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SHORT COMMUNICATION

A *Saccharomyces cerevisiae*-based bioassay for assessing pesticide toxicity

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Abstract This study evaluates the toxic effect of three pesticides (Azoxystrobin, Cymoxanil, and Diuron) on the yeast *Saccharomyces cerevisiae* for the development of a new bioassay based on inhibition of *S. cerevisiae* metabolic activity at the level of adenosine-5-triphosphate (ATP) synthesis, as compared with two different toxicity tests based on inhibition of *Daphnia magna* mobility (NF EN ISO 6341) and inhibition of *Vibrio fisheri* activity (NF EN ISO 11348). The *S. cerevisiae* bioassay is cheaper and 96 times faster than the *D. magna* toxicity bioassay, but has lower sensitivity. It is as fast as the *V. fisheri* bioassay and more sensitive. Thus, this new toxicity test can be proposed for rapid detection of pesticide residues in environmental samples as a complement to the more expensive and time-consuming *D. magna* toxicity test.

Keywords *Saccharomyces cerevisiae* · Pesticide residues · ATP · Toxicity · Bioassays

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Introduction

In recent years, there has been growing concern about the toxic effects of chemical pollutants present in the environment. Among these substances, pesticides are widely used in large quantities throughout the world to control pests and enhance agricultural production. Many of them are highly toxic and they form one of the main classes of chemical environmental pollutants that contaminate natural ecosystems [1].

Toxicity bioassays are used to detect and predict the detrimental effects of chemical pollutants on populations and ecosystems. Toxicity is assessed with in vitro bioassays or bioassays using vertebrates, invertebrates, bacteria or algae. Existing standard toxicity tests usually rely on eukaryotic species, such as fathead minnow and daphnids, that require long acclimatization times, making the methods labor intensive and expensive [2]. However, several publications have presented alternative rapid and cost-effective methods relying on microorganisms [3–5]. These use bacteria or microorganisms having biochemical pathways similar to those of higher organisms, as well as short life cycles, allowing rapid response to environmental changes [6, 7]. These tests are inexpensive. However, the majority of the existing microbial toxicity tests are not sensitive enough to detect low concentrations of pollutants [8]. Eukaryotes such as yeasts are potentially good models for assessing toxicity [9] as they are easy to maintain and culture under controlled conditions, thus avoiding the variability issues found with more complex organisms [10].

Inhibition of *Saccharomyces cerevisiae* by pesticides during alcoholic fermentation has been particularly studied [11]. For instance, the presence of four fungicide residues (cyprodinil, fludioxinil, glyphosate, and pyrimethanil) during fermentation affected the aromatic composition of

Vitis vinifera white wines inoculated with *S. cerevisiae* strains, causing a decrease in organoleptic quality [12–14]. Also, analysis of the *S. cerevisiae* gene expression pattern in response to lindane (an organochlorine pesticide) revealed mitochondrial dysfunction, oxidative stress, and ionic homeostasis [15, 16]. Razmovski and Pucarevic [17, 18] showed that flutriafol (a fungicide) decreased the protein and phosphorus cell content, as well as total ribonucleic acids, enzymatic activity (pyruvate carboxylase and isocitrate lyase), and respiration quotient of *S. cerevisiae*. Finally, Ribeiro et al. [9] also observed that *S. cerevisiae* specific respiration rates were reduced by other pesticides (Penconazol, Cymoxanil, and Dichlofluanid), showing that pesticides inhibit the yeast's respiratory metabolism.

Despite all of these studies and the demonstration of the usefulness of a yeast assay procedure for testing heavy-metal toxicity by Bitton et al. [7], only a few researchers have used *S. cerevisiae* in toxicity tests [19–22]. Nevertheless, Koch et al. [16] proposed yeast as an alternative organism to test acute toxicity of drugs and environmental chemicals, as tools for preliminary screening, and for inclusion in a test battery.

The goal of this study was to evaluate the effect of three pesticides on metabolic activity of *S. cerevisiae*. The three pesticides tested (Azoxystrobin, Cymoxanil, and Diuron) have different chemical structures and are used for different purposes (fungicide or weedkiller). The toxicity of the pesticides (half-maximal effective concentration, EC_{50}) was determined by monitoring *S. cerevisiae* ATP synthesis. The dose responses obtained were compared with standard toxicity tests (International Organization for Standardization) such as inhibition of *Daphnia magna* mobility bioassay (NF EN ISO 6341) and inhibition of *Vibrio fisheri* activity bioassay (NF EN ISO 11348).

Materials and methods

Chemical pesticides

The three pesticides used were: Azoxystrobin (methyl-2-cyanophenoxy-pyrimidin-4-yloxy-phenyl-3-methoxyacrylate), Cymoxanil (2-cyano-*N*-[(ethylamino)carbonyl]-2-(methoxyimino) acetamide), and Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) (Fig. 1), all supplied by Fluka. Azoxystrobin is derived from a group of natural fungicidals: b-methoxyacrylic acids. It inhibits mitochondrial respiration of fungi and disrupts the energy cycle and ATP production. Cymoxanil belongs to the amide family. It is a small aliphatic molecule used to control downy mildew diseases induced by fungal pathogens. Diuron belongs to the family of substituted ureas. It is used as a weedkiller. Diuron penetrates into plants via the roots and blocks

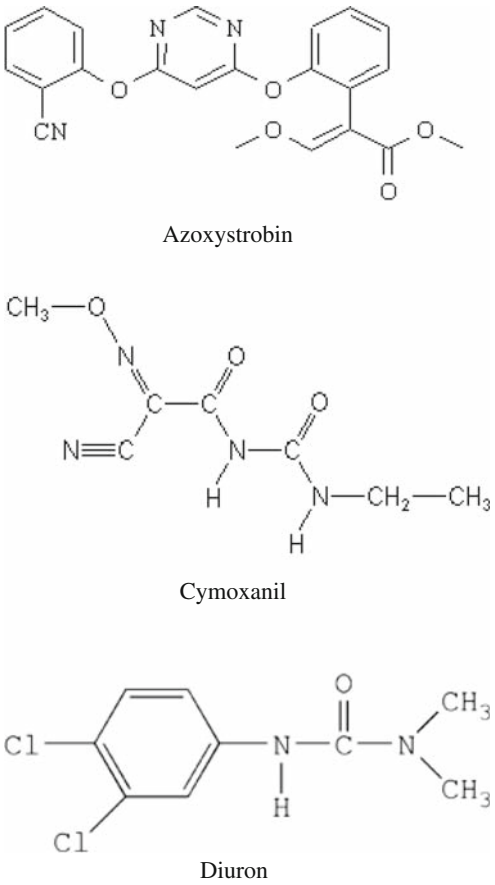

Fig. 1 Structures of the three investigated pesticides

electron transfer along the mitochondrial chain, stopping photosynthesis.

Daphnia magna and *Vibrio fisheri* toxicity bioassays

The toxicity effect of the pesticides was determined according to the NF normalized methods: inhibition of *Daphnia magna* mobility NF EN ISO 6341 [23] and inhibition of *Vibrio fisheri* activity NF EN ISO 11348 (Microtox®) [24].

Saccharomyces cerevisiae toxicity bioassays

Our toxicity test is based on the measure of adenosine-5-triphosphate (ATP) synthesis by *S. cerevisiae*.

Yeast

The yeast strain used throughout this study was *S. cerevisiae* var. *Bayanus* wild type (AWRI 350). The stump of yeast is marketed by AB MAURY Wine (Beziers, France). It comes from the collection of the Australian Wine Research Institute. This stump of active dry yeast is used

during vinification for the development of aromatic compounds in young red wines. It was selected for this survey because it has short growth-phase latency, excellent flocculation, and produces little foam.

Culture conditions

The dehydrated yeast was stored at 10°C. For culture, 10 g of yeast cells from the stock was incubated at 30°C for 30 min with 10 g D-glucose (SIGMA) dissolved in 100 ml mineral water. Rehydration of the yeast suspension was observed under an optical microscope (LEICA) at 400× magnification.

Growth assays

Each of the pesticides was dissolved in distilled water at the following concentrations: 0.1, 1, 10, 100, and 1,000 mg l^{-1}. One millilitre of cell suspension was grown in absence or presence of 4 ml pesticide solutions. The culture was maintained at 20°C for 30 min, and the amount of ATP in the culture was measured by using a bioluminiscence assay as described below.

Measurement of adenosine-5-triphosphate (ATP) rate

The technique is based on the capacity of *Photinus pyralis* beetle luciferase to emit light by an enzymatic reaction. In the presence of Mg^{2+}, ATP, and oxygen, the luciferase oxidizes luciferin into oxyluciferin and produces light [25]. The AQUAtrace kit (BIOTRACE Inc.) quantitatively estimates the amount of ATP in a sample. In practice, an AQUAtrace stick is placed in contact with the experimental medium. This stick contains all the reagents necessary to break the cell envelope, inhibit ATPases activity, and allow light production from ATP. The light is collected, measured at $\lambda = 562$ nm, and expressed in relative light units (RLU) by a Plain-Lite illuminometer NG (BIOTRACE Inc.).

Calculation of EC_{50} using the probit method

The probit method is a simple and fast method to calculate EC_{50} (the concentration that affects 50% of the population) from inhibition curves of logarithmic shape [26]. It has been used by Barata et al. [27] to quantify the toxicity of organochlorine pesticides and carbamates to the aquatic microorganism *Daphnia magna*. First, the percentage of inhibition is calculated as follows:

$$([\text{ATP}]\text{control} - [\text{ATP}]\text{assay}/[\text{ATP}]\text{control}) \times 100.$$

Then the percentage inhibition is plotted against the logarithm of the pesticide concentrations to obtain a linear regression. Finally, the value of EC_{50} is estimated, via a linear regression equation, by the calculation of the pesticide concentration that inhibits 50% of ATP synthesis in *S. cerevisiae*.

Reproducibility of results

All measurements were performed in triplicate and results are reported as mean ± standard error of mean (SEM).

Results

Toxic responses of the three pesticides using the new toxicity test were determined. Dose–response relationship and EC_{50} values are dependent on the chosen pesticide concentration range. Selection of an appropriate pesticide concentration permitted the toxicity of the sample to be established in a toxicity test. The effect of each pesticide on *S. cerevisiae* ATP synthesis, *Daphnia magna* growth, and *Vibrio fisheri* activity was evaluated, and the corresponding EC_{50} values were determined.

Effect of pesticides on *S. cerevisiae* ATP rate

Figure 2 presents dose–response histograms of the selected pesticides: Azoxystrobin (a), Cymoxanil (b), and Diuron (c), as measured by the ATP concentration of *S. cerevisiae* cultures after 30 min of contact with pesticide solutions at 0.08–800 mg l^{-1}.

For the three pesticides, increasing the pesticide concentration resulted in a stronger decrease of the *S. cerevisiae* ATP content.

The inhibition curves are of logarithmic type, not allowing direct calculation of EC_{50}. To determine exactly the differences of inhibition between the tested molecules, EC_{50} values were determined by using the probit method (Fig. 3) [26].

Comparison of relative pesticide toxicities using the new and traditional toxicity tests

The average EC_{50} values obtained for the three pesticides using the new toxicity test were compared with those obtained using the two standard toxicity tests based on *Daphnia magna* and *Vibrio fisheri* (Table 1).

The newly developed test based on *S. cerevisiae* has a contact time of only 30 min, which is the same as the bacterial test using *Vibrio fisheri* and much shorter than the mobility test using *Daphnia magna*, which requires 48 h. The effect of pesticides on *S. cerevisiae* is variable according to the nature of the pesticide (EC_{50} Cymoxanil < EC_{50} Diuron < EC_{50} Azoxystrobin). The same

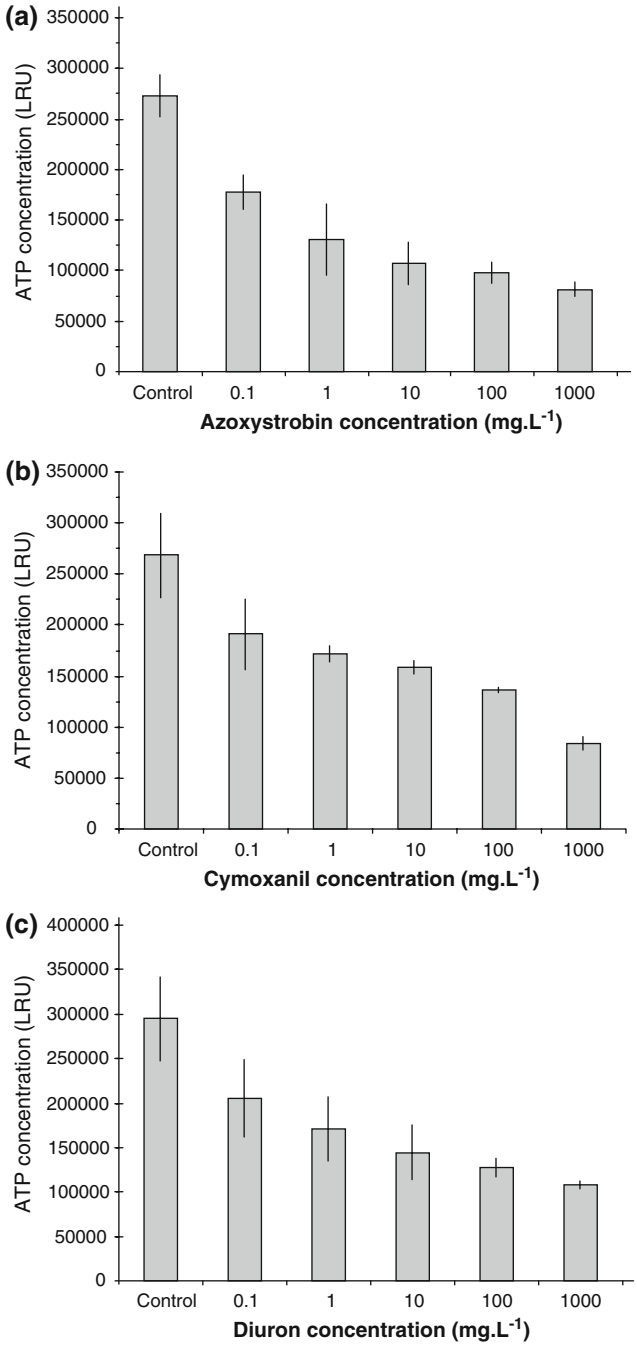

Fig. 2 Effects of Azostrobin (**a**), Cymoxanil (**b**), and Diuron (**c**) on *S. cerevisiae* ATP synthesis. Data represent the mean ± standard deviation (SEM) of three replicates

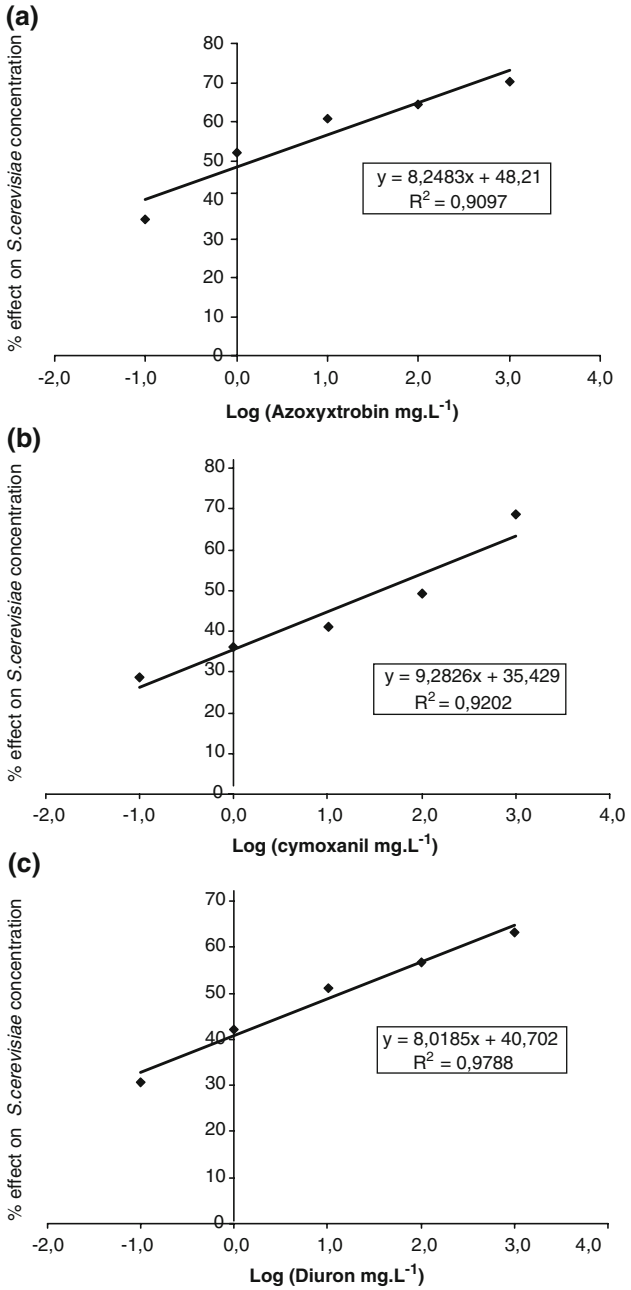

Fig. 3 Determination of the EC_{50} values of Azoxystrobin (**a**), Cymoxanil (**b**), and Diuron (**c**) by the probit method

observation is made for inhibition of *Vibrio fisheri* (EC_{50} Diuron < Cymoxanil EC_{50}) and of *Daphnia magna* (EC_{50} Cymoxanil < EC_{50} Diuron < EC_{50} Azoxystrobin). By comparing the sensitivity of the biotests, we notice that the EC_{50} values obtained with *Vibrio fisheri* are about three times higher for Diuron and similar for Cymoxanil. The developed *S. cerevisiae*-based test is thus more sensitive for phytosanitary products than is the current *Vibrio fisheri* test. The *Daphnia magna* test remains the most sensitive test, being about 1.5, 2.5, and 5 times more sensitive for Cymoxanil, Diuron, and Azoxystrobin, respectively.

Discussion

Traditional toxicity biotests are time consuming to operate and expensive [28]. They cannot be used extensively for the follow-up of treatment units or of contamination of

Table 1 Comparison of toxicity values obtained for Azoxystrobin, Cymoxanil, and Diuron with different biotests

Biotest	Azoxystrobin (mg l^{-1})	Cymoxanil mg l^{-1})	Diuron (mg l^{-1})
Yeasts: *S. cerevisae* EC_{50} 30 min	1.3	29.7	11.6
Bacteria: *Vibrio fisheri* EC_{50} 30 min	–	39.1	58
Aquatic invertebrates: *Daphnia magna* EC_{50} 48 h	0.3	27	5.7

agricultural machines. It thus seems useful to develop a reliable, easy to realize, fast, and inexpensive test. We propose to use the percentage inhibition of *S. cerevisiae* ATP synthesis to evaluate the toxic effect of pesticides. The proposed biotest was validated on three phytosanitary molecules with different chemical structures and by comparison with standardized tests (*Daphnia magna* and *Vibrio fisheri*).

The results demonstrate a toxic effect of the pesticides on *S. cerevisiae* that is variable according to the molecule concentration and nature. The inhibition of *S. cerevisiae* ATP synthesis is of exponential shape, and pesticide toxicity increases with the molecule's Log *P* value (coefficient of water–ethanol division). These observations are in agreement with previous publications. Ribeiro et al. [9] showed that the effect of Cymoxanil on *S. cerevisiae* IGC 3507 growth and oxygen consumption was of logarithmic shape for pesticide concentrations from 0 to 100 mg l^{-1}. Also, it has been noticed for *Daphnia magna* and *S. cerevisiae* that Cymoxanil is less toxic than Diuron and Azoxystrobin [29]. This result follows the Log *P* values of the chemicals, which represents the hydrophobic nature of the phytosanitary molecules. Indeed, Cymoxanil is an aliphatic molecule that has a very low Log *P* value of 0.66, in contrast to the other two tested pesticides (Log *P* value of $\sim$2.5). By comparing the effect of different strobilurines on aquatic species (*Daphnia magna* 48 h, green seaweeds 72 h, fishes 96 h) the authors demonstrated that an increase of Log *P* brings about an increase of toxicity [29, 30].

The EC_{50} values obtained for our *S. cerevisiae* test are on the order of mg l^{-1}, which is sensitive enough to estimate the efficiency of a treatment process of phytosanitary effluents or for the follow-up of agricultural machines. Indeed, our new test is more sensitive than the bioluminescence test using *Vibrio fisheri*. Kay et al. [31] already noticed that the *Vibrio Fisheri* test is not so sensitive for the estimation of toxicity in water. Also, Tixier et al. [32] have shown that the eukaryote *Tetrahymena pyriformis* (EC_{50} = 6.33 μg ml^{-1}) is ten times more sensitive than the bacterium *Vibrio fisheri* (EC_{50} = 58 μg ml^{-1}) for estimation of the toxicity of Diuron. Finally, the mobility test of *Daphnia magna* is still more sensitive than our *S. cerevisiae* test, but it requires a much longer contact time (48 h).

Conclusion

The results demonstrate that the effect of pesticides on *S. cerevisiae* is variable depending on their chemical structure. The toxicity to eucaryotic microorganisms is strongly influenced by the hydrophilic-lipophilic nature of the molecule, as quantified by Log *P*. Furthermore, the new biotest is more sensitive than the *Vibrio fisheri* test. On the other hand, the *Daphnia magna* mobility test, widely used in ecotoxicology, is more sensitive but requires a longer analysis time. In spite of this difference in sensitivity, the EC_{50} values obtained are on the order of mg l^{-1}, which is sensitive enough to estimate the efficiency of a treatment process for phytosanitary effluents or assure the follow-up of a purge over time. So, this new toxicity test can be employed for continuous evaluation of the elimination efficiency of the toxicity of residues of phytosanitary products during aerobic biological treatment by activated sludge.

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