
Review Paper:

Ecotoxicological Bioassays: An innovative tool for wastewater pollution control
Berrebaan I.*, Montassir L. and Bessi H.
Laboratory of Virology Microbiology Quality and Biotechnology/ Ecotoxicology and Biodiversity, Faculty of Sciences and Techniques, Hassan II University of Casablanca, Mohammedia, MOROCCO
*imane.berrebaan@gmail.com

Abstract
In recent years, water pollution is one of the most serious problems in the world. The release of many kinds of wastewaters is the main origin. Wastewaters have negative effects on aquatic ecosystem which are demonstrated across different organisms from basic to higher trophic levels. This arises concerns to ensure a high wastewater quality control. Current wastewaters quality assessment is usually regulated by traditional physical and chemical parameters.

However, this classical approach is not able to predict the effect of contaminant mixtures present in the wastewaters. As such, synergistic, antagonist and/or integrative effects combination affects contaminants. In addition, the analytical approach cannot address all contaminants present at trace concentrations in the wastewaters. The physicochemical analysis is not sufficient to explain the potential ecotoxicity in wastewaters. The whole effluent toxicity testing (WET) completes the shortcoming from routine screening. The goal of the WET is to expose freshwater organisms from different trophic level including primer producer (microalgae), primer and seconder consumer (invertebrates, fish) and/ or decomposers (bacteria) to toxic wastewaters. The current study highlights the benefit of using the WET approach as a supplementary tool for wastewaters treatment control as well as the effectiveness of wastewaters treatment plant (WWTPs) efficiency.

Keywords: Classical approach, WET, freshwater organisms, bioassay, wastewaters control.

Introduction
Water quality deterioration is fast becoming a major threat in the world. The direct and indirect high load’s industrial or municipal wastewaters release with huge main-made products such as toxic pollutants (e.g. metals), nutrients (e.g. ammonia, phosphorus) and/or emergent contaminants (e.g. pharmaceuticals) into water bodies is one of the major causes. The presence of such pollutants in the wastewaters may cause serious potential effects on aquatic biological balance even at low concentrations and consequently in human beings. Several wastewater treatments are conceived to decrease the environmental impacts of wastewater discharges. For this, significant advances in treatment process raise concerns about their potential depollution efficiency. In previous studies, pollution removal by classical or advanced wastewater treatment plants (WWTPs) has been proved incomplete. As such, Xu et al demonstrated that antibiotics are partially eliminated at the four WWTPs in the Pearl River Delta (south China). In their study, the highest elimination efficiency reached to 81%.

In another study, estrogenic chemicals are also partially degraded and generate ozonation by-products. So far, it is clear that a considerable portion of active contaminants remains prevalent into freshwaters and could induce adverse damages on aquatic wildlife. The need to improve the effectiveness of depollution process has increased the interest towards innovative control methods.

Current legislations on wastewaters control are limited to some traditional chemical and physical parameters [e.g. Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Potential Hydrogen (pH), or Total Suspended Solids (TSS)]. Such routine screening is laborious, expensive and produces toxic substances. Pollutants in wastewaters are identifiable and in relatively determined quantity. In fact, the signal analytical approach is not able to address all targeted micropollutants present in treated wastewaters.

An effluent could be toxic, even meeting standard discharges. In addition, the bioavailability of contaminants and their cocktails additive, antagonistic and synergistic effects are neglected in complex wastewaters. In the case of WWTP, the classical assessment does not detect all disinfection new by-products generated during the treatment process like ozonation or chlorination.

Several international standards and group researchers denote biological assessments of wastewater quality using standard ecotoxicological bioassays as complementary tools. The principle is based on the responses of the whole living organisms, cells, or tissues. This biological approach is usually recognized as Whole Effluent Toxicity (WET). The WET program was first formalized by the US Environmental Protection Agency (USEPA) in 1985. It completes some shortcoming from analytical methods. This biotesting is simple, sensitive, informative and cost-effective. The bioscreening provides concrete results on the overall water quality characterization.

Furthermore, the testing laboratory offers a direct measurement of pollutant’s effects on biological systems...
and produces further baselines and knowledge about discharge management⁶⁹.

On the basis of US EPA guidelines, many international agencies and countries have adopted bioanalytical approaches into their regulations⁵⁷. For example, Effect Directed Analysis (EDA) is a European adaptation of the WET approach since the 1980s. In recent years, effluents are regulated by WET test methods in South Korea’s Water Quality and Ecosystem Conservation Act since 2011⁵⁹. The toxicity evaluations in WET analyses are mainly performed by aquatic organisms from different food web which are convenient and sensitive. The most adopted organisms are microalgae, invertebrate and fish. All these species have specific strengths and weaknesses.

Meanwhile, other studies recommend a simultaneous use of suite ecotoxicological bioassays so called “battery of bioassays”¹⁹,⁴⁵,⁹¹. The advantage of using a multiple species testing resides in their unequal sensitivity to various pollutants in wastewaters⁷⁹. For example, Ra et al⁶⁰ suggested that green microalgae are sensitive to herbicides and fungicides, however, daphnids are more sensitive to pesticides.

This review provides an overview of most commonly ecotoxicological biotests used for wastewater’s quality assessment. However, it does not provide an exhaustive list of all available bioassays.

**Microalgae in wastewaters bioscreening:** Microalgae, as primary producers, constitute the basis of trophic chain. They play an important role in serving source of food to higher levels such as herbivorous zooplanktons³⁹. This means that any alterations to microalgae population may cause disturbances in the whole ecological pyramid⁷³. In aquatic ecotoxicology, microalgae is widely used for biomonitoring water quality due to its sensitivity, versatility, ease of culture, ubiquitous distribution and high sensitivity to spectrum toxicants including metals and organic contaminants⁶⁴. In addition, microalgae appear more sensitive to contaminants than invertebrates and fish due to direct contact between cells membrane and test medium⁶⁹.

Furthermore, algal biotests are simple and allow for the observation of chronic responses at multiple generations during short times⁴. In general, *P. subcapitata* (formerly known as *Selenastrum capricornutum* or as *Raphidocelis subcapitata*) has been considered as a reference organism standard toxicity tests¹⁶,⁴⁹,⁸². More species are also recommended (e.g. *Chlorella vulgaris*, *Desmodesmus subcapicatus*, *Dunaliella tertiolecta*).

The objective of biotest protocol is to determine the algal growth rate⁶⁶. Exponential cells are exposed to serial dilutions of toxicants or effluents under controlled conditions (light, temperature and shaking) at determining period (e.g. 72h and / or 96h) in Erlenmeyer flasks. Algal biomass are investigated at the end of the incubation period using direct counting (e.g. automatic particle counter) or indirect measurement (e.g. optical density). Results are expressed as 50% percentage inhibition (or stimulation) of algae population.

Some acute ecotoxicity endpoints including biochemical level (e.g. chlorophyll-a synthesis and enzyme activity), algal structure (e.g. cell membrane integrity) and/or metabolic activities (e.g. photosynthesis, respiration and/or ATP synthesis) are also recommended. According to Zhang et al⁹¹ algal growth and chlorophyll-a synthesis are more susceptible to nutrients while enzyme activity and cell membrane integrity are mainly influenced by some toxic substances that cause oxidative stress.

Methods using microalgae as test organisms are given a high priority for further optimizations. For example, a miniaturized and low-cost version bioassay in 96 well microplates is developed to encompass several constraints of classical procedure related to time, space and reproducibility⁷. The immobilization of microalgae in calcium alginate beads is another attractive alternative to classical algal growth bioassay⁴⁶,⁵⁵. Toxicity testing using immobilized microalgae as methods for monitoring wastewater quality has been optimized mainly to overcome limits of cells culture dependence in algal classical bioassays.

Owing to their importance, microalgae are very attractive scientific models to evaluate the ecotoxicity of aquatic matrices, especially industrial wastewaters. As an example, Blaise et al⁶ tested the phytotoxicity responses of *P. subcapitata* towards 46 effluent samples from pulp and paper sector (a major industrial force in the province of Quebec, Canada). As a second example, Yu et al⁶⁹ evaluated toxicity of pharmaceutical wastewaters using the microalga *Scenedesmus obliquus* growth inhibition after 72h. In addition, Raptis et al⁸¹ investigated the relationships between organic sum-parameters and chronic toxicity with *P. subcapitata* in kraft mill effluents.

In addition, algal biotests are commonly adopted for wastewater treatment performance in several studies. Zhang et al⁹⁴ evaluated the toxicity situation of municipal wastewaters as well as the toxicity removal performance of three WWTPs in the northeast of China. In their study, cell density, chlorophyll-a concentration, superoxide dismutase activity and cell membrane integrity of *Scenedesmus obliquus* are investigated. Algal growth inhibition endpoint is also employed in the waste waters treatment evaluation.

In fact, Tigini et al⁷⁵ suggested that *P. subcapitata* is the most sensitive organism to assess the effectiveness of remediation methods in the textile and tannery wastewaters. Table 1 summarizes different applications of the use of microalgae in wastewaters quality assessment.
**Water flea in wastewaters screening:** Water flea are zooplanktonic populations of Cladocera which represent an important trophic level in an aquatic food chain. They are principal grazer of microalgae, bacteria and protozoans and a primary forage for fish. So, they transfer contaminants to top consumers. The genus *Daphnia* is relevant in vivo model organism in aquatic ecotoxicology. They are therefore recommended in several standardized guidelines for acute lethality due to their availability, ease of handle, prolific, short life cycle and quite sensitive to many compounds, such as metals or herbicides. Many species of *Daphnidae* family are usually used for ecotoxicity bioassay very common such as *Daphnia magna* (D. magna), *Daphnia similis* (D. similis), *Ceriodaphnia dubia* (C. dubia) and *Daphnia pulex* (D. pulex).

The goal of bioassay consists to expose juveniles of *Daphnia* (<24h age) from parthogenetic generation to low doses of targeted contaminants or aqueous matrices during short period (2-4h or 48h). The biotests are run in glass tubes with final volume of 10 mL. The biotest is static and incubated under static conditions at 20 ± 2°C and in a complete darkness.

After incubation period, toxic effects (mortality and immobility) are recorded. For immobility, juveniles remove their antennules and did not swim within 15 min after gentle shaking considered as immobile. The acute toxicity is expressed as concentration that immobilizes 50% of population after 24/ and or 48h relative to a control. Several biological endpoints including physiological responses (e.g. ingestion and heart rates), enzymatic biomarkers related to neural function or antioxidant defense (e.g. acetylcholinesterase, superoxide dismutase, catalase and glutathione reductase and antioxidant defense and oxidative stress damage markers were also used as early response in ecotoxicity assessment. The use of *Daphnia* compartmental parameters (e.g. feeding, heart rate and swimming behavioral) is sensitive and early ecotoxicity indicator for wastewaters quality assessment.

Many studies highlight the applicability of acute toxicity with *D. magna* in wastewaters toxicity biotesting e.g. in biomining wastewaters, highly colored effluents (e.g. Sugarcane vinasse) textile and tannery effluents, municipal secondary WWTP effluents, disinfection of hospital wastewaters, industrial wastewater treatment, urban wastewater treatment and even in contaminated streams. Table 2 provides further acute toxicity wastewaters evaluation with *Daphnia*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Endpoints</th>
<th>Applicability</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. subcapitata</em></td>
<td>72h algal growth</td>
<td>Irrigation water for vegetables</td>
<td>Greater São Paulo watershed region.</td>
</tr>
<tr>
<td><em>P. subcapitata</em></td>
<td>72h algal growth inhibition</td>
<td>Cooking wastewaters</td>
<td>China</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>72h algal growth inhibition</td>
<td>Pharmaceutical wastewaters</td>
<td>China</td>
</tr>
<tr>
<td><em>Desmodesmus subspicatus</em></td>
<td>In vivo chlorophyll fluorescence, Cell density, Cell size (number of cells/colony) Cell ratio (length/width) after 24h</td>
<td>Washing cleaning wastewaters</td>
<td>Sweden</td>
</tr>
<tr>
<td><em>P. subcapitata</em></td>
<td>72h algal growth inhibition</td>
<td>Municipal wastewaters</td>
<td>Portugal</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>72h growth inhibition</td>
<td>River waters</td>
<td>Sava river SE Europe</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>72h Algal growth inhibition</td>
<td>Laboratory wastewaters</td>
<td>Portugal</td>
</tr>
<tr>
<td><em>D. tertiolecta</em></td>
<td>96h growth rate inhibition</td>
<td>Pesticide industrial wastewater</td>
<td>Pakistan</td>
</tr>
<tr>
<td><em>Desmodesmus subspicatus</em></td>
<td>72h Algal growth inhibition</td>
<td>Treated municipal wastewaters</td>
<td>Greece</td>
</tr>
<tr>
<td><em>P. subcapitata</em></td>
<td>72h Algal growth inhibition</td>
<td>Municipal wastewater effluents</td>
<td>Croatia</td>
</tr>
<tr>
<td><em>P. subcapitata</em></td>
<td>72h Algal growth inhibition</td>
<td>WWTP influents and effluents</td>
<td>Japan</td>
</tr>
</tbody>
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Table 2
Application of acute and sublethal toxicity bioassays with water flea in ecotoxicity biomonitoring of wastewaters.

<table>
<thead>
<tr>
<th>Species</th>
<th>Endpoints</th>
<th>Application</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. magna</td>
<td>48h immobility Sublethal oxidative stress</td>
<td>Textile and Leather wastewaters</td>
<td>Korea</td>
</tr>
<tr>
<td>D. magna</td>
<td>48h acute lethality</td>
<td>WWTP effluents</td>
<td>Iran</td>
</tr>
<tr>
<td>D. magna</td>
<td>Feeding rate Oxidative stress</td>
<td>Liquid crystal display effluent</td>
<td></td>
</tr>
<tr>
<td>D. magna</td>
<td>Immobilization after 24h and 48h</td>
<td>Urban WWTP effluents contamined with pharmaceuticals</td>
<td>Italy</td>
</tr>
<tr>
<td>D. magna</td>
<td>24h acute lethality</td>
<td>Sugarcane vinasse effluents</td>
<td>Brazil</td>
</tr>
<tr>
<td>D. magna</td>
<td>48h acute immobilization</td>
<td>Cooking waste waters</td>
<td>China</td>
</tr>
<tr>
<td>D. magna</td>
<td>24h acute toxicity</td>
<td>Raw and ozone treated textile wastewaters</td>
<td>Turkey</td>
</tr>
<tr>
<td>C. dubia</td>
<td>48h survival (acute)</td>
<td>Effluents from sewage treatment plant</td>
<td>Australia</td>
</tr>
<tr>
<td>D. magna</td>
<td>24h acute toxicity</td>
<td>Textile wastewaters</td>
<td>Mexico</td>
</tr>
<tr>
<td>D. magna</td>
<td>48h acute lethality 48h swimming inhibition rate</td>
<td>Wastewater effluents after disinfection (chlorination and ozonation)</td>
<td>Korea</td>
</tr>
<tr>
<td>D. magna</td>
<td>Acute immobilization after 24h, 48h, 72h and 96h</td>
<td>WWTP effluents</td>
<td>Turkey</td>
</tr>
</tbody>
</table>

**Fish toxicity in wastewater screening:** As compared with lower trophic level organisms, attempts about the protection of aquatic ecosystems towards wastewaters discharge is more focused to fish. This is due to their ubiquity in quasi totality of water bodies, their fundamental position in aquatic food chain (e.g. nutrient cycling), their similarity physiological responses similar to humans and their potential linkage to public health.

In aquatic ecotoxicology, one model organism is widely used. The first is Danio rerio, commonly known as zebrafish. It is a Cyprinidae originated from India and widely distributed in Europe, Africa, Asia and USA. Other species are recommended such as rainbow trout Oncorhynchus mykiss. Utility of using those species in aquatic ecotoxicology refers to their small size, high fecundity, simple laboratory maintenance, rapid development, short cycle life, external fertilization and transparent embryos.

The fish models are vulnerable of large numbers of biotest methods. The acute life fish stage bioassays are important to elucidate the early pollutants risks. During this acute toxicity, embryo, juveniles or adults were exposed to different dilutions of effluent samples. The bioassay is run in small beakers with 50 mL of synthetic medium without feeding. The toxicity criteria is lethality after a short period (e.g. 24h 48h, 72h and / or 96h). The bioassay was performed at controlled light, pH and temperature conditions.

In the case of embryo test hatching rate, total survival rate, survival rate after hatching and survival index were usually used as measurements of wastewaters toxicities. Sublethal parameters are more sensitive than lethality such as reproduction, swimming behaviors and locomotion.

In recent years, fish has been already used to investigate the toxicity of many compounds such as pharmaceuticals, pesticides and metals. Meanwhile, the use of acute toxicity with fish is accepted in extensive studies for wastewater’s quality control. At the moment, some researches have focused on assessing the detoxification efficiency of WWTPs.

In a previous study, Na et al evaluated detoxication efficiencies for an acrylonitrile wastewater treated by a combined anaerobic oxic-aerobic biological fluidized tank process in northeast China. In their study, the 96h acute toxicity was tested using Danio rerio (D. rerio) and the fish embryo toxicity zebrafish eggs. Table 3 gives more application of fish bioassay in the screening of wastewaters.

**Conclusion**
In conclusion, WET is an innovative approach mainly applied in wastewaters quality control and WWTPs efficiency. In fact, such bioscreening completes physicochemical assessment as they present some limits.
Many aquatic organisms are available for WET, however, the green microalgae (primer producers), Daphnia (primer consumers) and/ or fish (second consumers) are more vulnerable and widely applied in aquatic ecotoxicology research. The battery of bioassay is more suitable in the WET due to difference in sensitivity organisms toward contaminants present in the wastewaters.

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