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# *Toxicological risk assessment of complex mixtures through the WTox model*

# *Avaliação do risco toxicológico de misturas complexas através do Modelo WTox*

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**Abstract**

*Mathematical models are important tools for environmental management and risk assessment. Predictions about the toxicity of chemical mixtures must be enhanced due to the complexity of effects that can be caused to the living species. In this work, the environmental risk was accessed addressing the need to study the relationship between the organism and xenobiotics. Therefore, five toxicological endpoints were applied through the WTox Model, and with this methodology we obtained the risk classification of potentially toxic substances. Acute and chronic toxicity, citotoxicity and genotoxicity were observed in the organisms Daphnia magna, Vibrio fischeri and Oreochromis niloticus. A case study was conducted with solid wastes from textile, metal-mechanic and pulp and paper industries. The results have shown that several industrial wastes induced mortality, reproductive effects, micronucleus formation and increases in the rate of lipid peroxidation and DNA methylation of the organisms tested. These results, analyzed together through the WTox Model, allowed the classification of the environmental risk of industrial wastes. The evaluation showed that the toxicological environmental risk of the samples analyzed can be classified as significant or critical.*

*Modelos matemáticos são importantes ferramentas de gestão ambiental e gerenciamento de riscos. Previsões sobre toxicidade de misturas químicas ainda devem ser aprimoradas, devido à complexidade de efeitos que podem causar aos seres vivos. Neste trabalho, avaliou-se risco ambiental abordando a necessidade do estudo da relação entre organismo e xenobióticos. Para isto, cinco tipos de testes toxicológicos foram aplicados através do Modelo WTox, e com esta metodologia foi possível obter a classificação do risco de substâncias potencialmente tóxicas. Toxicidade aguda, crônica, citotoxicidade e genotoxicidade foram observadas nos organismos Daphnia magna, Vibrio fischeri e Oreochromis niloticus. Um estudo de caso foi realizado com resíduos sólidos provenientes da indústria têxtil, metal-mecânica e de papel e celulose. Os resultados demonstraram que diversos resíduos induziram mortalidade, efeitos na reprodução, aumentos na taxa de lipoperoxidação, mutações e alterações na metilação do DNA dos organismos. Estes resultados, analisados através do Modelo WTox, permitiram a classificação de risco ambiental dos resíduos industriais. A avaliação classificou as amostras como de risco ambiental toxicológico significativo ou crítico.*

Keywords: WTox Model; environmental risk; toxicity tests; complex mixtures.

Palabras clave: Modelo WTox; risco ambiental; ensaios toxicológicos; misturas complexas.

**Abbreviations**: $EC_{50}$: concentration of the test substance where 50% of the maximal effect is observed; $m^5dC$: 5-methyldeoxycytosine; MDA: malondialdehyde; NOEC: Non observed effect concentration; LOEC: Lowest observed concentration.

## 1 Introduction

In recent years, industrial activities have generated increasingly complex products; therefore, pollutants from these activities can be potentially impacting the environment. Solid waste and industrial effluents are composed of several toxic chemicals, so various techniques of chemical analysis were developed with the aim of identifying and measuring these substances. However, even if these techniques provide important data on pollutants, in the aquatic environment organisms are exposed not only to a single toxic substance, but to complex mixtures, and the synergistic and additive effects may become these mixtures dangerously powerful (Rand, 1995; Schwarzenbach et al., 2006).

Classical chemical analyzes are unable to detect synergistic additive and antagonic effects of complex mixtures, therefore, they are not suitable for evaluating the environmental risks of these substances (Lambolez et al., 1994). In this situation, bioassays are most recommended as they detect the presence of toxic substances and estimate quantitatively the damage that they cause to living organisms (Wolska et al., 2007). Several toxicological studies with aquatic organisms confirm the synergistic and additive effects of complex mixtures (Pavlaki et al., 2011, Cooper et al., 2009; Komjarova and Blust, 2008). Although it is possible to get a lot of data resulting from toxicity tests, one must give importance to its interpretation. The result itself is not the main objective of toxicity testing, but it is the basis for the gain of toxicological information, that can be used to make comparisons with other toxic agents and/or organisms (Rand, 1995). This toxicological information can be applied in methodologies that have the purpose of assessing environmental risks. In the American Regulatory Agencies like OECD and USEPA, the use of aquatic organisms from different trophic levels such as algae, microcrustaceans and fish is already mandatory in ecotoxicological studies for environmental risk analysis (Robbens, 2007).

Recently, the toxicological information has been used in computational toxicology, an area that integrates advances molecular biology and chemistry with modeling and computational science and that has been developed to increase the capacity of predictions in the field of toxicology (Kavlock et al., 2008). Therefore, several researchers have developed models of assessment and classification of hazard and environmental risks, based on toxicological effects. However, existing models which include toxicity data, like AQUATOX (Park et al., 2008) and DREAM (Reed and Hetland, 2002), also employ other variables such as flow rate and depth of the water body, the geology of the affected site, way of introduction of the toxic substance, data on the emission, and complete physical-chemical analysis of the substance analyzed. This amount of data (flow rate, depth of water body, etc...) can be difficult to obtain, and certain variables, like those mentioned before, may not make a link between the effects caused by toxic substances to the exposed organism. Moreover, models like AQUATOX (Park et al., 2008), and CREAM (Grimm et al., 2009), do not analyze the toxicity of complex mixtures, but only the substances that have their chemical composition known. Also, models are commonly used to estimate risks at sites previously known, or when an accident has happened. Despite advances in developing predictive models about the toxicity of chemical compounds, there is a need for developing predictive models about the toxicity of chemical mixtures, due to the complexity of the effects that these mixtures can cause in living cells (Silva et al., 2003).

Given this situation, and concerned with the problem of environmental contamination, this study aims to present a new model for environmental risk assessment, the Model WTox. This model proposes an assessment and a classification of the risk that substances or potentially toxic compounds presents to living things. The model was developed in order to address the relationship between the organisms and xenobiotic, namely how the individual behaves in the face of exposure to a substance with toxic potential. The toxic effect is the guiding variable of the model, and it is observed at the level of global and specific effects on organisms representing different trophic levels. The mathematical tool used to assess this relationship is based on Leopold Matrix Theory (Leopold at al., 1971). The matrix theory is a tool often used in environmental impact assessments, and the interaction matrix is a two-dimensional technique that counteract actions with environmental factors (Costa et al., 2005). The application of the results is done through software. In the case of samples of complex mixtures like industrial wastes (which are constantly bringing risks to the environment, either in the generation, storage within the industry, transport to final destination and even within the landfill), the WTox Model appears to be an alternative to environmental toxicological risk analysis. Thus, this work aimed to conduct a case study with industrial waste samples, to check the efficiency of the WTox Model, using five toxicological parameters for evidencing global and specific effects on organisms of different trophic levels.

## 2 Materials and methods

### 2.1 WTox Model-Toxicological Risk Assessment and Classification

The WTox Model was developed to evaluate and classify environmental risk, and its foundation is based on the results of the study of the relationship between the organism and complex mixtures, addressing parameters of global and specific toxicology. The mathematical tool applied in the model to assess this relationship is based on the Leopold Matrix Theory.

**Risk Matrix**

In environmental impact assessments the Leopold Matrix is a mathematical tool used to accomplish the combination of information from magnitude and significance of impacts, and which results in a numeric value (Leopold et al., 1971). The risk matrix of the WTox Model (Figure 1) was designed in order to obtain a numerical value derived from the crossing of the data resulting from toxicity tests (Toxicological Parameter) and the severity of effects found (Severity).

| **Toxicological Parameter** | | | | | |
|---|---|---|---|---|---|
| | A | 5 | 10 | 15 | 20 |
| | B | 4 | 8 | 12 | 16 |
| | C | 3 | 6 | 9 | 12 |
| | D | 2 | 4 | 6 | 8 |
| | E | 1 | 2 | 3 | 4 |
| | | IV | III | II | I |
| | | **Parameter** | | | |

Figura 1: Risk Matrix-interrelation of the toxicological parameter (toxicity level) with the severity induced by the xenobiotic.

**Toxicological Variables**

The criteria for selection of the variables used in the toxicological method were based on the application of the results from acute, chronic, genetic, oxidative and epigenetic effects. The selection of essays using representative organisms from different trophic levels was based on the fact that they are reproducible, accepted in the scientific community, provide known answers and have their methodologies consolidated (NBR 12713, 2003; ISO 10706, 2000; ISO 11348-3, 2007; Carvalho Pinto-Silva et al ., 2003; Matias et al. 1998a; Matias et al. 1998b).

The toxicological variables selected were the acute toxicity tests with Daphnia magna and Vibrio fischeri; chronic toxicity tests with D. magna; micronucleus test, lipid peroxidation test and DNA methylation test in Oreochromis niloticus'erythrocytes. Acute toxicity tests were applied to observe immobility effects on D. magna and inhibition of luminescence in V. fischeri. Chronic toxicity tests with D. magna were applied to evaluate effects on reproduction. The micronucleus test was applied in order to determine the genotoxic effect in erythrocytes of O. niloticus. The DNA methylation test was conducted to evaluate genotoxic and epigenetic effects by measuring the rate of 5-methylcytosine ($m^5dC$) in O. niloticus. The lipid peroxidation test was applied to evaluate oxidative effects on the membrane phospholipids by measuring the rate of MDA in O. niloticus.

**Toxicity levels**

Levels for toxicological endpoints were created to frame the results of toxicology tests. Each level corresponds to a particular risk classification. For each parameter, ranges of toxicological effect were established from outcome evaluations of researches already undertaken by LABTOX team members (Matias et al., 1998a; Matias et al., 1998b; Carvalho Pinto-Silva et al., 2003, 2005; Perreault et al., 2011; Melegari et al., 2012; Flohr et al., 2012a; Flohr et al., 2012b),and studies in the available literature (Lambolez et al., 1994; Villegas-Navarro et al., 1999; Knops et al., 2001; Backhaus et al., 1997; Lappaleinen et al., 2001; Çavas and Ergene-Gozucara, 2003; Rodriguez et al., 2006; Picado et al., 2008; Kang et al., 2010; Kostamo and Kukkonen, 2003; Grinevicius et al., 2009; Çavas and Ergene-Gözükara, 2005; Avci et al., 2005; Huang et al., 2007).

Depending on the result of the toxicity test applied, the toxicity level that the substance has for a particular species is determined. Each toxicological parameter proposed for the model has its $EC_{50}$ ranges for reproduction, frequency of micronuclei, MDA rate, and rate of $m^5dC$ (Table 1). Thus, toxicity is classified at levels ranging from A to E, i.e., extremely toxic to non-toxic.

Tabla 1: Interval rates of toxicological parameters of the WTox Model.

| **Level** | **$EC_{50}$** | **Reproduction** | | **Frequency of micronuclei** | **MDA rate** | **$m^5dC$ rate** | | **Classification** |
|---|---|---|---|---|---|---|---|---|
| | (%) | (%+) | (%−) | (n° of MN observed) | (%+) | (%+) | (%−) | |
| A | $< 5$ | >100 | 80−100 | $> 20$ | $> 120$ | $> 100$ | 80−100 | Extremely toxic |
| B | 5−10 | 60−100 | 50−80 | 10−20 | 90−120 | 70−100 | 50−80 | Highly toxic |
| C | 10−30 | 30−60 | 20−50 | 5−10 | 60−90 | 30−70 | 20−50 | Moderately toxic |
| D | 30−60 | 10−30 | 10−20 | 2−5 | 30-60 | 10−30 | 10−20 | Slightly toxic |
| E | 60−100 | 0−10 | $< 10$ | 0−2 | 0-30 | 0−10 | 0−10 | Non toxic or bland toxicity |

Note: The levels for the parameter $EC_{50}$ (%) were used for acute tests with D. magna and V. fischeri.
(%+): % of increase on the neonate number, compared to the negative control.
(%−): % of decrease on the neonate number, compared to the negative control.

**Severity categories**

The severity categories represent the physiological and behavioral observations of the biological reactive during testing. The definition of gravity is an essential step in the study, and should be performed only by an experienced professional in the field of toxicology. In this model, the severity categories were defined based on a Preliminary Hazard Analysis, as determined by the Technical Standard P4.261 (CETESB, 2003).

Thus, four categories of severity were defined and classified between I and IV, according to the effects observed during exposure. Gravity I is considered as Super Critical as irreversible physiological changes that causes cell or organism death are observed, affecting the food chain and therefore causing an ecological imbalance. Gravity II is considered as Critical as irreversible physiological changes are observed, though without causing cell or organism death, however, inducing chronic diseases that may influence the reproductive system, generate mutants, etc.. Gravity III is considered Marginal as reversible physiological changes are observed, not exceeding the body's ability to repair. Gravity IV is regarded as negligible, as unimportant physiological alterations are observed.

**Risk Classification**

Analyzing the proposed risk matrix to the model (Figure 1), it is apparent that insofar as it increases the degree of the relation between toxicological parameter and gravity, a proportional numeric increase occurs in the matrix. To classify these values the method establishes the following framework (Figure 2):

| |
|---|
| Critical Risk: $> 9$ |
| Significative Risk: $[6 - 9[$ |
| Reduced Risk: $[4 - 6[$ |
| Marginal Risk: $< 4$ |

Figura 2: Toxicological Risk Classification of the WTox Model.

However, when analyzing only one toxicological endpoint, we might be making misclassification of toxicological risk, because studies have shown that the set formed by acute effects, chronic effects, use of species representing different trophic levels, in addition to genetic and epigenetic effects can better support toxicological studies, consequently, the risk assessment becomes more accurate (Claxton et al., 1998, Crane et al., 2006; Pandard et al., 2006; Park and Choi, 2007).

The main reason for this possibility is rooted in the differences of mechanisms of toxic action. Thus, the methodology proposes that the toxicological risk assessment is only valid with the achievement of a set of three tests: one to show acute effects, one to show chronic effects, and one to show genotoxic and / or epigenetic effects. Due to differences between the availability of equipments in each laboratory, the methodology allows the use of different batteries of toxicological tests. This makes the WTox Model quite accessible, since it is an open method. To add a new toxicological endpoint, it is only necessary to establish the range of values of each toxicological level. Of course, this range of values should be established based on toxicological studies.

In models for environmental risk assessment security measures are used, such as the stipulation of different weights associated with the different toxicological tests applied. Thus, for the WTox Model, it was established that an acute effect has weight = 1, because according to Villela et

al. (2003), environmental contamination by agents that induce acute toxicity is easily detected due to the immediate effect which allows a rapid control of the emitting source. For a chronic or genotoxic and /or epigenetic effect, it was established a weight = 2 because, according to Villela et al. (2003), toxic substances that induce chronic effects produce long-term damage, which can reduce the survival of organisms, influence on the reproduction, or even change the genetic heritage. The authors also state that a source of genotoxic and /or carcinogenic activity may be detected only after many years, requiring preventive action.

The weight of the risks was considered as magnitude arithmetic, where the risk has marginal weight = 1, has reduced the risk weight = 2, the risk has significant weight = 3 and the critical risk has weight= 4. The crossing of the information occurs as follows: we use the result of the risk found for each parameter analyzed, and the weights are multiplied. Therefore, we add up the numeric values found in each of the three tests, and this value will define which is the toxicological risk of the sample analyzed (Table 2).

Tabla 2: Example: integration of the toxicological information.

| | **Classification** | | | | **Score** |
|---|---|---|---|---|---|
| **Parameter** | Marginal Risk (weight 1) | Reduced Risk (weight 2) | Significative Risk (weight 3) | Critical Risk (weight 4) | |
| Acute Toxicity (weight 1) | ✓ | | | | 1 |
| Chronic Toxicity (weight 2) | | | | ✓ | 4 |
| Genotoxicity (weight 2) | | ✓ | | | 8 |
| | | | | Total | 13 |

The integration of the results of the three tests applied provides the toxicological risk of the substance that was analyzed. The final risk can then be classified in four grades (Figure 3):

| **Risk Classification** | **Sum of scores** |
|---|---|
| Marginal | 1 to 5 |
| Reduced | 6 to 10 |
| Significative | 11 to 15 |
| Critical | 16 to 20 |

Figura 3: Final Risk Classification of the WTox Model.

In the example presented in Table 2, the sum of scores is equal to 13(1 + 4 + 8), and the substance is classified as Significant Risk.

## WTox Software

The WTox Software was developed to automate some steps of the classification methodology proposed by the WTox Model. It is freely available at http://www.labtox.ufsc.br/modelo-wtox. In Figure 4 is possible to see the main screen of the software. The first step is to select the toxicological variable to be evaluated, for example, $EC_{50}$ (%), and fill out the result found for this variable.

The next step is to set the severity of this result. The experience of the professional is essential for the correct judgment of the severity of each toxicological endpoint. Soon after, click on "Add". Thus, results of the variable are stored in the system. After this step we can calculate the risk of the selected variable by clicking on "Determinate Risk" or then add the results found in other toxicological variable. The methodology of the model proposes that a full risk assessment should include three parameters of toxicity: one to check for acute effects, one to verify chronic effects, and one to check for genotoxic effects. Thus, the procedure for including toxicological results of each variable is repeated until all the results of the battery of tests selected are stored in the system. Then click on "Determinate Risk", and the software calculates the toxicological risk for the selected variables and automatically generates the classification of risk for the studied sample.

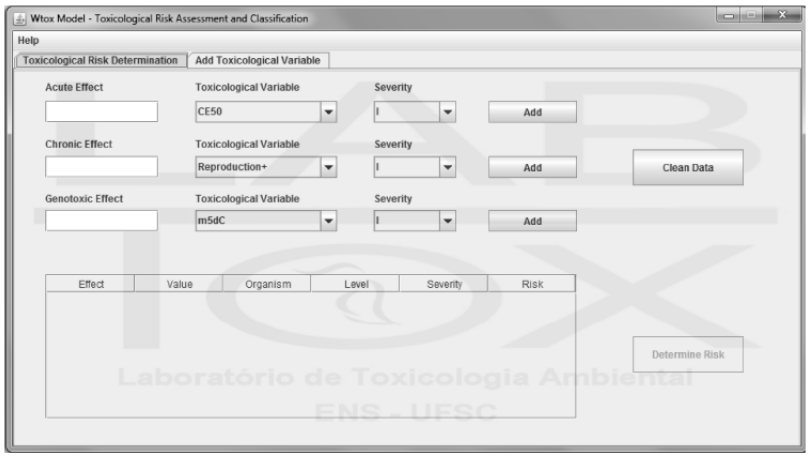


Figura 4: Initial window of the WTox Software.

## 2.2 Case Study

**Waste sampling**

Waste sampling was performed according to Flohr et al. (2012a). Briefly, ten samples of waste were collected at the entrance of the Industrial and Sanitary Landfill of Blumenau (Blumenau, SC - Brazil). Before entering the landfill, each sample must be previously classified according to NBR 10004 method (ABNT, 2004c). APHA methodologies (APHA, 2005) are used to obtain the chemical characterization of these wastes. Different samples of the same type of waste were chosen according to their chemical characterization. Only samples of the same type of waste with very close chemical composition were collected, so it was possible to obtain the average values of chemical parameters from each type of waste. Sample characteristics are described at Flohr et al. (2012a), according to their waste classification report. The wastes were characterized, according to NBR 10004 (ABNT, 2004c), as sludge from the treatment plant of the following industries: textile (samples TX1, TX2, TX3 and TX4), metal-mechanic (samples MM1, MM2 and MM3), and pulp and paper (samples PP1, PP2 and PP3).

**Waste sampling**

Soluble fraction of industrial waste sample preparation Sample preparation were based on NBR 10005 (ABNT, 2004b), with modifications, according to Flohr et al. (2012a). 100 g of raw

sample were introduced in a bottle which was filled with 2.0L of distilled water. The bottle was kept under shaking for 18 hours with a rotation of 30 rpm in a rotary shaker. After shaking, the samples had their pH and dissolved oxygen measured according to the methodology described in Standard Methods for Examination of Water and Wastewater (APHA, 1995). Samples of soluble fractions (leached) were used in the toxicity tests.

**Toxicity Tests**

Five different types of toxicity tests were selected for WTox Model. D. magna's acute and chronic toxicity tests, and V. fischeri toxicity tests were performed as described in Flohr et al. (2012a). Frequency of micronucleus, lipid peroxidation and DNA methylation tests were performed with O. niloticus, as described in Flohr et al. (2012b).

D. magna acute toxicity tests were performed according to NBR 12713 (ABNT, 2003). Samples were diluted with ISO medium, according to ISO 6341 (1996) in four concentrations: 100, 50, 25, and 12.5%, and a negative control (organisms exposed only to the ISO medium). Organism's immobilization was observed after a period of 48h. According to EPA 821-R-02-012 (USEPA, 2002), the Trimmed Spearman-Karber Method was used to calculate $EC_{50,48h}(\%)$.

Twenty one (21) days chronic toxicity tests were performed according to ISO 10706 (2000), Bianchinni and Wood (2002), and Knops at al. (2001), with modifications, as described in Flohr et al. (2012a). Each test was conducted with four sample dilutions, and a negative control (organisms exposed only to M4 medium). D. magna were fed with Scenedesmus subspicatus algae, and the media were changed every 48 hours. According to EPA 821-R-02-013 (USEPA, 2002), the results were analyzed using Dunett tests or t-test with Bonferroni adjustment, through the Dunnett Program version 1.5 (USEPA, 1999). A significance level of $P< 0.05$ was accepted.

Toxicity tests with marine luminescent bacteria V. fischeri were performed according to ISO 11348-3 (2007), and according to the methodology developed for the equipment Microtox© (Azur Environmental, 1997). Exposure time was 30 minutes, and $EC_{50,30min}$ (%) was calculated by the equipment's software Microtox Omni 4.0.

Frequency of micronucleus, lipid peroxidation and DNA methylation rates were measured in erythrocytes of O. niloticus (weight = 30.6 ± 0.3 g, length = 11 ± 2 cm), as described in Flohr et al. (2012b). Samples of soluble fractions (leached) were exposed to the test organisms in the following dilutions: 6.25, 12.5 and 25.0% for textile waste samples (adapted from Çavas and Ergene-Gözücara, 2003); 6.25 and 25% for metal-mechanic waste samples (adapted from Mishra and Mohanti, 2008, and from Chen et al., 2001); and a 25.0% dilution for pulp and paper waste samples (adapted from Wahbi et al., 2004). One organism exposed to the water of the acclimatization tank was used for the negative control, while one organism exposed to a concentration of 2.5 mg/L of potassium dichromate (sublethal concentration of $K_2Cr_2O_7$ adapted to body weight, based on Mishra and Mohanty, 2008) was used as a positive control.

The micronucleus test was performed according to Carvalho Pinto-Silva (2003). After the removal of fish's erythrocytes, two slides were prepared for each animal. The slides were gently washed with distilled water, and dried at room temperature. The slides were fixed in methanol for 10 min, and stained using the Feulgen/Fast-Green method. Cytogenetic analysis was performed through an optical microscope (Olympus BX40) in 10x100 magnification. Two thousand haemocytes per animal were counted and scored for micronucleus formation.

Lipid peroxidation was measured by MDA quantification. The analytical method for MDA quantification was performed according to Matias and Creppy (1998a), with modifications. 200 mL of blood from O. niloticus fish were collected and centrifuged. 100$\mu$L of plasma obtained were used for the test. MDA was quantified through HPLC (HP1050 HPLC System) with a Suplecosil LC-18 column (250x4.6 mm, 5$\mu$m). The amount of MDA-TBA quantified was related to the protein content of cellular homogenates, determined using the colorimetric Bradford method (1976).

DNA methylation was measured by $m^5dC$ rates. DNA methylation test was carried out based on Bardakci and Sibinski (1994) and Matias and Creppy (1998b), with some modification. 200 mL of blood from O. niloticus fish were collected and centrifuged. The pellet formed was used for DNA extraction. The quantification of $m^5dC$ was performed by HPLC (HP1050 HPLC System) and an UV detector (HP1050 Series Variable Wavelength Detector), using an Agilent Zorbax SB-Phenyl column (250x4.6 mm, 5$\mu$m).

**Utilization of the toxicological data in Model WTox**

For acute toxicity tests with D. magna and V. fischeri the $EC_{50}$ values found for each test were used. For chronic toxicity tests with D. magna the percent of increase or decrease in fertility rates observed in Non Observed Effect Concentration (NOEC) were used. When it was not possible to obtain the value of NOEC the value of the Lowest Observed Effect Concentration (LOEC) was used. To study the frequency of micronuclei it was considered the increased number of micronuclei in two thousand cells analyzed, regardless of this value is statistically significant. In lipid peroxidation and DNA methylation tests, the percentage increase or decrease in the values found in comparison with the negative control were used, regardless of whether this value is statistically significant.

# 3 Results

## 3.1 D. magna and V. fischeri toxicity tests

All samples of soluble fractions of industrial solid waste had pH and dissolved oxygen (DO) value in accordance with the recommendations for acute toxicity tests with D. magna (pH between 5.0 and 9.0, DO$\geq$ 2.0 mg/L). According to the proceedings of ISO 11348-3 (2007), all bacteria batches used in toxicity tests were sensitive to zinc sulfate ($ZnSO_4 \cdot 7H_2O$) and the correction factor (fk) was within optimal values for the viability of the toxicity tests. The results for the D. magna acute and chronic tests and V. fischeri tests are summarized in Table 3, and are expressed as defined for the application in the WTox Model. Details of the results and discussion of these tests can be seen in Flohr et al., (2012a).

Tabla 3: Summary of the results of D. magna and V. fischeri acute toxicity tests after exposition to soluble fractions of textile, metal-mechanic, and pulp and paper industrial solid wastes.

| **Industry** | **Sample** | **$EC_{50,30min}$(%) V. fischeri** | **$EC_{50,48h}$(%) D. magna** | **Reproduction effects D. magna** | |
|---|---|---|---|---|---|
| | | | | NOEC (%) | % of increase (+) or decrease (-) on the neonate number, compared to the negative control |
| Textile | TX1 | NT | 70.71 | 50 | +19.82 |
| | TX2 | 43.77 | NT | 6.25 | +37.4[a] |
| | TX3 | 12.08 | 11.26 | 0.78* | +32.50[a] |
| | TX4 | 17.99 | 48.29 | 12.5 | −18.68 |
| Metal-mechanic | MM1 | 73.86 | 84.86 | 0.78 | +5.85[a] |
| | MM3 | 17.47 | 2.21 | 0.52* | +25.75[a] |
| | MM2 | 48.73 | 70.71 | 12.5 | +31.1[a] |
| Pulp and Paper | PP1 | 91.93 | NT | 12.5* | +70.0[a] |
| | PP2 | 19.00 | NT | 12.5* | +35.61[a] |
| | PP3 | 18.61 | 51.76 | 6.25* | −40.33[a] |

* LOEC NT: Non Toxic
[a] Values which differ significantly from control ($P < 0.05$).

Among the organisms exposed to samples of the textile industry, it was observed that the sample TX3 induced the highest acute toxicity values both for V. fischeri as to D. magna. The effects on reproduction of the organisms exposed to this sample were significant, and there was an increase in the number of neonates as compared to negative control. Among the samples of the metal-mechanic industry, the greatest effect was observed in organisms exposed to the sample MM3, acute toxicity tests showed significant values of toxicity, and chronic toxicity test showed a significant effect on the increase of neonates generated by the organisms exposed to this sample. Among the samples of pulp and paper industry, it was observed that V. fischeri showed highest sensitivity when compared to D. magna. In chronic toxicity tests with D. magna samples PP1, PP2, and PP3 induced significant reproduction effects. Samples PP1 and PP2 induced an increase of neonates, and sample PP3 induced a decrease in the number of neonates when compared to negative control.

### 3.2 Cytotoxicity and genotoxicity tests with O. niloticus

The results of micronucleus frequency, lipid peroxidation and DNA methylation tests are summarized in Table 4, and are expressed as defined for the application on the WTox Model. Details on the results and discussion of lipid peroxidation and DNA methylation tests can be checked in Flohr et al. (2012b).

All samples of soluble fractions from the textile industrial waste induced significant increases in $m^5dC$ rates of O. niloticus, and the samples TX1 and TX2 induced significant increases in MDA rates of the exposed organisms. Samples of textile waste induced the greatest amount of micronuclei in erythrocytes from O. niloticus. Among the organisms exposed to samples of soluble fractions from metal-mechanic industrial waste, all organisms had a significant increase in MDA rates and only the sample MM3 induced significant increases in DNA methylation. Furthermore, the sample MM1 induced a decrease in the rate of $m^5dC$. Samples from the metal-mechanic industry induced the least amount of micronuclei in exposed fish. Among the samples

Tabla 4: Frequency of micronucleus, lipid peroxidation and DNA methylation rates in erythrocytes of O. niloticus exposed for 48h to soluble fractions of textile, metal-mechanic and pulp and paper wastes.

| **Sample** | | **Dilution** | **Micronucleus test** number of MN observed | **Lipid peroxidation test** % of increase on the MDA rates, compared to the negative control | **DNA methylation test** % of increase (+) or decrease (-) on the $m^5dC$ rates, compared to the negative control |
|---|---|---|---|---|---|
| Negative Control | | - | 2 | - | - |
| Textile | TX1 | 12,5% | 12 | 389,32 | +312,24 |
| | TX2 | 25% | 9 | 418,59 | +272,97 |
| | TX3 | 6,25% | 7 | 77,49 | +321,74 |
| | TX4 | 12,5% | 7 | 48,5 | +722,93 |
| Metal-mechanic | MM1 | 25% | 4 | 393,53 | −73,21 |
| | MM3 | 6,25% | 3 | 363,36 | +183,24 |
| | MM2 | 25% | 3 | 602,48 | +59,63 |
| Pulp and Paper | PP1 | 25% | 3 | 106,17 | +288,49 |
| | PP2 | 25% | 14 | 66,1 | +471,52 |
| | PP3 | 25% | 3 | 5,58 | +272,63 |
| Positive Control $K_2Cr_2O_7$ | | 2,5 mg/L | 31 | 521,40 | +165,90 |

from the pulp and paper industrial waste, only the sample PP2 induced a significant increase in the rate of $m^5dC$. None of these samples induce significant changes in MDA rates of the exposed organisms. Among the wastes from the pulp and paper industry, the sample PP2 caused the greatest amount of micronuclei observed in all ten samples tested, which was 14 micronuclei in 2000 cells counted. Samples PP1 and PP3 induced little micronucleus formation, only 3 in 2000 cells scored.

**Classification and risk assessment through the Model WTox**

According to the risk assessment methodology proposed by WTox Model, and with the aid of the software WTox, the environmental risk of the sample was obtained, using each of the five toxicological parameters employed. The values found for each toxicity test were applied in the software and severity was defined for each parameter. Through software, verification steps on toxicity level (Table 1) and risk classification by using of the risk matrix (Figure 1) occurs in an automated way, i.e., it is not necessary to consult the table and neither the matrix to classify the risk. Following these same steps, the environmental risk for each of the analyzed parameters was obtained in all samples from industrial waste. Tables 5, 6 and 7 show the results of the toxicity tests for each sample, the toxicity level found, the severity of the effect defined for each parameter and the risk classification found for each individual test. The soluble fractions of textile samples (TX1, TX2 and TX3 TX4) had, in general, Significant or Critical Risk to the environment (Table 5).

For samples MM1, MM2 and MM3 (soluble fractions of metal-mechanic industrial waste) the toxicological risk defined by WTox varies between Marginal, Significant and Critical (Table 6).

Tabla 5: Frequency of micronucleus, lipid peroxidation and DNA methylation rates in erythrocytes of O. niloticus exposed for 48h to soluble fractions of textile, metal-mechanic and pulp and paper wastes.

| | | Sample | Acute Test V. fischeri | Acute Test D. magna | Chronic D. magna | Micronucleus O. niloticus | Lipid peroxidation Test O. niloticus | DNA methydation Test O. niloticus |
|---|---|---|---|---|---|---|---|---|
| Textile Industry | Toxicity value | TX1 | 100.00 | 70.71 | 19.82 | 12 | 389.32 | 312.24 |
| | Level | | E | E | D | C | A | A |
| | Severity | | IV | IV | III | II | II | II |
| | Risk | | Marginal | Significative | Critical | Critical | Critical | Critical |
| | Toxicity value | TX2 | 43.77 | 100.00 | 37.4 | 9 | 418.59 | 272.97 |
| | Level | | D | E | C | C | A | A |
| | Severity | | I | IV | II | II | II | II |
| | Risk | | Significative | Marginal | Critical | Critical | Critical | Critical |
| | Toxicity value | TX3 | 12.08 | 11.26 | 32.5 | 7 | 77.49 | 321.74 |
| | Level | | C | C | C | C | C | A |
| | Severity | | I | I | II | II | II | II |
| | Risk | | Critical | Critical | Critical | Critical | Critical | Critical |
| | Toxicity value | TX4 | 17.99 | 48.29 | −18.68 | 7 | 48.5 | 722.93 |
| | Level | | C | D | D | C | D | A |
| | Severity | | I | I | II | II | II | II |
| | Risk | | Critical | Significative | Significative | Critical | Significative | Critical |

Tabla 6: Results of toxicity tests for samples of Metal Mechanic Industry (MM1, MM2 and MM3), level of toxicity found, severity of the effect defined for each parameter, and individual risk classification for each type of test.

| | | Sample | Acute Test V. fischeri | Acute Test D. magna | Chronic D. magna | Micronucleus O. niloticus | Lipid peroxidation Test O. niloticus | DNA methydation Test O. niloticus |
|---|---|---|---|---|---|---|---|---|
| Metal-mechanic Industry | Toxicity value | MM1 | 73.86 | 84.86 | 5.85 | 4 | 393.53 | −73.21 |
| | Level | | E | E | E | D | A | B |
| | Severity | | IV | IV | II | II | II | II |
| | Risk | | Marginal | Marginal | Marginal | Significative | Critical | Critical |
| | Toxicity value | MM2 | 48.73 | 70.71 | 31.10 | 3 | 602.48 | 59.63 |
| | Level | | D | E | C | D | A | C |
| | Severity | | I | IV | II | II | II | II |
| | Risk | | Significative | Marginal | Critical | Significative | Critical | Critical |
| | Toxicity value | MM3 | 17.47 | 2.21 | 25.75 | 3 | 363.36 | 183.24 |
| | Level | | C | A | D | D | A | A |
| | Severity | | I | I | II | II | II | II |
| | Risk | | Critical | Critical | Significative | Significative | Critical | Critical |

The soluble fractions of pulp and paper industrial waste (PP1, PP2, and PP3) had, in general, Marginal or Critical Risk (Table 7).

Risk assessments of the samples analyzed indicate that, for at least one of the toxicological parameters, all samples presented a Critical Risk. Among the 60 evaluations carried out, 37 resulted in Critical Risk. The Critical Risk includes consequences such as cell or the body death, which can influence the balance of the food chain. Also, the critical risk is associated with effects that do not cause cell or body death, but can influence the reproductive system, or also change the processes at genetic level.

According to the methodology proposed by the WTox Model the analysis of environmental risk must be performed with three toxicity tests. The use of a set of three tests should include a test to verify the acute effect (D. magna and V. fischeri), a test to verify chronic effect, and a test to verify specific effects to the organism (frequency of micronuclei, lipid peroxidation or DNA methylation).

Utilizing a battery of three toxicity tests there are six possible tests combinations tests to obtain an assessment of the toxicological risk. The possible test combinations are: (A) acute test with

Tabla 7: Results of toxicity tests for samples of Pulp and Paper Industry (PP1, PP2 and PP3), level of toxicity found, severity of the effect defined for each parameter, and individual risk classification for each type of test.

| | | Sample | Acute Test V. fischeri | Acute Test D. magna | Chronic D. magna | Micronucleus O. niloticus | Lipid peroxidation Test O. niloticus | DNA methydation Test O. niloticus |
|---|---|---|---|---|---|---|---|---|
| Pulp and Paper Industry | Toxicity value | PP1 | 91.93 | 100.00 | 70.00 | 3 | 106.17 | 288.49 |
| | Level | | E | E | B | D | B | A |
| | Severity | | IV | IV | II | II | II | II |
| | Risk | | Marginal | Marginal | Critical | Significative | Critical | Critical |
| | Toxicity value | PP2 | 19.00 | 100.00 | 35.61 | 14 | 66.10 | 471.52 |
| | Level | | C | E | C | B | C | A |
| | Severity | | I | IV | II | II | II | II |
| | Risk | | Critical | Marginal | Critical | Critical | Critical | Critical |
| | Toxicity value | PP3 | 18.61 | 51.76 | −40.33 | 3 | 5.58 | 272.63 |
| | Level | | C | D | C | D | E | A |
| | Severity | | I | I | II | II | II | II |
| | Risk | | Critical | Significative | Critical | Significative | Marginal | Critical |

D. magna, chronic test with D. magna, and micronucleus test with O. niloticus; (B) acute test with D. magna, chronic test with D. magna, and lipid peroxidation test with O. niloticus; (C) acute test with D. magna, chronic test with D. magna, and methylation test with O. niloticus; (D) acute test with V. fischeri, chronic test with D. magna, and micronucleus test with O. niloticus; (E) acute test with V. fischeri, chronic test with D. magna and lipid peroxidation test with O. niloticus; and (F) acute test with V. fischeri, chronic test with D. magna, and methylation test with O. niloticus.

By performing all combinations of tests including all the results found in toxicity tests, it was observed that the samples from textile industrial waste exhibit in general, significant or critical risk. In the case of sample TX1, all possible combinations of three tests classified the sample as having significant risk. For samples TX2 and TX3 all combinations of tests result in Critical risk. For the sample TX4 only the test sequence B acute (acute test with D. magna - chronic test - Lipid peroxidation test), results in a Significant risk. The other combinations of tests classify the waste as Critical Risk.

Samples of the metal-mechanic industrial waste presented, in general, Significant or Critical Risk. For Sample MM1, the two combinations that resulted in Reduced risk were those which included the micronucleus test (sequences A and D); the other four combinations resulted in significant risk. For Sample MM2, only the combination A resulted in significant risk, all other combinations of three tests resulted in Critical Risk. For sample MM3, all combinations resulted in Critical risk. For sample PP1, from pulp and paper industry, the combinations A and D resulted in Significant Risk, while the other four combinations resulted in Critical Risk. For sample PP2, all combinations of tests resulted in critical risk. For sample PP3, combinations of the two tests which included the lipid peroxidation test (B and E) resulted in significant risk. The other test combinations resulted in critical risk. Table 8 summarizes the results of the toxicological risk classifications found for the six combinations of tests.

## 4 Discussion

In this study, it is evident that a battery of toxicological tests is more appropriate to assess the environmental risk of industrial waste (complex mixtures). Fenske et al. (2006) stated that a combination of several tests using organisms of different levels of complexity, provide reliable

Tabla 8: Toxicological risk classifications found with six test combinations (A, B, C, D, E and F) for each sample of textile (TX1, TX2, TX3 and TX4), metal-mechanic (MM1, MM2 and MM3) and pulp and paper wastes (PP1, PP2 and PP3).

| Samples | Test combinations | | | | | |
|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** |
| TX1 | Significative | Significative | Significative | Significative | Significative | Significative |
| TX2 | Critical | Critical | Critical | Critical | Critical | Critical |
| TX3 | Critical | Critical | Critical | Critical | Critical | Critical |
| TX4 | Critical | Significative | Critical | Critical | Critical | Critical |
| MM1 | Reduced | Significative | Significative | Reduced | Significative | Significative |
| MM2 | Significative | Critical | Critical | Critical | Critical | Critical |
| MM3 | Critical | Critical | Critical | Critical | Critical | Critical |
| PP1 | Significative | Critical | Critical | Significative | Critical | Critical |
| PP2 | Critical | Critical | Critical | Critical | Critical | Critical |
| PP3 | Critical | Significative | Critical | Critical | Significative | Critical |



results for the evaluation of toxicity in several parts of the ecosystem. Likewise, Pandard et al. (2006) state that a battery of tests with organisms of different trophic levels is more appropriate for estimating the ecotoxicity of industrial and municipal waste. Zurita et al. (2007) performed a battery of toxicological tests in vivo (D. magna, V. fischeri and C. vulgaris) and in vitro (two fish cell types), observing effects through twelve parameters. The researchers concluded that a single bioassay will never provide adequate information for an ecotoxicological evaluation.

Employing the methodology of the WTox Model, it is confirmed that a set of three tests (one to verify the acute effect, one to verify chronic effect, and one to verify specific effects to the organism) provides better results of risk classification when compared with risk assessments that uses only one toxicological parameter.

Other researchers have proposed methodologies for risk assessments or environmental hazard of complex mixtures that resemble WTox Model for using the information resulting of batteries of toxicological tests, and integrate these results, classifying the risk or hazard levels (Tigini et al., 2011; Piva et al.; 2011, and Constan et al. 1993). Tigini et al. (2011) have proposed a methodology for assessing toxicity, genotoxicity and environmental risk from the textile industry waste. The methodology suggests the use of acute toxicity tests with organisms of different trophic levels like bacteria, aquatic and terrestrial plants and microcrustaceans. By the evaluation method proposed by the researchers, the samples showed toxicity values between 10 and 40%, representing an environmental risk ranging between high and very high. In this work, the integration of the toxicological endpoints resulted in Significant or Critical Risk for samples of textile industry.

When we use the results of this study on the methodology proposed by Tigini et al (2011), there is an indication that the environmental risk is similar. Although the methodology of Tigini et al. (2011) is able to integrate results from different toxicity tests classifying the risk based on these values, only the acute toxicity is observed. According to Robbens et al. (2007), the ecotoxicological risk assessment has long been based only on the acute and the effective or lethal concentration ($EC/LC_{50}$) in organisms of different trophic levels, and these tests are insufficient to properly assess the risk associated with several chemical products.

Robbens et al. (2007) also claim that the introduction of advanced molecular techniques would lead to a more adequate risk assessment. Based on this statement, it is considered that the

WTox model is the most appropriate for toxicological risk assessments, compared with the methodology proposed by Tigini et al. (2011), since the use of the micronucleus frequency test, and the lipid peroxidation and DNA methylation tests (specific toxicity) complements the results found with the assessment of acute effects.

Another method which can be compared with the WTox model is the one developed by Piva et al. (2011). These researchers proposed a method that provides a hazard classification of sediments, by evaluating various toxicological endpoints and, in the end, makes the integration of all of them. In this methodology, the hazard assessment takes place by four lines of evidence: 1) chemical characteristics of the sediment, 2) bioavailability in fish, 3) biomarkers and 4) laboratory bioassays. Integration is performed according to the results observed in each line of evidence. The classification is separated into five levels of hazard: absent, mild, moderate, high, and severe. This methodology considers several important toxicological endpoints, but unlike the WTox Model, the chemical characterization of sediment is required. Through a case study, the researchers evaluated the environmental hazard of sediment from the petrochemical industry, exposing *Anguilla anguilla* eels in the sediment of a river that receives effluent from this type of industry. The authors state that the sediment contains large amounts of metals such as iron, lead and zinc, which makes this material similar to the metal-mechanic industrial waste evaluated in this study (MM1, MM2 and MM3, details in Flohr et al., 2012a ).

In the case study conducted by Piva et al. (2011), the integration of all lines of evidence resulted in a Severe hazard classification for sediments of the petrochemical industry. Using the data found in the samples of metal-mechanic industrial waste of the present study in the methodology of Piva et al. (2011), we obtain a hazard classification between Light and High to samples MM1 and MM2, and between High and Severe for sample MM3.

A methodology that can also be compared with WTox Model is the one designed by Constan (1993), called PEEP Index (Potential Ecotoxic Effects Probe). This index is used to evaluate and compare the ecotoxic potential of effluents and industrial waste. The methodology uses a scale of 0 to 10 to rate the hazard of an industrial pollutant and a battery of toxicological and genotoxicity tests. Still, requires the use of the variable flow rate ($m^3/h$), and considers the biodegradability of the sample.

The PEEP method basically uses the non observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of the sample in each test and transforms this data into a toxic unit, which is further included in an equation comprising the sum of the toxic units and the emission flow of the sample. The PEEP index is represented by the letter P in a formula that combines all the data found in toxicity tests and the flow. The need to know the emission flow and biodegradability of the sample is an obstacle, since other data are required in addition to the toxicity variable.

The results observed for samples from the pulp and paper industrial waste (PP1, PP2 and PP3) were compared to Blaise and Ferard (2005). These researchers use the PEEP index to assess and compare the ecotoxic potential of several industrial effluents, including those from the pulp and paper industry. In the study, these researchers found values between 4 and 7.5 for effluents from the pulp and paper industry. The toxicity classification proposed by the PEEP method varies from practically non-toxic to extremely toxic. Values greater than 5 are already considered extremely toxic. An essential difference between the PEEP method and WTox Model is the use of the flow rate ($m^3/h$) variable, which is not necessary for the WTox Model.

To compare the two methods, PEPP and WTox, we carried out a simulation of the data that

was not observed in this study, and we adapted the data found in the tests. A flow of 3000 $m^3/h$ was established, because this was the flow employed by Blaise and Ferard (2005). The PEEP value found for the sample PP1 was P = 4.83; for sample PP2, P = 6.45, and for the sample PP3, P = 6.65. By the PEEP methodology, the sample PP1 can be classified as highly toxic, and samples PP2 and PP3 as extremely toxic. Comparing the two methods is possible to verify the closeness of PEEP values regarding toxicity classification, as well as the similarly about the alert to the danger and environmental risk from pulp and paper industrial waste.

Comparing the WTox Model with other models (Table 9), it was observed that the WTox Model can perform an efficiently environmental risk assessment by using a battery of three toxicological tests (involving global and specific toxicology) without the necessity to use other data (physico-chemical variables, flow rate, etc.), achieving similar results toward other models, in the same environmental risk situation.

Tabla 9: Comparison between information included in Models of Environmental Risk Assessment.

| Models | Information included in Models of Environmental Risk Assessment | | | | | |
|---|---|---|---|---|---|---|
| | Global Toxicology | Specific Toxicology | Phisico-chemical Data | Flow Rate Data | Geological Data of the Environmental Compartment | Complex Mixtures Assessment |
| WTox | *** | *** | – | – | – | + |
| Tigini et al., 2011 | *** | * | – | – | – | + |
| Piva et al., 2011 | *** | *** | + | – | – | + |
| PEEP (Constan, 1993) | *** | * | – | + | – | + |
| AQUATOX (Park et al., 2008) | *** | – | + | + | + | – |
| CREAM (Grimm et al., 2009) | *** | ? | + | ? | + | – |
| DREAM (Reed and Hetland, 2002) | *** | *** | + | + | + | + |

(*) One type of toxicity test applied (**) Two types of toxicity test applied (***) More than two types of toxicity test applied
(–) Not included (+) Includes (?) Not defined

In the research to find models of environmental risk assessment that also include toxicological data, it was noted that most of them consider the integration of global and specific effects. However, most models consider the inclusion of physicochemical data, discharge flow rate, and/or geological data of the environmental compartment. Moreover, models like AQUATOX and CREAM can only be used to evaluate the risk of previously known substances. The application of a methodology that does not require the inclusion of these types of data is more representative because it focuses on the interaction between toxic substance and organism, and allows the analysis of synergistic, additive or antagonistic effects of complex mixtures.

Due to the enormous complexity of ecosystems is not possible to obtain details on all processes related to the behavior of pollutants. These processes should be described in a consistent level of complexity to provide appropriate responses to the issues highlighted, however, when it comes to risk assessment methodologies, the goal is to develop a simple model, which involves all the variables of a given problem (Schwarzenbach et al., 2006). Suter (1993) affirms that the use of one-dimensional models with only one variable, for example, concentration, simplifies the risk assessment, since the answer is determined by the relative exposure concentration and the effective concentration.

The development of tools that allow accurate qualitative and quantitatively the evaluation of chemical, physical and biological risks has become a priority since risk assessment is increasingly seen as a way to integrate science, policy and management, to respond the huge amount of environmental problems (Hacon, 2003; CENR, 1999).

In Brazil, the problem in predicting risk of damage is the fact that the system of classification of industrial waste follows the NBR 10004 (ABNT, 2004a), and the methodology of this system

classifies waste based primarily on their physical-chemical composition. Studies show that not always the physical-chemical factors demonstrate the toxic potential of chemicals, as these factors do not translate correctly the relationship between living organisms and contaminants (Flohr, 2012b; Silva et al., 2011).

In this sense, the toxicological analysis of complex waste, which can cause synergistic and additive effects, presents itself as an important ally in risk assessment and decision making in the process of environmental management. The computational toxicology can translate more efficiently the hazards determination of various environmental stressors that should be monitored, and, from this, one can decide which types of information are the most necessary to reduce uncertainty in the protection of the environment and human health (Kavlock et al., 2008).



## 5 Conclusions

The results observed in this study confirm that all samples of waste presented for any of the toxicological endpoints, an important toxic potential, indicating that a risk assessment that considers global and specific toxicological effects is more appropriate and representative. Beyond of evidencing biological phenomena (which includes the additive, synergistic, and antagonistic effects of complex mixtures), the application of the WTox Model showed that the response to exposure can be readily demonstrated without the need of using techniques to find out the composition of the sample, since this method showed similar results to models that utilizes physicochemical variables. Thus, the WTox Model is indicated for the protection of aquatic organisms and the environment as a whole, because it can be applied in any scenario and in any situation, reducing costs and time.

The integration of toxicological data is essential to define the risk associated with environmental pollutants. A computer program can act as a tool to provide and to facilitate this integration. The results of this study confirm that WTox model is able to perform an adequate environmental risk assessment, based only on toxicological data. Thus, the WTox Model can be used as an auxiliary tool in decision-making processes that involve the preservation of the environment and in situations of environmental toxicological risk.

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