from a single species may not offer protection for all species exposed to a broad range of contaminants (Milam *et al*., 2005).

Planarian species and populations from distinct regions show great divergences regarding sensitivity to metals (see Box 1). And besides, through combinatorial metal testing, the synergistic and antagonistic effect of these metals on the survival of the animals can be observed (Chu *et al*., 2005). Thus, the use of regional populations or species to evaluate the environmental risk requires previous standardized toxicity tests. The importance of using early life stages of this regional species for the evaluation of environmental impact has also been emphasized (Preza & Smith, 2001). Therefore, the development of standardized tests in freshwater planarian is a first step to facilitate the evaluation of the contaminant's impact on the ecosystems.

Useful biomarkers for planarians

Biomarkers refers to a measured biological responses which may be used as an indicator of some biological state or condition. They can detect early responses and pre-pathological alterations before other disturbances as disease, mortality, or population changes occur. The use of biological responses is a promising approach in the assessment of ecosystem health (Vasseur & Cossu-Leguille, 2003; Zagatto & Bertoletti, 2006). The multiparametric approach that uses different and/or complementary responses enables the

Box 1- A summary of toxicity test in planarians for evaluation of susceptibility at metals.

Species	Metal ($mg.L^{-1}$)	pH	LC_{50} ($mg.L^{-1}$)					Ref.
			24 h	48 h	72h	96 h	7 days	
Dugesia etrusca[b]	Al^{3+}	6.8	27 – 81	13 – 27	13 – 27	13 – 27	27	[1,2]
Poycelis felina[b]	Al^{3+}	5.2	80 – 100	-	-	-	-	[3]
Girardia tigrina[a,b]	Al^{3+}	-	-	47	47	47	-	[4]
Dugesia etrusca[b]	Cd^{2+}	6.8	0.45 – 0.67	0.45 – 0.67	0.45 – 0.67	0.45 – 0.67	0.28 – 0.67	[1,2]
Polycelis tenuis[a]	Cd^{2+}	-	-	-	-	-	10.2 - 6.00	[5]
Girardia tigrina[a]	Cd^{2+}	7.25	0.54	0.15	0.10	0.10		[4]
Polycelis felina[b]	Cu^{2+}	-	3.17 – 6.35	-	-	-	-	[6]
Girardia schubarti[a]	Cu^{2+}	-	2.44; 1.23;	-	-	-	0.95; 0.48	[7, 8]
Girardia schubarti[b]	Cu^{2+}	-	1.23	-	-	-	0.48	[9]
Girardia tigrina[a]	Cu^{2+}	-	0.46 – 0.76	0.33 – 0.50	0.33 – 0.50	0.33 – 0.48	-	[10]
Girardia tigrina[b]	Cu^{2+}	-	0.32 – 0.60	0.36 – 0.60	0.32 – 0.59	0.32 – 0.59	-	[10]
Girardia tigrina[c]	Cu^{2+}	-	0.24 – 0.32	0.19 – 0.24	0.11 – 015	0.11 – 0.12	-	[9]
Dugesia etrusca[b]	Cr^{3+}	6.8	26.0 – 78.0	26.0 – 78.0	26.0 – 78.0	26.0 – 78.0	-	[1,2]
Girardia tigrina[a]	Cr^{3+}	-	6.37	5.9 – 40.0	6.81 – 40.0	9.0 – 40.0	-	[4]
Girardia tigrina[b]	Cr^{3+}	-	4.79 – 30.0	9.1 – 20.0	6.6 – 20.0	5.3 – 20.0	-	[4]
Girardia tigrina[a]	Cr^{6+}	-	-	2.00	-	1.31	-	[11]
Girardia tigrina[a]	Cr^{6+}	-	-	-	-	-	7.96	[12]
Girardia tigrina[c]	Cr^{6+}	-	-	9.27	-	4.56	1.80	[12]
Poycelis felina[a]	Zn^{2+}	-	327	-	-	-	-	[13]
Girardia tigrina[a]	MMS	-	0.002	-	-	0.001	-	[14]
Girardia schubarti[a]	MMS	-	-	-	-	0.004	-	[15]

[a] Intact; [b] Regenerating; [c] Newborn (1 to 10 days of eclosion 10 days); [d] asexual; MMS = methyl methane sulfonate. [1] Calevro *et al.*, 1998; [2] Calevro *et al.*, 1999; [3] Kalafatić & Tomaskovic, 1999; [4] Knakievicz & Ferreira, data not published; [5] Indeherberg *et al.*, 1999; [6] Franjević *et al.*, 2000; [7] Guecheva *et al.*, 2001; [8] Guecheva *et al.*, 2003; [9] Prá *et al.*, 1999; [10] Knakievicz & Ferreira, 2008; [11] Sáfari, 1993; [12] Preza & Smith, 2001; [13] Franjevic *et al.*, 2000; [14] Knakievicz *et al.*, 2008. [15] Best & Morita, 1991.

assessment of the effect of different contaminants in the aquatic environment (Bebianno *et al*., 2004). Using planarians in tropical environments as test organisms, however, calls for the standardization of optimized methodologies. In this way, some methodologies in use are decribed bellow to orient future studies.

Histo-cytopathological alterations

Morphological or physiological alterations at the cellular or organ levels for acute toxicity have been measured as exposure responses. A wide range of histopathological and cytological damages and developmental and behavioral changes have been evaluated in planarians exposed to toxic agents (see Box 2). Theses measurable alterations are useful biological responses for the assessment of water-borne toxicants, and although promising, they are still in the experimental stage.

Mobility or behavioral assay

Flatworms are soft-bodies organisms, unprotected on the environmental and without supportive skeleton. Most vital functions, including maintenance of body shape, depend on the muscular system and are associated to an extracellular network of filaments. Definitive smooth muscles and distinctive characters seem to relate to the dimensions of the worm and its energy requirements, as well as to functional aspects of the particular organ (Silveira, 1998). Typical behavior range from simple movements such as locomotion and feeding, to more complex actions, including peripheral reflexes, integration of multiples stimulus modalities and even rudimentary forms of learning (Blair & Anderson, 1996).

Metals, such as Al^{3+}, cause changes in the serotonergic system of manner depending on the exposure period in mammals (Kumar, 2002). The quantitative assessment of locomotion activity changes on planarians have been successfully used to assess the effect of dopaminergic agonists, antagonists, or neuronal reuptake inhibitors (Buttarelli *et al*., 2008; Risso *et al*., 2012). This method provides a sensitive quantifiable approach for studying neurosubstances in a simple *in vivo* system (Raffa *et al*., 2001). The use of planarians' locomotion as a functional and sensitive endpoint also proves to be applicable to the study of neurotoxic agents (Knakievicz & Ferreira, 2008; Pagán *et al*., 2009, see Box 3).

Regeneration assay

Regeneration in adult planarians gives the opportunity to meet developmental biomarkers for aquatic pollutant. Planarian regeneration is based in blastemas, which share striking similarities between very distant phyla (Sánchez-Alvarado, 2000). For the blastema formation, the nervous system and the dorso-ventral interactions are evoked by

Box 2 - A summary on the use of histopathological and cytological biomarkers of sublethal effect in planarians.

Species	Metal	pH	Dose (mg.L^{-1})	Exposure time	Histopathological and cytological acute damages	Ref.
Polycelis felina[a]	Al^{3+}	7.1	80 – 100	24 h	2nd and 3rd day – disordered locomotion and rhabdites and disintegrated epidermal layer. 7th day – recovery of structures and depigmentation	[1]
Polycelis felina[a]	Al^{3+}	5.2	80 – 100	24 h	1st, 2nd and 3rd day – many lesions and depigmentation zones on the bodies. Abrupt movement of head. 7th – lesions were partially regenerated but still lacking pigment	[1]
Polycelis felina[a]	Cu^{2+}	-	3.17 – 6.36	24 h	Rhabdites and mucous layer in epidermis were damaged on the larger potion of the body. Endoplasmatic reticulum and mitochondria in the cells were considerably damaged.	[2]
Polycelis felina[a]	Cr^{6+}	-	300	3 and 6 h	3 h – reduction of mitotic index from 4.2% (control) to 3.6% (treatment) 6 h – increase of mitotic index from 4.5% (control) to 7.9% (treatment)	[3]
Poycelis felina[a]	Zn^{2+}	-	16.3 – 64,4	24 h	1st, 2nd day – Damaged auricles, lesion and depigmented areas on the body surface, disturbed locomotion. Mitochondria with altered structure. 3rd day – wounds closed, recovery of normal mobility and diminution of depigmented areas. Larger quantity of disintegrated cells. 4th and 5th day – similar to controls	[4]

[a] Intact planarian. [1] Kalafatić & Tomaskovic, 1999; [2] Franjević *et al.*, 2000; [3] Kalafatić & Taborsak, 1998; [4] Franjević *et al.*, 2000.

Box 3 - A summary on the use of behavioral and developmental biomarkers in planarians. Neurotoxicity, carcinogenesis and teratogenesis in planarians.

Species	Agent	Dose (mg.L^{-1})	Exposure time	Behavioral and cellular changes	Ref.
Dugesia dorotocephala[b,d]	MMC	0.02 and 0.04	14 days	Three different behavioral tests: a) righting response, b) motility, and c) prey capture. Effect on synaptic regeneration: Larger proportion of simples synapses and smaller proportion of complex synapses	[1]
Dugesia dorotocephala[b,d]	DMBA	-	2 – 12 weeks	Developed tumors and supernumerary eyes and heads	[1]
Dugesia dorotocephala[b,d]	BP and BA	-	2 – 12 weeks	More symptoms of acute toxicity	[1]
Dugesia etrusca[b]	Al^{3+}	13.0 – 27.0	6 days	Inhibited or disturbed development of eyespots and auricles	[2,3]
Dugesia etrusca[b]	Cr^{3+}	26.0 – 78.0	6 days	Inhibited or disturbed development of eyespots and auricles	[2,3]
Dugesia polychroa[b]	Mg^{2-}	0.50	-	Regeneration inhibition. Strongly impaired wound closure because of muscle relaxation	[4]

[a] Intact; [b] Regenerating; [c] Newborn (1 to 10 days of eclosion 10 days); [d] asexual. MMC – methylmercuric chloride; DMBA – dimothylbenzanthracen; BP – benzopyrene; BA – benzathracene; [1] Best & Morita, 1991; [2] Calevro *et al.*, 1998; [3] Calevro *et al.* 1999; [4] Shürmann & Peter, 1998.

wound close and cell proliferation (Martelly, 1984; Schumann & Peter, 1998). The blastema may have the most distal characteristics and functions as a center to send positional cues for the rearrangement of positional identity of the undifferentiated cells in the mesenchymal space (Agata & Watanabe, 1999; Baguñà, 2012).

Decapitated planarians rapidly regenerate their head, such as 72 to 96 hours in diploid *Girardia tigrina*, mixoploid *Girardia schubarti* (Knakievicz *et al*., 2006) and *Dugesia etrusca* (Calevro *et al*., 1999). The beginning of the regeneration presents active neoblast division (above basal levels) and is kept in high rates until complete reconstitution of lost structures, such as eyes and auricles (Newmark & Sánchez-Alvarado, 2000).

Hence, a better understanding of the effects of toxic substances on this process is of particular interest. Besides that, regeneration provides an opportunity to study the developmental effect of toxins on adult organisms (Calevro *et al*., 1998, 1999; Knakievicz & Ferreira, 2008), and it has been demonstrated that compounds that affect the development of vertebrates also affect decapitated planarians regeneration process (Best & Morita, 1982, 1991; Kalafatić *et al*. 2004, see Box 3).

Comet assay

The alkaline single-cell gel electrophoresis or comet assay is an elegant and sensitive technique for the detection of deoxyribonucleic acid damage at the level of the individual eukaryotic cell (Collins, 2004). It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet. The comet assay is expanding in application because of its rapidity and ability to discriminate cell types regarding the degree of DNA damage and DNA repair level. The comet assay has been used to evaluate the susceptibility of planarians to copper sulphate (Guecheva *et al*., 2001) and environmental samples (Prá *et al*., 2005). This assay detected similar response in planarians and *Golden mussel*

Box 4 - A summary on the use of DNA damage biomarkers in planarians.

Species	Assay	Agent	Exposure time	Concentrations of effect	Ref.
Mixoploid *Girardia schubarti*[a]	Comet	MMS	2 h	0.4 µM, 0.8 µM and 1.6 µM	[1,2]
Mixoploid *Girardia schubarti*[a]	Comet	Cu^{2+}	2 h	1.9 $mg.L^{-1}$ and 3,17 $mg.L^{-1}$	[1]
Mixoploid *Girardia schubarti*[a]	Comet	Cu^{2+}	7 days	0.16 $mg.L^{-1}$ and 0.32 $mg.L^{-1}$	[1]
Mixoploid *Girardia schubarti*[a]	MN	MMS	24 h	1.2 µM and 1.6 µM	[3]
Diploid *Girardia schubarti*[b]	MN	MMS	24 h	0.8 µM, 1.2 µM and 1.6 µM	[4]
Diploid *Girardia tigrina*[b]	MN	MMS	24 h	0.8 µM, 1.2 µM and 1.6 µM	[3]
Diploid *Girardia tigrina*[b]	MN	Cu^{2+}	24 h	0.10 mg Cu L^{-1}	[4]
Diploid *Girardia tigrina*[b]	MN	Cu^{2+}	96 h	0.80 mg Cu L^{-1}	[4]
Mixoploid *Girardia schubarti*[b]	MN	Gy	-	1.00 and 1.25 Gy	[4]
Diploid *Girardia tigrina*[b]	MN	Gy	-	0.50 and 1.25 Gy	[4]
Diploid *Girardia tigrina*[b]	CA	MMS	24 h	0.4 µM and 0.8 µM	[5]
Diploid *Girardia schubarti*[b]	CA	MMS	24 h	0.4 µM and 0.8 µM	[5]
Diploid *Girardia schubarti*[b]	CA	CP	72 h	100 $mg.L^{-1}$ and 200$mg.L^{-1}$	[5]
Diploid *Girardia tigrina*[b]	CA	CP	72 h	100 $mg.L^{-1}$ and 200$mg.L^{-1}$	[5]
Diploid *Girardia schubarti*[b]	CA	Gy	-	0.50 and 1.00 Gy	[5]
Diploid *Girardia tigrina*[b]	CA	Gy	-	0.50 and 1.00 Gy	[5]
Polycelis felina	CA	Cr^{6+}	3 and 6 h	0.3 $g.L^{-1}$	[6]
Polycelis felina	CA	Dicuran 500 FL	4 h	10 mM and 25 mM	[7]

[a] Intact; [b] Regenerating. [1] Guecheva *et al.*, 2001; [2] Prá *et al.* 2005; [3] Knakievicz *et al.*, 2008; [4] Knakievicz & Ferreira, 2008; [5] Lau *et al.*, 2007; [6] Kalafatić & Taborsak, 1998; [7] Milic-Strkalj & Kalafatić, 1997.

Box 5 - DNA damage biomarkers. Genotoxicity test in invertebrates.

Species	Assay	Agent	Exposure time	Concentrations of effect	Ref.
Golden mussel (*Limnoperna fortunei*)[a]	Comet	Cu^{2+}	2 h	3.75 $mg.L^{-1}$ and 20.00 $mg.L^{-1}$	[1]
Golden mussel (*Limnoperna fortunei*)[a]	MN	Cu^{2+}	24 h	3.75 mg. L^{-1} and 7.50 mg. L^{-1}	[1]
Golden mussel (*Limnoperna fortunei*)[a]	MN	Cu^{2+}	48 h	3.75 mg. L^{-1}	[1]

[a] Intact. [1] Villela *et al.*, 2006.

when exposed to environmental samples (Prá *et al*., 2003; Villela *et al*., 2006; see Box 4 and 5), which indicate that planarians can be useful for biomonitoring.

Micronucleus assay

Micronuclei are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division. Their formation indicate mutant daughter cell. The micronucleus (MN) assay is widely used to estimate cytogenetic damage induced by chemical or physical agents. As the MN test consists in the comparison of micronuclei frequencies, it is important to know exactly what is being compared, thus the cell cycle should be known (Schmid, 1975; Luzhna, 2013). For MN assay, it is also recommended to use cells with fewer and larger chromosomes (Udroiu, 2006), such as in *G. tigrina* (2n =16) and *G. schubarti* (2n = 8) (Knakievicz *et al*., 2007; 2008).

As planarian seems to respond to xenobiotics in the same way as mammals, they can be used to test the possible genotoxic properties of chemical and physical agents (see Box 4). Because, the developmental stage have been considered in intact and regenerating planarians, which had shown different sensitivities in MN assay, probable due to neoblast division activation (Reddien *et al*., 2004; Knakievicz *et al*., 2008). Thus, the neoblast MN assay in regenerating planarians can be useful for monitoring damages caused by both acute and chronic exposure to aquatic environmental pollutants with mutagenic potential.

Chromosome aberrations assay

Exposition of cells with DNA-damaging agents can result in unrepairable lesions in both strands of DNA. This leads to chromosome breakage, a phenomena that can be visually detected with the help of a microscope in metaphase cells, most easily identified in proliferating cells, such as plant meristematic cells, animal epithelial and hematopoietic cells, and planarian neoblasts. The analysis of chromosomic aberration (CA) is a classic method for direct mutation measure in systems exposure to mutagenic agents. This assay is indicated for the assessment of the new organism-test sensibility to mutagens, because it is perceptive and well-established, and can be applied in any organism with proliferative cells (Carrano & Natarajan, 1988). Planarians with mitotically activated neoblasts and treated with chemical mutagen agents of direct and indirect action and physical mutagen agents show an increase in chromosome aberration frequency in comparison to non-treated planarians (Lau *et al*., 2007; see Box 4). CA assay and MN assay are

equivalent, however the last assay is easier and faster than first (Smith, 1975).

Biomarkers in biochemical level

Toxic effects are also manifested at the molecular–subcellular level by impaired biochemical function (see Hyne & Maher, 2003). Catalase activity, heat shock protein and metallothionein induction are well-know biomarkers suitable to assess organism stress in environmental changes at a subcellular level. Theses endpoints are widely used to monitor the impact of water-borne toxicants on invertebrate and vertebrate (Viarengo *et al*., 2000; Guecheva *et al*., 2001; Arts *et al*., 2004; Plusquin *et al*., 2012).

The catalase activity, non-specific biomarker, is an important endpoint because it indicates if an organism has been submitted to a particular oxidative stress (Bebianno *et al*., 2004). Catalase is a ubiquitous antioxidant enzyme that is present in nearly all living organisms. The determination of a catalase activity in planarians is very easy to achieve, it does not require expensive chemicals, and produces results in a short time (Aebi, 1984). It functions to catalyze the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. There are commercial catalase assay kits that provide highly sensitive, simple, direct and ready assay for measuring catalase activity in any biological samples. In the assay, catalase first reacts with H_2O_2 to produce water and oxygen, the unconverted H_2O_2 reacts with a probe to produce a product, which can be measured at 570 nm (Colorimetric method) or at 535/587nm (fluorometric method). Thus, it is worth performing studies on this organism with other ROS-inducing substances to extend the applicability of the assay in ecotoxicology. In planarians, the induction of catalase activity show controversial results. The catalase activity in *G. schubarti* was a very sensitive indicator to acute copper exposure. Concentration as low as 0.040 mg Cu L^{-1} to 0.160 mg Cu L^{-1} stimulated the activity of the enzyme after 24 hours of incubation (Guecheva *et al*., 2003). However, regenerating *Girardia tigrina* treated by 96 with 0.05 mg Cu L^{-1} to 0.80 mg Cu L^{-1} did not show changes in catalase activity (Knakievicz & Ferreira, 2008). Different responses of catalase activity have been described in aquatic organisms exposed to copper in both field and laboratory experiments (Doyotte *et al*., 1997; Teisseire *et al*., 1998; Varanka *et al*., 2001; Guecheva *et al*., 2003). The enzyme activity pattern in acute and chronic exposure, however, should be investigated to elucidate the kinetic of the catalase activity induction.

The heat shock, or stress proteins (HSPs) are families of proteins, including stress-inducible and constitutively expressed members, classified according to their apparent molecular weight into four major groups denominated hsp90, hsp70, hsp60, and low-molecular weight stress proteins (Morimoto *et al*., 1990; Nover, 1991). The detection of stress protein induction in organisms, in particular hsp60 and hsp70, has been suggested as a suitable biomarker to evaluate environmental conditions in their surroundings. In *G. schubarti*, an induction of hsp60 was observed after a heat shock treatment; however, the hsp60 content cannot be considered a valuable biomarker of copper exposure (Guecheva *et al*., 2003).

Metallothioneins (MTs) are proteins found in virtually all major invertebrate phyla as well as in all vertebrates. They are water soluble, heat stable, and have a molecular mass of approximately 6 – 7 KDa. This protein presents a high affinity for metal ions and can selectively combine with them under very low intracellular concentrations. The major physiological role of MTs is to serve as a reservoir of cations such as copper and zinc, which are used to synthesize apoenzymes (Virarengo & Nott, 1993). And their inductive capabilities serve a key role in heavy-metal detoxification when intracellular metal concentrations exceed those necessary for metabolic functions (Ahearn *et al*., 2004). *G. tigrina* planarians probably have some type of Cu^{2+} inducible detoxification mechanism; because they gather Cu^{2+} solution at first, but in a second stage rapidly remove the Cu^{2+} gathered in their bodies when they are exposed to Cu^{2+} solution (Knakievicz & Ferreira, 2008). This detoxification mechanism is probably mediated by MTs (Ahearn *et al*., 2004), but there is no knowledge about MT induction in planarians.

In summary, biochemical techniques offer the possibility of rapidly detecting the initial stages of resistance in a population, and the mechanism(s) of resistance involved, however, there are important endpoints to predict effects on field populations of the freshwater contaminants from effects at the individual level (see Hyne & Maher, 2003).

Sexual reproductive assay

Studies at individual and cellular levels provide specific information about pollutant's effect on the action site, but they rarely provide information about the result in higher level of biological organization. Responses in higher biological organization levels (e.g. population and community) can vary from organism to organisms, and know little about your relationships with ecosystem health. Because, alterations in this level are more difficult to determine, less specific and only manifest at a late stage when environmental damages have already occurred (Connell *et al*., 1999). Each pollutant can interfere with hormone regulation involving different mechanisms (Corrêa *et al*., 2005). Thus, the development of suitable biomarkers of reproductive disruption in aquatic invertebrate by contaminant compounds is recommended (Fur *et al*., 1999; Rotchell & Ostrader, 2003). The knowledge of reproductive mechanisms in planarians can underpin biomarkers development, and subsequently make use of new molecular and cellular biology techniques for the enlargement of this understanding area.

A factorial study is carried out to determine the effects of environmental conditions on reproduction and growth and to examine differences among populations, generally made prior to using this species for toxicological studies. Heavy metals and organic compounds have been found in studies

with invertebrates to negatively affect hormonally-regulated functions, such as reproduction among others (Fingerman *et al*., 1998). The effects of pollutants on hormonally-regulated processes appear due at least in part to the impacting variable release of a neurohormone, possibly by affecting release of the neurotransmitter that normally stimulates release of that particular neurohormone. Neurotransmitters, including serotonin and dopamine, have been identified in planarians, involved in the proliferation of neoblasts, germinative cell precursors (Marqueti & franquit, 2002; Rossi, *et al*., 2012). Hence, alterations in reproductive performance can also be used as biomarker of water-borne toxicants in sexual planarians. The fecundity, cocoons production, in general, decreased with increasing exposure concentration. The peak of cocoons production occurred at the first days of exposure, following the gradual decline (Indeherberg *et al*., 1999; Knakievicz & Ferreira, 2008). The fertility, offspring production, can be affected by the quality of embryos produced. In marine invertebrates, five heavy metals were ranked in decreasing order of toxicity as follows: Cu > Zn > Pb > Fe > Mn (Virarengo & Nott 1993). Only zinc or manganese could cause specific malformation of embryos. However, the effects of zinc were intensified by the presence of the other metals such as manganese, lead, iron, and copper. (Kobayashi & Okamura, 2005). Thus, chronic pollution may also impair reproductive success and hence population sustainability.

The ecological relevance of this physiological response is very high, considering that hormonal alterations or endocrine disrupter compounds (EDCs) may cause variations in sex ratio of a wild population, its reproductive capability, and even the presence of the species in the investigated areas. Hence, these population responses are a direct measure of the ecosystem health, and relevant to the environmental management. Besides, the use of this development biomarker can produce additional information about reproductive strategies in distinct populations and species of planarians. Recently simultaneous direct determination of six endocrine disrupter compounds in wastewater samples in ultra trace levels has been made by liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ES-MS/MS) (Komesli, *et al*., 2012). And effect of these xenobiotic endocrine disrupters in the upregulation of sexual gene expression can be investigated by quantitative real time-PCR assay and immunohistological analysis (Miyashita *et al*., 2011), complemented the observations of endpoint already used in planarians.

Fission assay

Fissioning provides another simple but useful behavioral measure. The close interrelationship between fission and the ability of planarians to regenerate may result from the co-option of asexual reproduction mechanism for regeneration events. Nevertheless, there is little information about the difference in regeneration between fission fragment and artificially amputated worms (Sánchez-Alvarado, 2000). Hori and Kishida (1998) observed externally and anatomically the features of regeneration blastema in decapitated and fission planarians appeared to be similar, but the distribution of cellular types was quite different.

Many factors are known to accelerate the rate of fission, including water temperature, feeding condition, circadian clock and decapitation (Morita *et al*., 1987; Hori and Kishida, 1998; Itoh *et al*., 1999), probably because neurosecretions, such as melatonin, have implicated in the control of fission (Morita & Best, 1984) and neurotoxic substances are known for abolishing fission (Best & Morita, 1991). Thus, the amplification or inhibition in fission rate can be a suitable biomarker of the presence of aquatic contaminants with neurotoxic effect.

CONCLUSIONS

Planarians are suitable organisms for monitoring because they play different roles in the trophic web, undergo bioaccumulation and respond to toxin xenobiotic at low concentrations. Asexual planarians also are organisms of long time, easy and inexpensive culture in laboratory (Knakievicz, 2007).(Knakievicz, 2007). However, one important fact observed in those populations is the loss of allelic diversity, that occur mainly through genetic drift bottlenecks, especially of small sized populations resulting in populations less representative of natural populations of one species (Stohler *et al*., 2004; Templeton, 2011). But this can be an advantage, since genetically monogeneas populations are recommended as test organisms, because a higher genetic stability which allows obtaining uniform organisms, necessary to ensure precision and accuracy of the assays (Zagatto & Bertoletti, 2006).

This study demonstrated the advantageous and disadvantageous quality of planarians as bioindicator organisms. Planarians such as *G. schubarti* and *G. tigrina* have a wide distribution in unpolluted streams, lakes, and estuaries in site where the studies were conducted (Carbayo & Froehlich, 2008) so that field results of local to be monitored can be related to laboratory and *in situ* assays. In contrast to mobile species during part of their lives, planarians, as sedentary organisms, are more likely to exhibit sensitivity differences among populations, which can be explicated by physiological adjustments (Indeherberg *et al*., 1999) and/or the occurrence of bottlenecks (Weinzierl *et al*., 1999). Then, especially in countries like Brazil where there is a wide variety of differing ecosystems it is necessary to conduct experiments with regional species for more meaningful evaluation of the ecological potential impact. Nevertheless, there is little reference available or published on freshwater planarians distribution in Brazil, except from southern States and Coastal Plain (see Preza and Smith, 2001; Knakievicz *et al*., 2007; Carbayo & Froehlich, 2008 and Kawakats's work at 1970 and 1980 decade see Knakievicz 2007).

This study demonstrated the range of biological responses, potential biomarkers, in planarians available for biomonitoring. They constitute an early warning system

of chemical stress in organisms. Biormarkers are different in their significance and terminology, i.e. biomarkers of exposure, stress, defense, and damage (Bebianno *et al*., 2004). The usefulness and applicability of each one of nine biological responses were examined and evaluated from the data already available in the scientific literature, against a number of objective criteria, including: ecological relevance, sensitivity, specificity, dose–response relationship, confounding factors, technical difficulties and cost-effectiveness, when available in each case. In conclusion, the measurement of biomarkers in planarians is a promising approach to monitoring the contamination of the environmental because they provide complementary information about pollutants obtained otherwise. Thus, these data contributes to the future establishment of standardized methods in tropical planarians with basis on internationally agreed protocols on biomarker-based monitoring programmers.

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