Monitoring of Paralytic Shellfish Toxins Using Biological Assays

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Abstract

The enrichment of nutrients in aquatic ecosystems is an important factor that leads to eutrophication and to accelerated growth of cyanobacteria. Some species produce toxins as secondary metabolites, which can impact ecosystems, animal, and human health. In this chapter, we presented some tools to assess paralytic shellfish toxins (PSTs) and their bioaccumulate effects in fish to bioassay and in field studies. An in vitro method to evaluate the effects in fish primary neuron culture is also described. Besides the analysis of the presence of the PSTs, biological assays in vivo and in vitro studies are important tools to assess the mechanism of actions in cellular and tissue target.

Keywords

Saxitoxins · Bioassays · In vitro studies · Biomarkers · Eutrophication · Cyanobacteria bloom

26.1 Introduction

Eutrophication can occur naturally but has been exacerbated by anthropogenic actions and can result in cyanobacterial blooms (Yan et al. 2017). This process compromises water quality (O'Neil et al. 2012) and increases the cost of treatment to

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make it potable (Chorus and Bartram 1999). This phenomenon is caused by the excessive enrichment of organic matter and nutrients, especially phosphorus and nitrogen that are limiting factors for the growth of algae and aquatic plants. The proliferation of algae and plants contributes to the reduction in dissolved oxygen, death of aquatic biota, and decrease in species richness (Misra and Chaturvedi 2016). Among comprising the phytoplankton algae, cyanobacteria are causing increased environmental and economic problems mainly due to the production of toxins. These toxins, called cyanotoxins, are classified according to their toxicity and can be harmful to aquatic organisms and human beings.

Fish can bioaccumulate these toxins when present in the aquatic environment, causing loss of balance and even death of the animal. Calado et al. (2017) reported poisonings in humans cause burning sensations, salivation, vomiting, diarrhea, or even lethal reactions dependent on the amount ingested (Carmichael 2012). These toxins can cross the blood–brain barrier and cause effects to the brain and alteration in behavior and neural development (O'Neill et al. 2016).

Biological analyses should be added to water monitoring, allowing an assessment that integrates water quality and its toxic effects on human population and aquatic communities (Altenburger et al. 2019). The implementation of more effective strategies in program evaluation of water quality in reservoirs is important, as some methodologies in the water treatment system have been ineffective in removing some contaminants such as drugs and heavy metals and may even increase genotoxicity of these compounds (Palma et al. 2010).

The use of this type of strategy allows an overall assessment of the effects of substances in the water, in addition to their synergistic and/or antagonistic effects. Therefore, monitoring of water bodies from biological analysis can determine the real risks to human health and the environment (Calado et al. 2018).

26.1.1 Saxitoxin (PSTs)

The saxitoxins (STXs) paralytic shellfish toxins called—PSTs—are neurotoxins that block sodium channels, preventing the transmission of nerve impulse (Carmichael 2012). Moreover, the blockade of sodium channels can damage the permeability of the membrane, altering cellular homeostasis (Stevens et al. 2011). There are over 20 analogs described STX divided into four groups of magnitude and toxicity (Oshima et al. 1993). This grouping will depend on the group linked to its chain, being decarbamoyl, hydroxyl, and sulfate (Oshima et al. 1993; Chorus and Bartram 1999). The first group is considered the most toxic and understands the saxitoxins (decarbomoilsaxitixona, saxitoxin, dercabamoilneosaxitoxina, and neosaxitoxin). The second group consists of sulfated toxins; these are considered less powerful than saxitoxins (gonyautoxins 1-6). Therefore, they are the most abundant toxins in shellfish. The epimers and GTX2 GTX3 are highly absorbed by the gut epithelium; in humans, this absorption occurs by simple diffusion, facilitating the contamination when these toxins are present in the environment. The third group consists of toxins doubly sulfated; they are less powerful than the previous two groups (C-toxins 1-4).

The last group consists of varieties of American Lyngbya strains wollei 1-6. The LD50 in mice via intraperitoneal to saxitoxin is 10 μg kg^{-1} and orally is 260 μg kg^{-1} (Chorus and Bartram 1999). A study carried out in rats at the concentration of 3 μg L^{-1} (equiv.STX) showed a significant effect on chronic antioxidant defenses and oxidative stress induction (Ramos et al. 2014).

The *Raphidiopsis raciborskii* (also called *Cylindrospermopsis raciborskii*) is a planktonic cyanobacteria in inland waters that can produce of some of these variants (Calado et al. 2019). This is a filamentous cyanobacteria alga, highly invasive, and, in addition, cosmopolitan, occurring in tropical, subtropical, and temperate regions; this causes water contamination by the presence of this species which is a major problem for global health (Zanchett and Oliveira-Filho 2013).

Raphidiopsis raciborskii can produce various toxic secondary metabolites, including the hepatotoxic alkaloid represented by cylindrospermopsin (CYL), the neurotoxic represented by saxitoxins (STX, neoSTX, gonyautoxins, and C-toxins) (Lagos et al. 1999) and toxoids (ANA-a) (Chorus and Bartram 1999) and unidentified analogs.

The most severe case of poisoning in humans by these cyanotoxins was in 1979 "Palm Island Mystery Disease" affecting 138 children and 10 adults. The intoxicated people showed hepatoenterite, renal dysfunction, and diarrhea (Byth 1980). Another case of public health involving *Raphidiopsis raciborskii* occurred in 1995 at a reservoir in Queensland and caused the interruption of the public water supply of the city of Brisbane (Hawkins et al. 1997). In Brazil in 1991, in Lake Paranoá, Brasilia, flowering non-toxic strains have also caused great damage in water use for recreation and public supply (Branco and Senna 1991).

26.1.1.1 Reservoir

The reservoir has been widely used by human for power generation, public water supply, recreation, and landscaping. Therefore, these water bodies receive the input of various toxic substances due to agricultural practices, sewage discharge, and leaching. Alagados Reservoir located in the state of Paraná, Brazil, has been detected cyanobacterial blooms, such as *Raphidiopsis raciborskii* and *Dolichospermum* spp. (Table 26.1).

Table 26.1 Physical and phytoplankton analysis of water samples from Alagados Reservoir, Brazil, in 2015–2016

Sampling	Temperature (°C)	pH	DO (mg/L)	*C. raciborskii* (cell/mL)	Total phytoplankton (cell/mL)
Aug S1	17.9	6.8	4	33,570[a]	458,417
Aug S2	18.3	6.7	3	1020	34,389
Aug S3	18.3	6.6	3	31,540[a]	153,622
Feb S1	25.0	7.4	5	111,095[a]	518,443
Feb S2	25.4	6.7	5	0	29,401
Feb S3	22.1	6.5	6	172,940[a]	439,385

Adapted by Calado et al. (2019)
[a]Above to the limit recommended by Brazilian law (20.000 cells/mL)

Table 26.2 Total concentrations of PSTs and saxitoxin equivalent (Eq. STX) in the Alagados Reservoir, Brazil, during winter 2013 until Fall 2014

	Total PSTs (μg/L)	Eq. STX (μg/L)
Winter	<LQ	—
Spring	<LQ	—
Summer	24.21	13.18
Fall	6.66	3.62

Adapted by Calado et al. (2017)
<LQ under the quantification limit of the method

These cyanobacteria are potentially producing neurotoxins, and a study reported concentrations of these toxins in the water and in fish muscle (Table 26.2). The reservoir is heavily used for recreation, and such human activities have caused the degradation of this water body.

As a result of eutrophication since 2002 are reported intense blooms of potentially toxic cyanobacteria in this reservoir, especially persistent blooms of *Cylindrospermopsis raciborskii*. The results of studies (Clemente et al. 2010; Wojciechowski et al. 2017; Calado et al. 2017) show the need for effective monitoring of water quality.

In light of the problems caused by cyanobacteria, it is necessary to detect these organisms in the environment and the monitoring of its abundance. To detect the presence of cyanobacteria is the technique used to measure the number of photosynthetic pigments and make the recognition and counting cells under a microscope. However, none of these techniques allows whether the cyanobacteria are producing toxins; thus, chemical water analysis should be performed (Merel et al. 2013).

The cyanobacteria density established by the Brazil Ministry of Health (No. 2914/2011) is 20,000 cells mL^{-1} for primary contact waters and established as acceptable limit in water sampling point for human consumption. When the blooms are above, this value is needed for the weekly monitoring of cyanobacteria and cyanotoxins.

26.1.2 Bioaccumulation, Biomagnification, and Biotransformation of the Cyanotoxins

The toxic compounds such as cyanotoxins in aquatic ecosystems can remain for a long time in the environment. Therefore, these compounds are called persistent and may have a high potential for toxicity to aquatic biota. According to the persistence of these substances contributes to their adsorption in the sediment, bioaccumulation in aquatic biota, and biomagnification in the food chain.

The bioaccumulation is the process in which a substance is absorbed and accumulated in the tissues of organisms, and this process may occur through water or food intake. However, the bioaccumulation occurs when the assimilation rate exceeds the rate of elimination of the compounds in the body. Therefore, this process can result in biomagnification, which is the increasing concentration along the food chain; i.e., the chain top organisms can accumulate very high concentrations (Van

Der Oost et al. 2003). The biotransformation of these compounds occurs primarily in the liver with chemical reactions generally mediated by enzymes in order to yield water-soluble metabolites that are more readily eliminated. The biotransformation can decrease the toxicity of compounds and may also convert for more toxic substances. Calado et al. (2018) found PSTs in water and fish samples, and they were estimated as a potential risk to humans, mainly for children. In addition, toxins were accumulated, biotransformed to other analogs, and excreted by the fish. After 90 days, the toxins were still present in the water and fish muscle. Therefore, PSTs can remain for a long period in water, and fish can be a carrier of these neurotoxins.

26.1.3 Bioindicator and Biomonitor

The organisms that respond to environmental changes are called bioindicator or biomonitor. They provide information on ecosystem health and assist in environmental impact assessments. Being constantly exposed, the physical and chemical conditions of the environment the biota of aquatic ecosystems can be considered a good bioindicator of environmental quality. Fish are widely used and considered good biological indicators of water quality because they are in direct contact with the aquatic environment, present wide distribution, body size suitable for the analysis, and be distributed in different trophic levels (Yamamoto et al. 2017). They are constantly exposed to environmental conditions and can carry contaminants over the food web (Aguilar-Betancourt et al. 2016; Cerveny et al. 2016; Guiloski et al. 2017; Silva et al. 2018).

26.1.4 Biomarkers

The biomarkers can describe the effects of environmental stress on different biological levels. The biomarkers are often used to identify changes caused by sublethal contaminants. The use of them is considered advantageous for allowing the presence of contaminants to be detected before they can cause adverse effects on organisms, thus enabling preventive action to conserve ecosystems.

There are several types of biomarkers, such as biochemical, genetics, morphological, and hematological, which have been used as important tools to assess effects of exposure of organisms to pollutants (Guiloski et al. 2013; Silva de Assis et al. 2013).

26.1.4.1 Biochemical Biomarkers

Acetylcholinesterase is a biochemical biomarker which can be used to evaluate neurotoxicity, because this enzyme acts on nerve synapses hydrolyzing acetylcholine (ACh) and aiding in the transmission of nerve impulses. Many studies using fish have shown inhibition of this enzyme when exposed to organophosphate pesticides, even in small concentrations (Guiloski et al. 2013). Another contaminant that can also inhibit this enzyme is the cyanotoxin anatoxin (Rodríguez et al. 2012).

The biotransformation mechanism is mediated by enzymes and the cytochrome P450 enzymes responsible for the major components phase I. The main subfamily responsible for the biotransformation of xenobiotics is CYP1A. The catalytic activity of this subfamily can be measured by ethoxyresorufin-*O*-deethylase (EROD). Many studies have shown the induction of CYP1A activity by exposure to organic pollutants as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans (PCDF) (Van Der Oost et al. 2003). The proteins of the cytochrome P450 family are known to be involved in the biotransformation of various xenobiotics in aquatic invertebrates, such as bivalve species (Ruiwen et al. 2018).

The GST enzyme is essential in the phase II biotransformation mechanism performing the conjugation with the involvement of GSH in hydrophilic lipophilic compounds, facilitating elimination by the cell (Kurutas 2016).

The metabolism in normal conditions or when the body is exposed to some stress produces the so-called ROS or reactive oxygen species. These include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical (HO). Biomarkers involved in metabolism control the production and degradation of ROS in order to avoid oxidative stress causing damage by molecules such as DNA. Some of these biomarkers are the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and non-enzymatic compound reduced glutathione (GSH).

SOD is a metalloenzyme engaged in degradation of O_2, forming H_2O_2, and oxygen (O_2). This result requires CAT and GPx enzymes involved in degradation of H_2O_2 into H_2O and O_2. The CAT is present in peroxisomes of most cells (Kurutas 2016).

GPx degrades other types of peroxides besides H_2O_2. This is a selenium-dependent enzyme and the main peroxidase in fish. It is important in the degradation of lipid peroxides avoiding the reactions involved in lipoperoxidation. The cofactor for their activity is the GSH (Van Der Oost et al. 2003).

GSH is a non-enzymatic compound cofactor of the GPx activity and also takes part in reactions in phase II metabolism through the activity of GST. Changes in GSH levels are related to detoxification mechanisms of xenobiotics by organisms (Van Der Oost et al. 2003).

The biotransformation mechanism is divided into two phases. Phase I is one where oxidation, reduction, and hydrolysis reactions will occur. The products formed by these reactions may be more reactive than previous compounds. In phase II, conjugation reactions occur which aim the elimination of these compounds (Mels et al. 2011).

The lipid peroxidation (LPO) is a major damage caused by oxidative stress. This damage occurs in the phospholipids of cell membranes which are very susceptible regions to oxidation reactions. When ROS react with the lipid membrane, it affects the function of cell membranes causing the increased permeability reaching total breakage and cell death (Kurutas 2016).

The protein carbonylation (PCO) is also molecules in damage caused by oxidative stress. The damage consists in protein modification to ketone and aldehyde

groups and the loss of some function enzymes such as pyruvate dehydrogenase (Suzuki et al. 2010).

26.1.4.2 Genetics Biomarkers

The toxic components can also cause genetic damage as breaks in the DNA molecule resulting in biological effects in organisms in the population and in the community. Genetic biomarkers are those that detect DNA damage and some of the methods used to identify such damage is the micronucleus test and analysis of morphological changes as well comet assay (Carrasco et al. 1990; Speit and Hartmann 2005).

The concern of contamination of water bodies by cyanotoxins is growing requiring the use of tools to identify the real damage they cause in the ecosystem. The use of different biomarkers is important to understand the effects of these toxins and they area a useful tool in environmental monitoring programs.

26.2 Biomonitoring: Since Field Assessment to Bioassay

Field study is very important to evaluate the environmental quality such as the water and its aquatic ecosystems. Several authors have been showed that PSPs are frequently found in mussel, oysters, and other filter-aquatic organisms and marine fishes (Ben-Gigirey et al. 2012; Xie et al. 2013). Furthermore, it was reported that freshwater fish present in public supply reservoirs with microalgae blooms may contain PSPs and that they can endanger human health when consumed (Clemente et al. 2010; Calado et al. 2020).

However, the field research little quantity elucidates the mechanisms of action of these xenobiotics and their toxic effects on exposed organisms and thus making a simple indicator of presence, or absence, inside, or outside acceptable limits. In this way, how to measure the problem to animal health? How to assess the real effects on the biota directly exposed to PSPs or through the trophic chain?

The answer to these questions can be the bioassay for PSP animal exposure. In field study, the organisms suffer environmental influences and the action and toxic effects to many xenobiotics over PSPs. The results found in the field study not revealed only PSP effects, but the synergism of several substances presents in the aquatic ecosystem. The bioassay is performed in controlled conditions at laboratory and thus the results can be considered trustworthy of PSPs effects. It is possible to measure the capacity of bioaccumulation, the toxic effects on gill, kidneys, liver, brain, blood, and others.

Fish's bioassay can be regarded as an indispensable tool to evaluate the health of ecological and public health. For example, a bioassay realized with fishes on saxitoxins extracts from *Raphidiopsis raciborskii* strain revealed that the liver that until then was not considered target this xenobiotic has damaged several enzymatic level and tissue, suggesting that PSPs are able to provoke hepatological changes (Silva de Assis et al. 2013).

Similar results were found in fish bioassay by Silva et al. (2011) in brain, suggesting that PSPs are not only able to block the sodium channel but damaged the tissue and leading the neuron cells to death and provoke genotoxic effects.

Bioassays can be performed of different methods such as direct exposure to PSPs extract or purified substances into water or through flow trophic chain.

The PSP concentration and the time exposure depend on the aims of the study. The animal's exposure to PSPs dissolved in water has the disadvantage that the substances present can undergo oxidation and change their toxicities, leading to variation in results. To mitigate these variable effects due to molecular instability of substances it is suggested to perform a dynamic bioassay with constant water changes and measuring the periodic PSPs concentration. It is also possible to evaluate the effects of water from site contained PSPs since be realized chemical water analysis. The water test should be carried out in aquarium and performed the exposure with fish or other aquatic organisms that be sensitive to PSPs. The choice of bioindicator or biological model depends on the experimental design.

To assess the PSPs bioaccumulation recommends chronic or subchronic PSPs exposure considering the lifelong time of the model organism. Low concentration of PSPs test can make the bioaccumulation not detectable or a false absence result.

The flow trophic chain has the vantage of ensuring that the organism is exposed to the PSPs and the flux of this substance is metabolized and its effects should be measured during the bioassay. This type of bioassay can be performed using feed animal with PSPs included when the animal is not predominantly carnivorous or directly applied in small specie and offered as food to the top of the chain specie.

Flow trophic experimental design recommends this method:

1. Choose a top of the chain specie.
2. Choose small specie that serves food to the top–base trophic chain.
3. Define the condition of the experiment: time of exposure, PSP concentration, doses, temperature water, and others.
4. Define the experimental groups according to concentration or dilution of PSPs extract or water containing PSPs.
5. Introduce intraperitoneal PSPs in small specie.
6. Food to the top with the small specie contained PSPs.
7. Monitor the experiment during the time previous defined.
8. Sacrifice the animals exposed.
9. Analyze the kidneys, liver, brain, blood, and others using chemical and biochemical analysis such as biomarkers.
10. Analyze the presence of PSPs and their analogs in the muscle.

It is recommended that the bioassay is performed using top and basic native's biological models and the doses should be according to the lapse's temporal food to the top chain, example: one dose every four days. The total time of the experiment should be defined according to the number of doses.

Uses of biochemical, genotoxic, histopathological, morphological, and hematological biomarkers with chemical analysis are recommended to get a better interpretation of results and clarification.

26.2.1 New Perspective to Monitoring PSPs: In Vitro Bioassay

Although safety limits for PSPs are reported in the literature (Chorus and Bartram 1999), the direct effects of toxins on target cells still require elucidation and particularly to define and search antidotes and treatments.

On the other hand, in vivo studies considered the environmental influences such as the field assessment which can lead to false diagnosis for animal life and ultimately to public health.

In this way, how to ensure that the limits on exposure to PSPs are safe for animals and their ecological relationships?

The safety limits for biota should be not only based on human health because the aquatic toxins are able to danger the ecological equilibrium and result in several damages to ecosystem aquatic with consequences to support of quality of water and organism's life.

Studies in vitro can provide knowledge about the mechanisms of the damages and detoxification of saxitoxin and other PSPs on brain tissue, provided that a purification of the cells is performed in order to avoid conflicting results due to various cell types which may be nonspecific responses to the test substance.

Several types of animal organisms have been used to cellular donor to primary culture (Martins et al. 2011; Southam et al. 2013; Ribas et al. 2014; Schnell et al. 2015; Oliveira-Mello et al. 2021). However, it is important that the in vitro bioassay be carried in a specie that has evolved exposure to environmental toxins containing and, in this case, rats are not good biological models for assessing the harmful effects of PSPs on neuronal cells as never had contact natural with these substances, despite this specie can be used to understand the mechanisms of toxins in mammals, but do not express the real damage caused by PSPs in aquatic organisms.

Fish is reported as a good biological model to elucidate the effects of many aquatic pollutants and the water quality (Mela et al. 2010; Katsumiti et al. 2013; Calado et al. 2018, 2020). In vitro, freshwater specie fish such as *Hoplias malabaricus* has cited in several types of in vitro studies (Bussolaro et al. 2010; Liebel et al. 2011; Ribas et al. 2014; Silva et al. 2014) and can be to monitor PSPs on biological assay using cells cultivated.

The problems found in fish primary culture is the microbial contamination, but this can be mitigated by pre-washing with 70% ethanol throughout the body of the fish having been anesthetized (benzocaine 0.02%) in laminar flow sterile hood.

For the brain, for example, after being sacrificed by medullar section and decapitated the fish head should be again rinsed with chlorhexidine 2%. Then, the brain can be removed of cranium and transferred to Hank's balanced salt solution (HBSS) supplemented with 2 mM glucose and antibiotic penicillin/streptomycin (ATB; 50 U mL^{-1} Pen, 50 μg mL^{-1} Strept) and the meninges have been cleaned

before slices the brain at 0.5 mm using a scalpel to avoid the cell excess impurities, following the mechanical dissociation using a sequence flamed Pasteur pipette.

Many types of proteases can be used for cellular dissociation. To fish brain is recommended Papain 30 U mL^{-1} at 30 °C for 30 min with occasional gently mixing in Neurobasal with B27 supplement (B27; 1:50) medium for digestion. The tissue was triturated with a flame-polished Pasteur pipette for 1 min followed by another digestion for 15 min at 30 °C. This procedure should be repeated twice followed by a new triturating step with a new flame-polished Pasteur of a minor diameter.

A segregation cellular is important to choose the cells type which will be used in the bioassay such as the density gradient prepared with Optiprep and Neurobasal/B27 medium (Brewer and Torricelli 2007) and centrifuged at 800 × *g* for 15 min at 22 °C. To remove the debris and other cells types that such as oligodendrocytes the fractions should be aspirated and discarded. Then, the fraction contained neurons will be transferred to a new tube and added Dulbecco's modified Eagle's medium (DMEM/F12) and cells were centrifuged at 800 × *g* for 5 min at 22 °C. The supernatant, which contained debris, was discarded and pelleted cells should be washed in DMEM/F12 and collected by a new centrifugation at 800 × *g* for 5 min at 22 °C. Then, cells should be re-suspended in culture medium appropriated and total and viable cells counted by Trypan blue exclusion in Neubauer chamber (Hu and Fakahany 1994) and diluted before seeded in Petri dishes.

To increase the adherence and development and cell differentiation, in vitro studies with fish neurons is necessary several types of nutrients into culture medium. Silva et al. (2014), for example, recommend the use of DMEM/F12 medium supplemented with B27 + 5% fetal bovine serum (FBS) + glutamine (0.29 mg mL^{-1}) + ATB + fibroblast growth factor (bFGF) (from bovine pituitary glands; 3 ng mL^{-1}) and seeded for experimental condition (recommended: 24 °C and 1.7% CO_2 at humidified atmosphere) in plate coating substrate for the cellular adhesion with poly-L-lysine (15 μg mL^{-1}) for overnight. Before use, plates should be washed three times with phosphate-buffered saline (PBS). The whole procedure is summarized in the flow diagram shown in Fig. 26.1.

But how to monitor PSPs using in vitro bioassay? See next section how to apply this innovative method to assess these substances in fishes

26.2.1.1 Evaluating PSP Effects by In Vitro Bioassay

In order to evaluate the toxic effects of PSPs substances, the cells after counted by Trypan blue exclusion in Neubauer chamber should be seeded at 2 × 10^5 cells at 96-well dish treated with poly-L-lysine coating substrate and added DMEM/F12 medium supplemented with B27 + FBS + glutamine + ATB + bFGF according to section above should be incubated for 24 h for adhesion.

After incubation of neuronal cells from fish, the medium should be removed and added new medium contain PSPs interest test such as saxitoxins (STX) or its analogs, or cyanobacterium extracts, or water test for 1 day at experimental condition 24 °C and 1.7% CO_2 at humidified atmosphere.

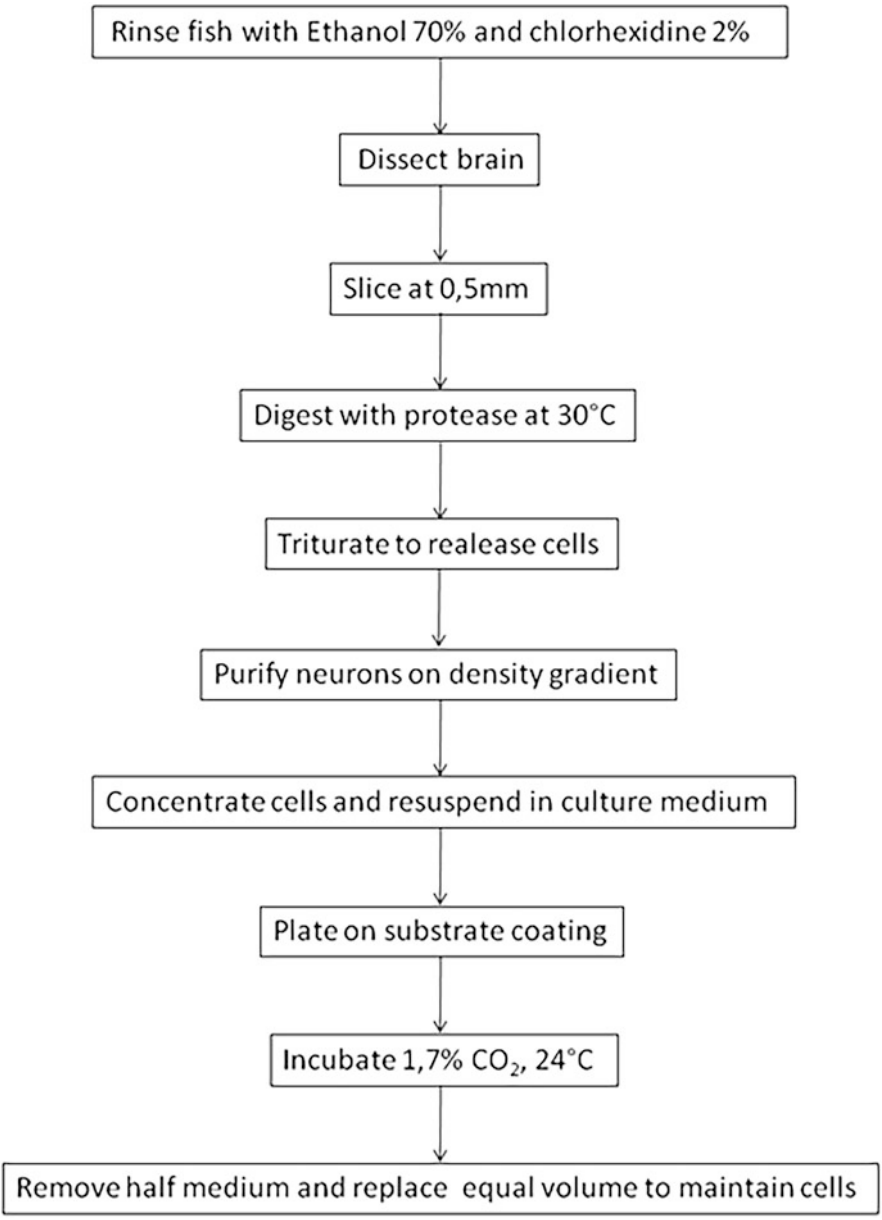

Fig. 26.1 Diagram for brain cellular obtention. (Source: authors' collection)

The PSP concentrations for experimental design depend on the degree of toxicity from substances to be tested and which tests are carried out. It is suggested that they carry a pilot experiment before of the bioassay.

After 24 h incubated in conditions described above, it is possible to assess the effects of the PSPs in target cells using several tests and analysis such as biochemical and genotoxic biomarkers. In this case, the plates should be washed three times with PBS before starting the carry out the analysis. It suggests five repetitions for each experiment.

It is recommended that the viability of cultured cells be determined by MTT method. This assay is based on the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, soluble form) into dark blue formazan crystals by mitochondrial dehydrogenases. The accumulation of formazan reflects directly the activity of mitochondria and indirectly the number of viable cells can be taken expressed as optical density (OD) at 570 nm, according to the method described by Sarmento et al. (2004).

Indeed, it is necessary to quantify the neuron cells in primary culture using immunocytochemistry test. For this trial, it is suggested to use III β-tubulin antibody (1:200) and anti-mouse Ig Texas Red to mark neuron protein specific.

To the biochemical biomarkers, the cells were plated after washing with PBS; the plate should be frozen at −80 °C for lysis cellular.

Then, the cells lysed can be homogenized in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 3400 × *g* for 30 min at 4 °C using rotor plate. The supernatants should be carried out to biochemical biomarkers such as superoxide dismutase, glutathione peroxidase, caspases, and other enzyme activities. In addition, supernatants can be used to assess the concentration of hydroperoxides and protein carbonylation. It is recommended to use the Bradford protein test to normalize the results of the tests applied.

Beyond that, the potential genotoxic actions of PSPs can be evaluated by comet assay detaching cells of the plate rising PBS/EDTA and carrying out the cells to procedure described by Singh et al. (1988) modified by Ferraro et al. (2004). For each brain slide, 100 cells should be visually analyzed and scored as belonging to one of five classes from undamaged (0) to maximally damaged (4) pre-defined with reference to the tail intensity.

The diagram shown in Fig. 26.2 summarizes the exposure PSPs procedure for bioassay in vitro.

26.2.2 Advantage and Disadvantage of In Vivo Versus In Vitro Studies

Monitoring PSPs by bioassay allows identifying risks to animal and public health and establishing safe limits according to the results observed in the exposure organisms. However, what kind of bioassay chooses: in vivo or in vitro methods?

Both have advantages and disadvantages. Bioassay in vivo, for example, enables checking the animal behavior when exposed to high or low concentration of PSPs. Moreover, it is possible to evaluate the bioaccumulation and metabolites and biodegradation or detoxification and their toxic effects.

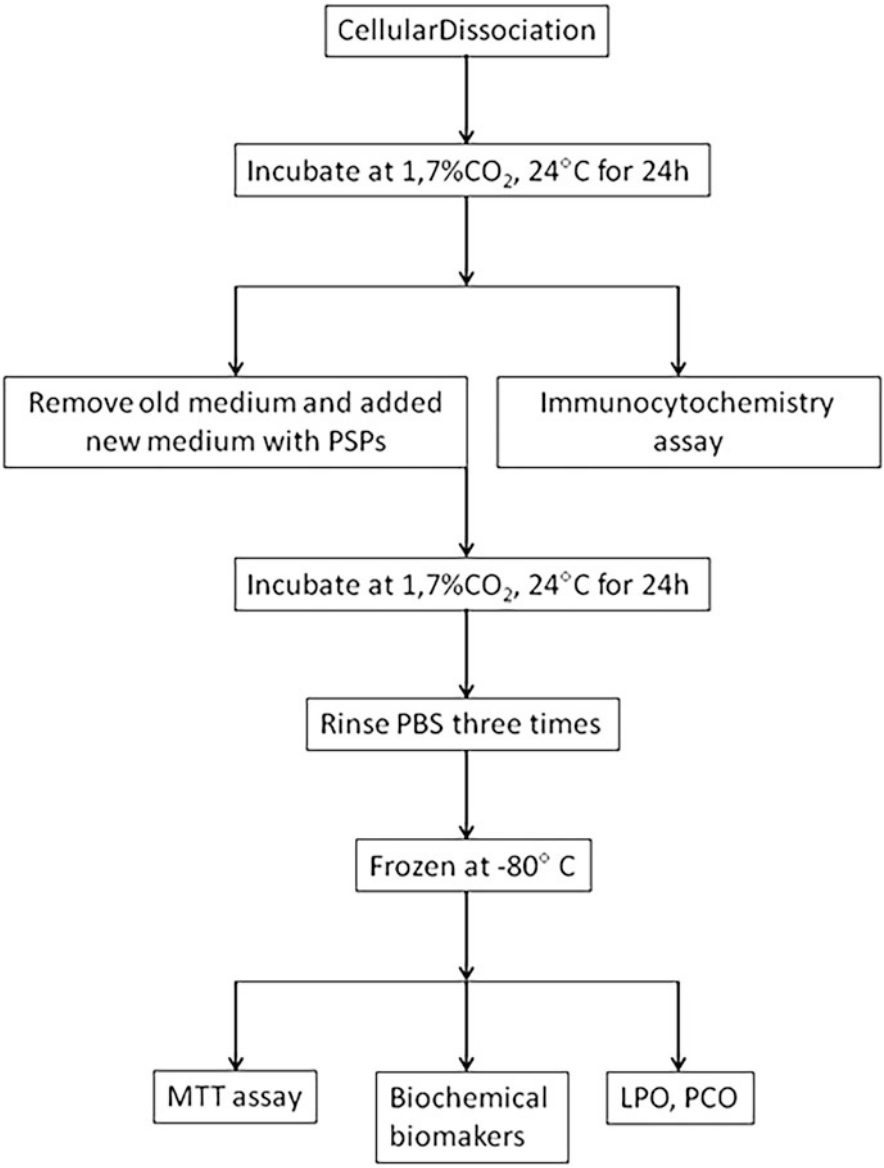

Fig. 26.2 In vitro bioassays to assess the PSP exposure. (Source: authors' collection)

The exposure time can also be controlled in accordance with lethal or sublethal concentration in acute, subchronic, or chronic assay, making possible a valuable observation of the damage caused by exposure and its respective bioaccumulation.

Allow yet the observation of the trophic flow of PSPs by direct exposure to water through the tegument and gills of the animal or due to application in food offered to the organism.

On other hand, the PSPs metabolizing during in vivo bioassay can mask the mechanism of action of the PSPs testing leading to false clarification about the starting and endpoint effects.

In addition, for in vivo studies is needed several types of chemical analyzes in the test water during the exposing and use high concentration of PSPs strain toxic extracts to expose the animals. Apart from that, the number of individuals is an important factor to consider to in vivo bioassay.

Ethics committees have denied authorization depending on the manner and the number of individuals used in the tests which can make unfeasible in vivo bioassays.

Additionally, depending on the quantity of experimental groups will be necessary lot of space for aquariums, especially if working with a top species the trophic chain that sometimes they can have territorial behavior and will need individual aquariums.

In contrast, the in vitro bioassay provides several types of results using a bit number of individuals considering the independents experiments. It is possible to perform the test using the total number of cellular from the tissue selected, and thus, it is a population of cells and not a sample like an in vivo bioassay. One individual can be donator of many millions of cells (Silva et al. 2014).

The environmental interference can be controlled and monitored. Because the target cells are cultivated from a tissue and applying PSPs directly over them, the effects are evidenced with no changes in molecular test substance.

In vitro studies allow to elucidate the mechanisms of the action of PSPs such as expression of proteins.

By the fact that there are no digestive metabolic processes of the organism donor cells due to exposure to xenobiotic, the time of exposure can be reduced and the toxic effects observed in a short period as some hours or minutes. In addition, the concentration can also be abridged because of the direct action on the cellular structures.

However, these studies are more expensive than in vivo bioassay and do not provide assessing the bioaccumulation in tissues or organisms.

Finally, the primary culture does not provide information about the interactions between tissues and organs from animals, and this method should be avoided for clarification about the interaction's mechanisms.

Table 26.3 shows the main characteristics of the in vivo and in vitro bioassays for the best choice between the two methods.

Table 26.3 Principal characteristics of the in vivo and in vitro studies

	In vivo bioassay	In vitro bioassay
PSP concentration	High concentration is required	Response to low concentration
Bioaccumulation test	Yes	No
Number of individuals	High. It is necessary about 20 individuals per group	One individual can be enough
Statistical test	Sample	Cell population
Death during test	It is probable death of individuals in acute or chronic bioassay	No. Under optimal conditions, the cells can survive up to 30 days
Ecosystem clarification	Yes. It is possible to estimate results observed in bioassay to aquatic ecosystems	No
Ecological interaction	Yes. It is possible to elucidate some interaction about ecological interaction	No
Tissues and organ interaction	Yes. It is possible to elucidate interaction between tissues and organs from animal exposure and toxic effects	No. The toxic effects are measured for only tissue or organ donor of cells
Mechanism of PSP metabolism	Yes. The bioassay allows clarification about the PSP metabolism in organs and tissues	No. It is only possible to estimate the PSP influence about cellular metabolism
Mechanism of biodegradation or detoxification	Yes. It is possible to estimate the biodegradation in tissues and organs	Yes. Biodegradation and detoxification in cellular and molecular levels
Expression of specific proteins and molecular	Yes. However, the analytical methods are expensive and difficult	Yes. A simple method can elucidate the expression of proteins
Mechanism of action of PSPs	Yes. It is possible to estimate the action mechanism to tissue and organs	Yes. It is possible to clarify the mechanisms about target cellular and receptors
Apoptosis clarification	Yes. It is possible to estimate the probability of the tissue, organ, or cells which are dying by apoptosis or necrosis	Yes. It is possible to clarify the mechanism of cellular death, including the caspase cascade
Time of bioassay exposure	High	Low. Some minutes or hours
Staff for bioassay	Recommends trained staff	Trained staff is required
Cost	Low or medium	Expensive or medium

Source: authors' collection

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