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Predictive effects (study *in silico*) of cadmium/fungicide cocktail on biomarkers of snail oxidative stress: *Cantareus aspersus* (Müller, 1774) using the forecasting grey model GM (1, N)

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Key words: Cadmium, Fungicide, *Cantareus aspersus*, Python, Grey model, Modeling

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Abstract

In this study, we were interested in the physiological and biochemical predicted effects of a copper-based fungicide (Vacomil-PLUS) (0.5, 1 and 2g/l), cadmium (200, 400, 800µg/l) and their mixtures (CdCl2 + Copper fungicide, 1/1, V/V) on bio-indicator organisms of pollution: the snail *Cantareus aspersus*. Our work consists in predicting the long-term effect of these xenobiotics using the forecasting grey model (1, N) as a predicting model. To our knowledge, this is the first study evaluating the predictive effects of toxicants on the snail *Cantareus aspersus*, using a computer prediction model. Our predictive results obtained from the modeling of predictive values, using the Grey model show that the presence of cadmium and/or fungicide causes growth inhibition of the treated animals, thus reducing the weight of the digestive gland. In addition, more disturbances that are significant also noted in the biochemical composition of the hepatopancreas of *Cantareus aspersus* (total proteins) after treatment with the cadmium/fungicide mixture. Monitoring of oxidative stress biomarkers shows disturbances due to contamination by these pollutants. We revealed an induction of MDA as well as a depletion of the GSH level, testifying to the occurrence of lipid peroxidation. Finally, cadmium, copper-based fungicide and their mixture significantly inhibits AChE activity.

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Introduction

The development of analytical methods in toxicology, over the last thirty years has been based on the concept of the 3Rs (replaces, reduces, refines). This aims to reduce animal experimentation as much as possible, and to replace it with in vitro or in silico methods, when these are capable of assessing hazards to humans and the environment as well as conventional in vivo studies. The methods *in silico* use mathematical modelling of toxicological data obtained *in vitro* and *in vivo*. These methods can be purely statistical and/or use the knowledge of expert toxicologists who associate the functions and structural patterns of chemical molecules with specific toxic responses.

A variety of computer tools for data analysis is typically used. They are based on computational algorithms (mathematical, chemical and biological), designed to produce either predictions of toxicity or actual toxicological experiments related to data for use in the hypothesis of scientific tests or safety analyses. The introduction of *in silico* methods in the modern toxicological approach should allow limiting a heavy, expensive animal experiment, and which is not necessarily representative of human situations (Wu *et al.,* 2019). In order to better understand the effects of the xenobiotics, we completed our work with an *in silico* study, it consists in predicting the long-term effect of the xenobiotics using the forecasting grey model (1, N) as a predicting model. The grey system model with time power, which is often called the GM, appeals considerable interest of research due to its effectiveness in time series forecasting (Jiang *et al.,* 2022).

There are several studies using this model in various fields: in financial circles and in management, to improved manage the workers of hotels and restaurants (Joyce *et al.,* 2022); in economics and engineering, to know the planned natural gas consumption (Ding *et al.,* 2018) and the emission of carbon dioxide (Wang *et al.,* 2019). This initiated us to apply this model in biology to better understand the effects of environmental toxicants.

Our main objective was to predict the long-term effects of cadmium, fungicide and their mixtures on the morphophysiological, biochemical and enzymatic parameters of the snail *Cantareus aspersus* without resorting to animal experimentation, and draw attention to the long-term and mixture result on the living beings.

Materials and methods

Biological material

The biological material is the snail *Cantareus aspersus* (Müller, 1774). The snails used in this study are adults (8 ± 1g). In the laboratory they were adapted to the controlled conditions described by Vaufleury *et al.* (2006) (temperature 20 ± 2°C, photoperiod 18hL/6hD, relative humidity 80 to 90%) for two weeks. They divided into four (04) batches of three snails/batch (Control; batch treated by the Cd alone, batch treated by fungicide alone and batch treated by the cocktail Cd + F).

Chemical materials

The chemical materials used in our study are cadmium, in the form of cadmium dichloride CdCl2 (N° CAS 10108-64-2), fungicide and their mixtures CdCl2 + fungicide, 1/1, V/V), cited in the following table (Tab 1). The fungicide chosen, used in the commercial form VACOMIL-PLUS® from the company VAPCO, it comprises two active ingredients; metalaxyl (N° CAS 57837-19-1) in 15% and copper oxychloride in 35% (N° CAS 1332-65-6).

Table 1. Concentrations of mixtures used.

	Cadmium (µg/l)	Fungicide (g/l)
Control	0	0
M1	200	0,5
M2	400	1
M3	800	2

Methods

Experiment was conducted for 15, 30 and 90 days under previously controlled laboratory conditions. After each treatment period, three snails from each group weighted and sacrificed by decapitation. The digestive gland removed, weighted and recovered in appropriate buffer for the evaluation of the biochemical parameters (Protein, MDA, GSH and Ach E activity).

Protein analysis

Total protein levels of control and treated snails were quantified according to the method of Bradford (1976), using Blue Brilliant of Coomassie (G250, Merck) as a reagent and bovine serum albumin (BSA, Sigma) as standard protein. The absorbance was read at a wavelength of 595 nm.

Malondialdehyde analysis

MDA determination was carried out in control and treated snail digestive gland using the colorimetric method of Draper and Hadley (1990), which is based on the reaction of thiobarbituric acid with MDA. MDA levels were estimated at 532 nm. The concentration of lipid peroxidation expressed as µg of MDA per mg of proteins.

Glutathione analysis

Content of GSH in control and treated snail quantified according to Weckberger and Cory (1988). Glutathione levels estimated at 412nm and expressed as µM of glutathione per mg of proteins.

Acetylcholine esterase activity

Activity of AChE in control and treated snail digestive gland performed using a method described by Ellman *et al.*, (1961) with the use of acetylthiocholine (ASCh) as substrate. The activity rate was measured aschange in absorbance/min at 412 nm (extinction coefficient1. 36x104 M-1.cm-1). Activity expressed as nmol/min/mgprotein.

Statistical tests

Data were expressed as mean ± standard deviation (SD). All statistical calculations were performed with the MINITAB Software (Version 16, Penn State College, PA, USA). Data were tested using two-way analysis of variance (ANOVA) and Tukey' s test. A significant difference was assumed when p< 0.05.

Computer equipment

We carried out a Python language implementation of a "Grey Model" forecasting method that best suits our case study. This implementation performed on an I3 PC, running windows 10 at 4 GB of RAM.

In recent times, Python is the most fashionable programming language. It is associated with a shell available for different OS (Windows, Linux, Mac OS X, etc.). Recently developed, one of Python's strengths is its expressive richness as well as its high ability to model natural phenomena. It refers to a set of tools and libraries (Numpy, pandas, matplotlib, etc.), for manipulating and analyzing data, implementing most artificial intelligence algorithms, such as decision trees, Baysian networks and neural networks (witten *et al.*, 2000).

The GM model (1, N)

The Grey model used to predict the behavior of nonlinear time series. This is a non-statistical forecasting method, particularly effective when there are a small number of samples (Wu *et al.*, 2019) or when the number of observations (results) is reduced (Che-Chiang and Chia-Yon, 2003).

It contains a system of variable behavior. It can analyze the effect of several influence variables on the behavior of the system. System behavior variables can be predicted, knowing the changing trend of influence variables.

The modeling process is defined as:

$$x_1^{(0)} = x_1^{(0)}(1)\, x_1^{(0)}(2), \ldots\ldots\ldots\ x_1^{(0)}(n)$$

Definition 1: is assumed to be the date series of the system feature.

After determining the initial sequences, the GM (1, N) model can be implemented in three steps:

Step 1. Cumulative production operation

Where,

1. $x_2^{(0)} = x_2^{(0)}(1)\, x_2^{(0)}(2), \ldots\ldots\ldots\ x_2^{(0)}(n)$
   $x_3^{(0)} = x_3^{(0)}(1)\, x_3^{(0)}(2), \ldots\ldots\ldots\ x_3^{(0)}(n)$
   $x_N^{(0)} = x_N^{(0)}(1)\, x_N^{(0)}(2), \ldots\ldots\ldots\ x_N^{(0)}(n)$, are the date series of relevant factors,

2. $X_i^{(1)}$ is the series (i=2,3,..... ,N) generated by the accumulation of the first order $(1 - AGO)$,

3. $Z_1^{(1)}$ Represents the date series for the nearest weighlor mean of $x_1^{(1)}$ and is expressed as: $x_1^{(0)}$ which is the grey multi variable model and measured as GM (1, N).

Step 2. Determination of driving parameters.

The series of parameters $\hat{\propto} = [a, b2, \dots\dots\dots, bN]$, supposing:

a- $x^{(0)}_1 = (x^{(0)}_1(1), x^{(0)}_1(2), \dots\dots\dots, x^{(0)}_1(n))$ is the series of date series of the system features,

b- $x^{(0)}_i\ (i = 2, 3, \dots\dots\dots, N)$ is the data series of relevant factors,

c- $x^{(1)}_i$ is the 1-AGO series of $x^{(0)}_i$,

d- $Z^{(1)}_1$ is the data series of NNM of $x^{(1)}_1$

Then: the series of parameters (∝) =[a, b2,.....,bN] can be solved by Least Square Method:

$\hat{\propto} = [a, b_2, \dots\dots\dots, b_N] = (B^TB)^{-1}\, B^T_y$

Where,

$$B = \begin{Bmatrix} z^{(1)}_1(2)\ x^{(1)}_2(2), \dots\dots\dots, x^{(1)}_N(2) \\ z^{(1)}_1(3)\ x^{(1)}_2(3), \dots\dots\dots, x^{(1)}_N(3) \\ \vdots \\ z^{(1)}_1(n)\ x^{(1)}_2(n), \dots\dots\dots, x^{(1)}_N(n) \end{Bmatrix}$$

$$Y = \begin{Bmatrix} x^{(0)}_1(2) \\ x^{(0)}_1(3) \\ \vdots \\ x^{(0)}_1(n) \end{Bmatrix}$$

Supposing: $\hat{\propto} = [a, b_2, \dots\dots\dots, b_n]^T$, we obtain : $\frac{dx^{(1)}_1}{dt} + ax^{(1)}_1 = b_2\, x^{(1)}_2 + b_3\, x^{(1)}_3 + \dots\dots\dots\ b_N\, x^{(1)}_N$

Supposing: $x^{(0)}_i, x^{(1)}_i$, (i = 1, 2, ..., N), B, Y,

$\hat{\propto} = [a, b_2, \dots\dots\dots, b_n]^T = (B^TB)B^TY$

Step 3. Prediction using the cumulative reverse generation operation

$\hat{x}^{(1)}_1(k+1) = \left[x^{(1)}_1(0) - \frac{1}{a}\sum_{i=2}^{N} bi\ x^{(1)}_i(k+1)\right] e^{-ak} + [\frac{1}{a}\sum_{i=2}^{N} bi\ x^{(1)}_i(2+1)]$

Where,

$x^{(1)}_1(0)$ is set to $x^{(1)}_1(1)$ by conducting an inverse operation of cumulative generation (-1 AGO), the predicted values are obtained by the equation:

$x^{1(0)}_{(1)}(k+1) = x^{1(1)}_{(1)}(k+1) - x^{1(1)}_{(1)}(k)$

Methods

In this study, we exposed snails to different concentrations of cadmium, fungicide and their mixtures, but in the natural environment snails exposed to different pesticide mixtures throughout their life and it is therefore necessary to check whether these responses are similar (Wang and Rainbow, 2005). This initiated us to use an *in silico* model to predict the long-term effects of cadmium, fungicide and their mixtures on physiological, biochemical and enzymatic parameters of the snail *Cantareus Aspersus*. The study of the toxicity of these xenobiotics was carried out using the forecasting grey model (Liu *et al.* 2010; Vijayan and Bindu, 2017); following a history of results of the various parameters studied. We select the changes in physiological parameters, changes in total proteins and biomarkers of toxicity (GSH, MDA and AChE).

Results

Evolution of average weight of Cantareus aspersus

Figs. 1, 2 and 3 shows the predictive evolution of mean weight of control snails and treated with increasing concentrations of cadmium and/or fungicide. We see an increase in the weight of the animal in the controls, but in those treated by the different concentrations of the two xenobiotics, we notice a decrease and then a stagnation of weight compared to the controls.

Changes in mean weight of hepatopancreas

Figs. 4, 5 and 6 illustrate the predictive evolution of the mean weight of the digestive gland of control snails treated with increasing concentrations of cadmium and/or fungicide over time. We notice a decrease then a stagnation in the weight of hepatopancreas of the treated compared to the controls.

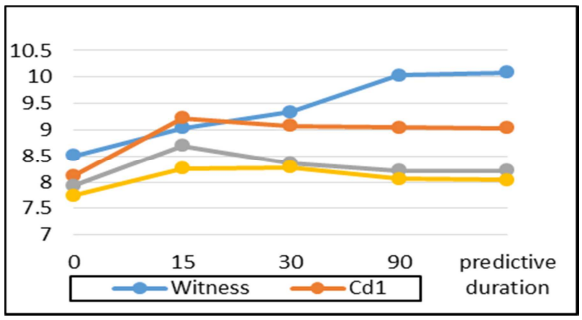

Fig. 1. Predictive effect of increasing cadmium concentrations on the evolution of Mean weight of *Cantareus aspersus* over time.

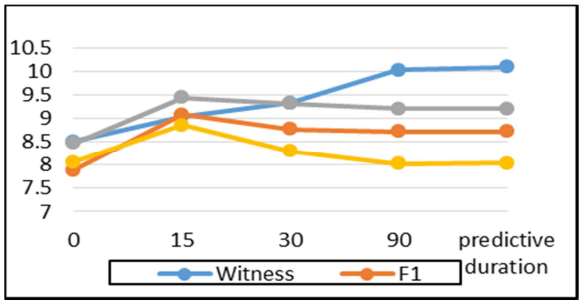

Fig. 2. Predictive effect of increasing fungicide concentrations on the evolution of Mean weight of *Cantareus aspersus* over time.

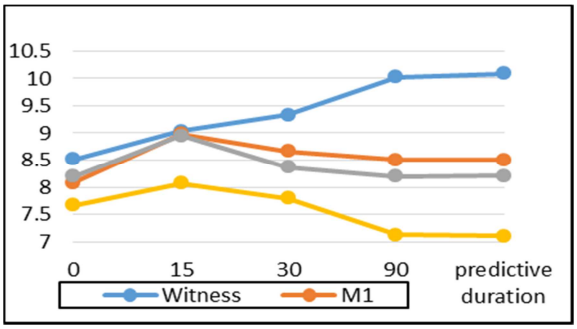

Fig. 3. Predictive effect of increasing concentrations of the cadmium/fungicide cocktail on the evolution of Mean weight of *Cantareus aspersus* over time.

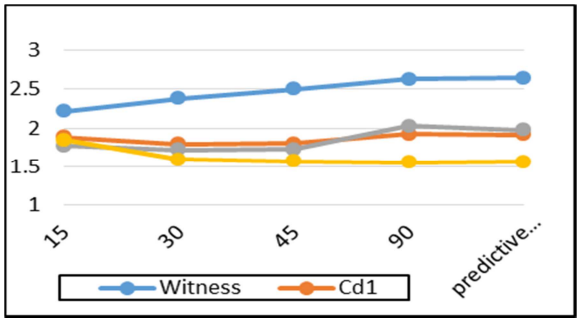

Fig. 4. Predictive effect of increasing cadmium concentrations on the change in mean weight of the digestive gland in *Cantareus aspersus* over time.

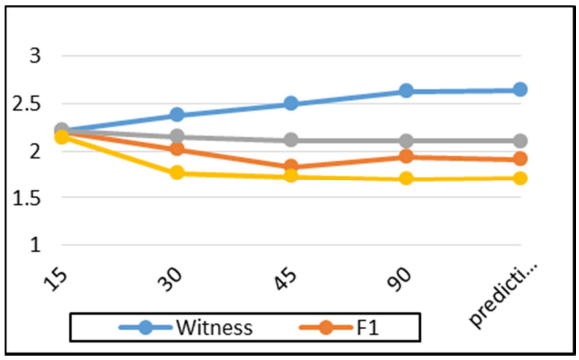

Fig. 5. Predictive effect of increasing fungicide concentrations on the evolution of mean digestive gland weight in *Cantareus aspersus* over time.

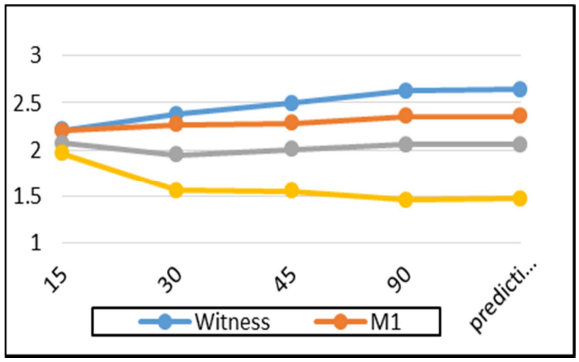

Fig. 6. Predictive effect of increasing concentrations of the cadmium/fungicide cocktail on the evolution of the mean weight of the digestive gland in Cantareus aspersus over time.

Predictive study of total protein levels

Figs. 7, 8 and 9 illustrate changes in total digestive gland protein levels. We note a decrease in this rate in those treated by the two concentrations of cadmium 200 and 400 µg /l, but an increase is noted in those treated by the higher concentration 800 µg / L.

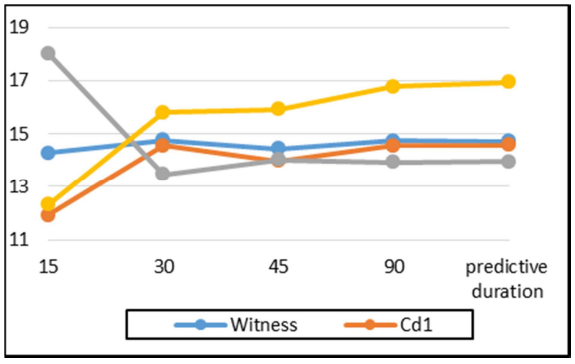

Fig. 7. Predictive effect of increasing cadmium concentrations on hepatopancreatic total proteins in *Cantareus aspersus*.

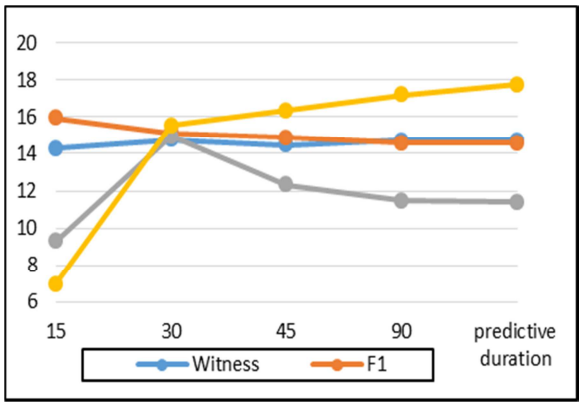

Fig. 8. Predictive effect of increasing fungicide concentrations on hepatopancreatic total protein levels in *Cantareus aspersus*.

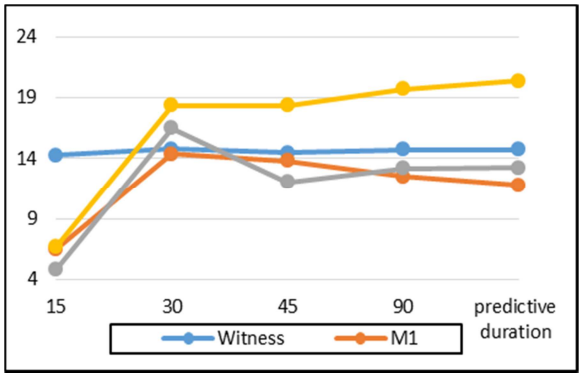

Fig. 9. Predictive effect of increasing cadmium/fungicide cocktail concentrations on hepatopancreatic total protein levels in *Cantareus aspersus*.

Also in those treated with the fungicide alone. We note a decrease in the total protein level for the 1g/l dose, and an increase in this level for the highest concentration 2G/L. The same note for the cadmium/fungicide mixture, there is an increase in this level in those treated with the highest dose M3 (800µg + 2g), and a decrease for the lowest doses M1 and M2.

The level of glutathione (GSH)

Figs. 10, 11 and 12 show the predictive variations of control snails treated with increasing concentrations of cadmium or/and fungicide on hepatopancreatic GSH levels. We noticed a decrease in GSH levels in all treated snails compared to controls. This decrease more pronounced in those treated with the mixture and specifically the mixture M2, which reaches the value of 0.259µM/mg of protein, compared to controls with a level of about 0.8319µM/mg of protein.

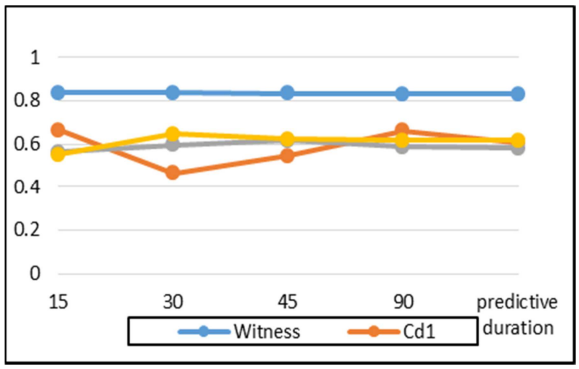

Fig. 10. Predictive effect of increasing cadmium concentrations on hepatopancreatic GSH levels in *Cantareus aspersus*.

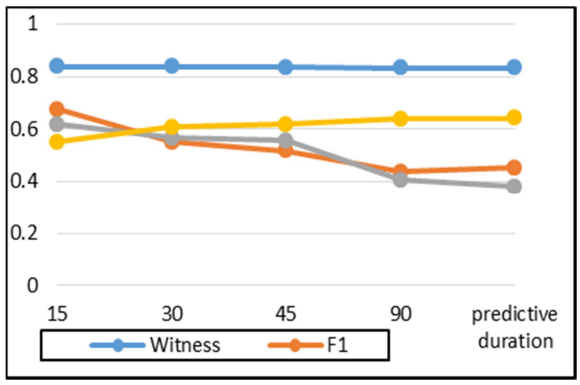

Fig. 11. Predictive effect of increasing fungicide concentrations on hepatopancreatic GSH levels in *Cantareus aspersus*.

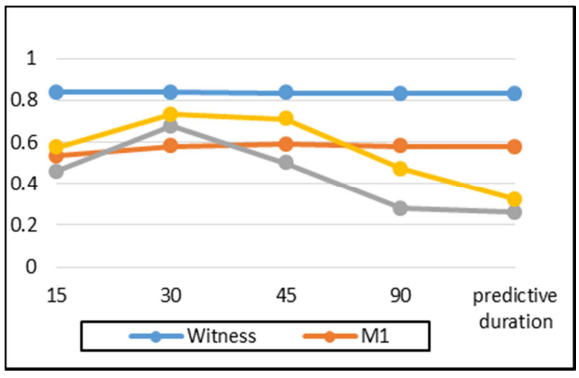

Fig. 12. Predictive effect of increasing cadmium concentrations on hepatopancreatic GSH levels in *Cantareus aspersus*.

The level of malondialdehyde (MDA)

Figs. 13, 14 and 15 illustrate predictive variations in hepatopancreatic MDA levels in control snails treated with increasing concentrations of cadmium or/and fungicide. We see an increase in this rate among all treaties compared to controls.

On the other hand, we note a slight decrease in the predictive rate of MDA in the treated by the concentrations F3 (2G), M2 (400µg + 1G) and M3 (800µg + 2G), compared to the previous treatment duration (90 days).

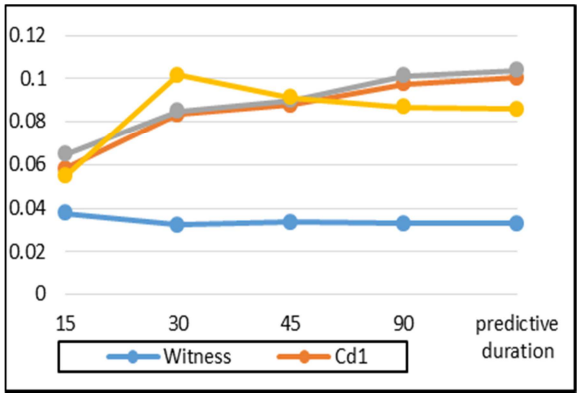

Fig. 13. Predictive effect of increasing cadmium concentrations on hepatopancreatic MDAs in *Cantareus aspersus*.

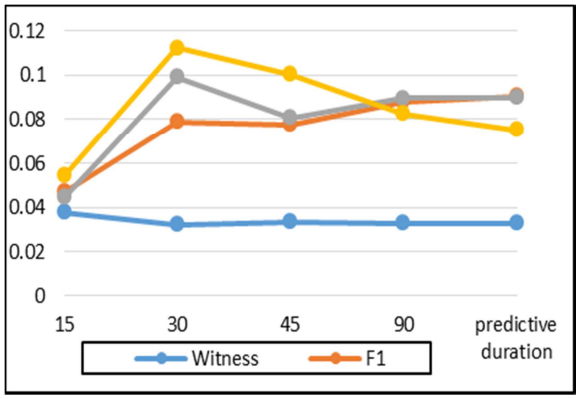

Fig. 14. Predictive effect of increasing fungicide concentrations on hepatopancreatic MDA levels in *Cantareus aspersus*.

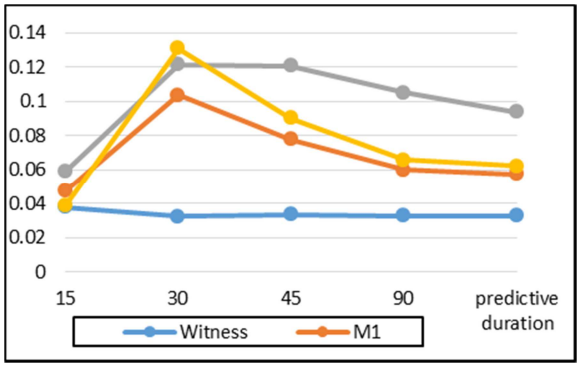

Fig. 15. predictive effect of increasing cadmium/fungicide cocktail concentrations on hepatopancreatic MDAs in *Cantareus aspersus*.

Acetylcholine esterase (AChE) activity

Figs. 16, 17 and 18 illustrate an inhibition of acetylcholine esterase activity, in all treated snails compared to controls. Similarly, we note a decrease in this activity in those treated with cadmium alone and fungicide alone, and more significant in those treated with mixtures compared to the previous treatment duration.

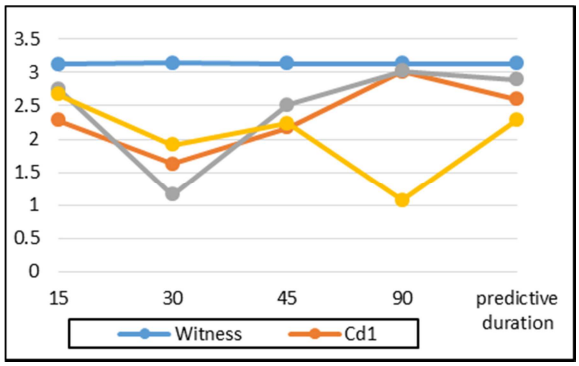

Fig. 16. Predictive effect of increasing cadmium concentrations on variations in AChE activity in *Cantareus aspersus*.

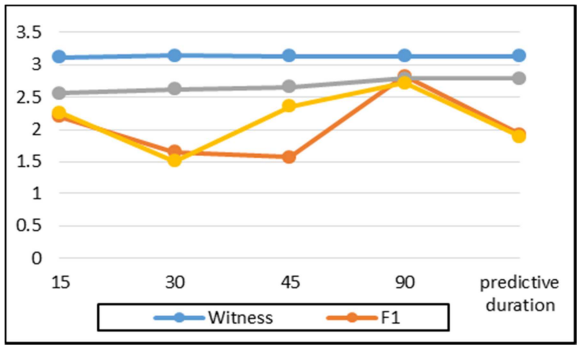

Fig. 17. Predictive effect of increasing fungicide concentrations on variations in AChE activity in *Cantareus aspersus*.

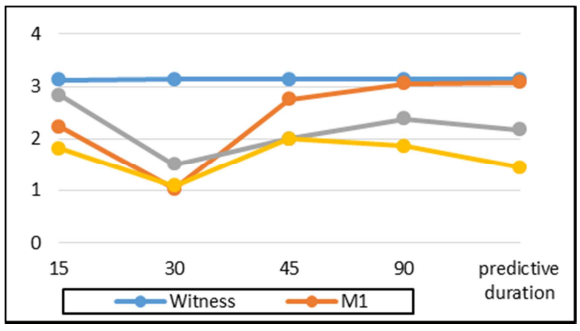

Fig. 18. Predictive effect of increasing cadmium/ fungicide cocktail concentrations on variations in AChE activity in *Cantareus aspersus*.

Discussion

Time series forecasting is an exciting area of research. One of the main objectives of applied statistics is to establish forecasting models, based on observed data, without using the same tools or methods. Case studies of logistics demand forecast indicate that the Grey model (1, N) can solve the problem of factor choice and data sample size determination with high accuracy, and can fully utilize the sample information (Hui *et al.,* 2013). In order to fill this research gap, the GM makes it possible to model with a small amount of samples and to predict the results of several parameters studied.

Currently, the prediction of nonlinear time series firmly established in various fields. In financial circles and in management, to better manage the workers of hotels and restaurants (Chen *et al.,* 2022). In economics and engineering, to know the expected natural gas consumption (Ding *et al.* 2018) and the emission of carbon dioxide (Wang *et al.,* 2019).

In biology Ren *et al.,* 2013 used the Grey model to predict biohydrogen yield under rare data conditions. They used pH, glucose and iron sulphate concentration as independent variables and biohydrogen yield as dependent variables. Their results show that the proposed method makes it possible to predict biohydrogen yield under rare data conditions and that the effect of factors influencing yield also identified. Based on the comparison with the results predicted by the artificial neural network, they could conclude that the Grey model has better predictability with rare data.

More recently, employed by Jingzheng Ren (2017), to model an anaerobic digestion system to achieve a higher methane yield. These results demonstrate that the GM model (1, N) can effectively simulate an anaerobic digestion system in cases of misinformation with less computational time. This type of proposed model has the following two most significant advantages: Users do not need to know the mechanism of the variables studied; and requires less data with better accuracy.

This opens a privileged framework for our study of modeling predictive values following a temporal history of our results of morphometric parameters or biochemical assays. From the predictive results obtained, using an implementation in Python and the GM Grey model (1, 1), it is clear that the evolution of morphometric and biochemical parameters is similar to the evolution of our results (*in vivo*).

Conclusion

Identification *in silico* of toxic effects caused by toxic products would be highly desirable, as it not only addresses the protection of human health, but also generates a variety of ecological benefits and the sustainable management of resources. *In silico* studies are generally less expensive than clinical trials.

In our work, when cadmium combined with a copper-based fungicide, our results reveal:

- ✓ Decrease in GSH levels, similar to cadmium alone and fungicide alone after 15 and 30 days of treatment, and synergistic to M2 after 90 days;
- ✓ Induction of MDA levels, similar to that of both pollutants at 15 and 30 days of exposure, and antagonist at 90 days for M1 and M3;
- ✓ Significant inhibition of AChE activity at M2 and M3, synergistic at M2 and M3 antagonist after 90 days of treatment.

By observing the results obtained through the use of the computer tool Prediction, in particular the GM Grey non-linear time series model (1, 1), we can conclude that these products (cadmium or fungicide), have more significant effects when combined in the long term.

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