biolutoxR: An R-Shiny package for easy performing data analysis of a toxicity test based on bacterial bioluminescence inhibition

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Abstract.

In the 21st century, in the context of an environmental crisis, chemical pollution has become a major global concern. In addition to chemical analysis, many bioassays have been developed and have proved to be interesting tools for considerably improve our understanding of the effects of human activities on species and ecosystems. Compared with animal or plant bioassays, bacterial bioassays based on bioluminescence inhibition have the advantage of being relatively economical and reproducible. This toxicity test uses the bioluminescent metabolic response of bacteria exposed for a short time to a solution of interest in the aim of assessing and quantifying its toxicity. Although routine tools are available to pre-process the data obtained, to our knowledge no flexible research tool is available for the data analysis following these tests, which could limit the access to the results for novices or experienced people unfamiliar with data processing software. To overcome this lack of tools for this bioassay, an R-Shiny package is proposed to generalise data analysis following a toxicity test based on bacterial bioluminescence inhibition. The traditional paper-based working environment is reproduced digitally in this package, which ultimately facilitates data entry and cleaning, makes the creation of relevant dynamic graphs, and simplifies access to toxicity data (e.g. dose-response curve and median effective concentration, i.e. EC_{50}). The aim of this tool is to provide the target community with a high-performance tool that can be used to obtain toxicity test results based on the inhibition of bacterial bioluminescence.

Code metadata.

Current Code Version	version 0.1.0
Permanent link to code / repository used for this code version	https://github.com/bbellier/biolutoxR_package
Permanent link to reproducible capsule	*NA*
Legal code license	GPL ≥ 3
Code versioning system used	Git
Software code languages, tools and services used	R
Compilation requirements, operating environments and dependencies	R (≥ 4.1.2); dependencies: bslib, DT, openxlsx, shiny, shinythemes, shinyMatrix, tidyverse
If available, link to developer documentation/manual	https://bbellier.github.io/biolutoxR_website/
Support email for questions	coralie.lepicard@yahoo.com

Keywords. Environmental pollution, Chemicals, Ecotoxicology, Bioassay

1. Motivation and significance

Six of the nine planetary limits - limits that cannot be exceeded to ensure the processes necessary to preserve the stability and resilience of the earth system - have been exceeded, including the limit for chemicals [1]. Indeed, for more than two centuries, human domestic and industrial activities have contributed to an increase in the discharge of chemical substances into aquatic environments, contributing to a deterioration in the functioning of ecosystems [2,3]. In response to the ecological emergency caused by environmental pollution, standardised methods have become essential for identifying toxic effects and, ultimately, helping to ensure the resilience of the environment [4]. Among the existing standardised methods, chemical analysis of aquatic environments appeared to be one of the most relevant, but ultimately proved to be expensive and limited, which finally complicated routine monitoring [5–7]. Thus, the use of bioassays - experimental methods used to study the toxic effect of a substance on model organisms - has proved to be complementary and less restrictive [8].

Nowadays, numerous bioassays are used, using different types of organisms: animals (e.g. mammals, fish, invertebrates), photosynthetic organisms (e.g. plants, algae) and microbiological organisms (e.g. bacteria, fungi). However, bioassays performed on animal and photosynthetic species have been confronted to some major disadvantages (costly experiments, large sample volume, long manipulation time, standardisation of the species, specific equipment, qualified operator and lack of reproducibility), which is reduced for bioassays on bacteria, offering the possibility of assessing the toxicity of a solution of interest with speed, simplicity and reproducibility [9].

Bacterial bioassays using bioluminescence inhibition assess the bioluminescent response of bacteria following exposure to a solution of interest at a given exposure time, generally a short time, i.e. 5, 15 or 30 minutes [10]. This type of test uses particularly some bacteria, among others : *Aliivibrio fisheri* (e.g. Backhaus and Grimme [11]), *Photorhabdus luminescens* (e.g. Masner et al., 2017 [8]), *Photobacterium leiognathi* (e.g. Muneeswaran et al., 2021 [12]) and *Vibrio harveyi* (e.g. Thomulka et al., 1997 [13]). Under optimum conditions, these bacteria produce bioluminescence, i.e. an enzymatic reaction catalysed by luciferase which releases light (see Supplementary materials n°1).

This bioluminescence is directly linked to the respiratory metabolic process of bacteria [14]. In other words, in a favourable environment, these bacteria emit light. However, when a bacterium is exposed to a toxic substance, this metabolic process is disrupted, resulting in the inhibition of bioluminescence. This reaction is therefore used in this type of test to study the direct link between light intensity and the level of toxicity of a sample of interest compared with a control sample. This inhibition is often calculated as follows (e.g. Si et al., 2024 [15]; Westlund et al., 2018 [16]) :

- I_{biolu} (%) = (B_{Ct} – B_{St}) / B_{Ct} * 100

Where I_{biolu} represents the inhibition of bioluminescence of the bacteria studied (in %); *t* represents the exposure time (in min); $B_{Ct,rb,rt}$ represents the average of the bioluminescence values of the bacteria exposed, during a time *t,* to the control solution; and B_{St} represents the average bioluminescence values of the bacteria exposed, during a time *t,* to the solution of interest.

The toxicity of the solution of interest is then visualised on a dose-response curve, providing a visual representation of the percentage of inhibition (in %) as a function of the log dilution (in %). This curve can be used to obtain a reference toxicity value: the EC_X, which is the effective concentration of a toxicant causing an X% reduction in light intensity of bacteria [17]. The equation for the 5-parameter dose-response curve is as follows :

- $y = c + (d-c) / (1 + \exp(b * \log(x) - \log(e)))$

Where y represents the inhibition of bioluminescence of the bacteria studied (in %); x represents dilution (in %); b represents the curvature parameter; c represents the first plate of the curve; d represents the last plate of the curve; e represents the EC_{50}.

More specifically, and in a non-exhaustive way, the toxicity test based on the bacterial bioluminescence inhibition can be performed experimentally as follows (Fig. 1): (i) creation of a matrix document to record the position of a sample on the bowl holder, for subsequent data retrieval; (ii) sample preparation: negative control, positive control, blank and substance of interest (see Table 1 for an explanation of the samples); (iii) rehydration and acclimatisation of the lyophylated bacteria; (iv) exposure of the bacteria to each sample; (v) bioluminescence measurements with a luminometer for each sample and for each exposure time (generally: 5 min, 15 min and 30 min); and (vi) fill in the matrix document, created previously, with the bioluminescence values. For statistical reasons, the experiment should include several 'biological replicates' for each sample of the substance of interest (at least 3) and several 'sets' (i.e. the whole experiment should be repeated several times). Many studies use this type of approach, resulting in the development of commercial solutions. (e.g. Microtox®, CheckLight, BioTox, see Kokkali & Van Delft, 2014 [18]).

The toxicity test based on the bacterial bioluminescence inhibition therefore produces a large amount of data in a very short time, with a high level of reproducibility. However, at the end of the test, a significant number of bioluminescence values need to be recorded and processed to obtain the final test results. Some machines can be used to produce pre-processed results, sufficient for repeated routine analysis, but offering little freedom to the user, particularly, for research work. To our knowledge, there is currently no package or application in the R environment that facilitates the analysis of data following this test. For these reasons, a Shiny-R package was created, simplifying data entry and automating data processing in an application with an interface specially designed for toxicity tests based on the bacterial bioluminescence inhibition. Using the conventional annotation format, this Shiny-R package facilitates data entry, automates data cleaning and makes results visualisation dynamic, particularly for obtaining the dose-response curve and reference toxicity values (i.e. EC_X), which are often complex and subject to errors. In the long term, this tool could be generalized/standardised for the data analysis of toxicity tests data based on bacterial bioluminescence inhibition, with the aim of increasing its reproducibility by its widespread use.

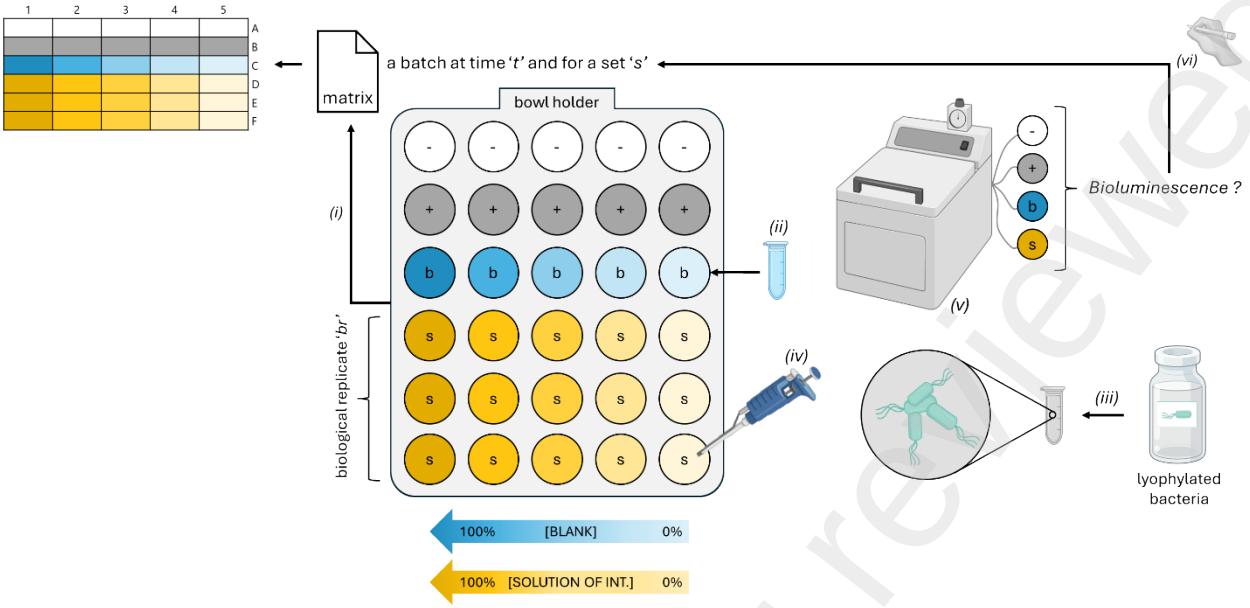

Figure 1. *Main steps toxicity tests based on bacteria bioluminescence inhibition. The negative control is represented by a '-', the positive control is represented by a '+', the blank is represented by a 'b' and the solution of interest is represented by an 's'. The steps represented by letters in brackets (i, ii, iii, iv, v, vi) are explained in the 'Motivation and significance' section.*

Table 1. *Description of samples used in toxicity tests based on bacteria bioluminescence inhibition.*

Dependencies (code used in Fig.1)	**Description**
Negative control (-)	Non-toxic sample used as a reference to show normal bacterial behaviour.
Positive control (+)	Toxic sample used to confirm correct detection and determination of toxicity by the test.
Blank (b)	Non-toxic solution that has been subjected to the same operations as the samples of the solution of interest. It provides assurance that the steps performed during manipulation do not introduce toxic biases. It can be diluted to assess its dose-response relationship.
Solution of interest (s)	Solution of interest, i.e. an ecologically/ecotoxicologically relevant solution to be analysed. The test based on inhibition of bacterial bioluminescence is used to determine its potential toxicity. It can be diluted to assess its dose-response relationship.

2. Software description

This R-Shiny package is written in the R [19] and Shiny framework [20] and hosted on a github repository (available at https://github.com/bbellier/biolutoxR_package). This Shiny App used only a few dependencies (Table 2). To use this Shiny application package, the following commands can be used:

(1) To install the *biolutoxR* package:

R> install.packages("devtools")
R> devtools::install_github("bbellier/biolutoxR_package")
R> library(biolutoxR)

(2) To run the *biolutoxR* Shiny App:

R> run.biolutoxR()

(3) To run the *biolutoxR* Shiny App example:

R> example.biolutoxR()

(4) To cite the *biolutoxR* package:

R> citation.biolutoxR()

Table 2. *biolutoxR Shiny App dependencies.*

Dependencies	Functions	CRAN Reference
bslib	Use to customize Shiny CSS styling	https://cran.r-project.org/web/packages/bslib/index.html
DT	Use to table management in Shiny	https://cran.r-project.org/web/packages/DT/index.html
openxlsx	Use to generate and read .xlsx file	https://cran.r-project.org/web/packages/openxlsx/index.html
shiny	Use to generate an R-Shiny application	https://cran.r-project.org/web/packages/shiny.exe/index.html
shinythemes	Use to customize Shiny aspect	https://cran.r-project.org/web/packages/shinythemes/index.html
shinyMatrix	Use to generate matrix in Shiny	https://cran.r-project.org/web/packages/shinyMatrix/index.html
tidyverse	Useful set of packages (e.g. ggplot package for graphical data visualization)	https://cran.r-project.org/web/packages/tidyverse/index.html

The operating principle of the *biolutoxR* package application is summarized in Figure 2. Therefore, this Shiny App is divided into different tabs that can be navigated using a navigation bar (Fig. 3). The application is therefore composed of 8 tabs: "*Presentation*", "*Scheme*", "*Time 5*", "*Time 15*", "*Time 30*", "*Table*", "*Plot*", "*Exit*" and 4 main uses: R-Shiny package presentation, bacterial bioluminescence toxicity test data input, final data table construction and final data visualization.

The "*Presentation*" tab is necessary to help users understand (1) how the bacterial bioluminescence toxicity test works and (2) the relevance and operation of the Shiny application. Indeed, the first paragraph succinctly describes the main stages of a bacterial bioluminescence toxicity test, its usefulness, and the analysis that can be made of it. The second paragraph guides the user through the mechanisms and steps in inputting data and understanding the final constructed variables.

The '*Scheme'* tab contains a matrix used to define the identifiers of the manipulation (Fig. 4). In this tab, the user must define the name of the manipulation (called '*manip_name*' in the final data table), the number of matrices required (i.e. one matrix represents one set, '*Number of matrices*'), the size of the matrices ('*Number of rows*' and '*Number of columns*', which allow to define the position of a sample in the matrix, which is given in the '*id*' variable in the final data table) and the name of the negative control ('*Name of negative control*'), which is then used to correct the bioluminescence values (called biolu in the '*Time*' tabs and in the final data table) in the final data table. Each cell is filled to position and characterise the samples in the matrix. Each cell of the matrix must contain values in this format: '*sol,dil,rep_bio,sol_sh*'; where '*sol*' corresponds to the full name of the solution (e.g. tire and road wear particles of France), '*dil*' corresponds to the dilution level (from 0% to 100%), '*rep_bio*' corresponds to the biological replicate and '*sol_sh*' corresponds to the short name of the solution (e.g. TRWP-FR).

The '*Time 1*', '*Time 2*' and '*Time 3*' tabs each contain a matrix which works in the same way (Fig. 5). For each *Time* tab, the exposure time ('*Time (in min)*') is entered by the user to be considered in the final data table. Each cell in these matrices is filled with bioluminescence data from the bioluminescence bacterial toxicity test.

The '*Table*' tab displays the final data table obtained, which can be exported in '*.csv*' or '*.xlsx*' format. All the data generated by completing the 'Sheme' and 'Time' matrices is included in this final data table: '*manip_name'*, '*set*', '*time*', '*id*', '*sol*', '*sol_sh*', '*dil*', '*rep_bio*', '*biolu*', '*biolu_mean_neg_control*', '*biolu_corr*', '*perc_inhib*', '*perc_inhib_corr*'). The final value used to display the results is '*perc_inhib_corr*' (i.e. corrected inhibition percentage, i.e. bioluminescence inhibition corrected values), which requires the calculation of '*biolu*' (i.e. bioluminescence measured values), '*biolu_mean_neg_control*' (i.e. mean of bioluminescence measured values for the negative control sample), '*biolu_corr*' (i.e. bioluminescence corrected values) and '*perc_inhib*' (i.e. bioluminescence inhibition calculated values).

The '*Plot*' tab provides a simple and dynamic way of viewing and modifying plots and generating standardised toxicity plots/data (i.e. the dose-response curve and EC_X). Firstly, in '*Final data plot*' part, the user can modify the visualisation of the results as desired (choice of x variable, y variable, time, solutions, dilutions and colour variables). Secondly, in '*Dose-response curve*' part, the dose-response curve is plotted (by setting the the *c* parameter to 0 and *d* parameter to 100), using the appropriate dilution(s) and substance

of interest. Furthermore, the equation of the dose-response curve, the significance of the parameters (b, i.e. the curvature parameter and e, i.e. the EC_{50}) and the Pearson's linear coefficient of determination (R^2, i.e. measure to assess the efficiency of a linear regression model; an R^2 of 1 indicates a robust model) are calculated. Also, any desired EC_X (with its standard deviation and 95% confidence interval) could be calculated and printed.

The "*Exit*" tab serves to quit the Shiny App.

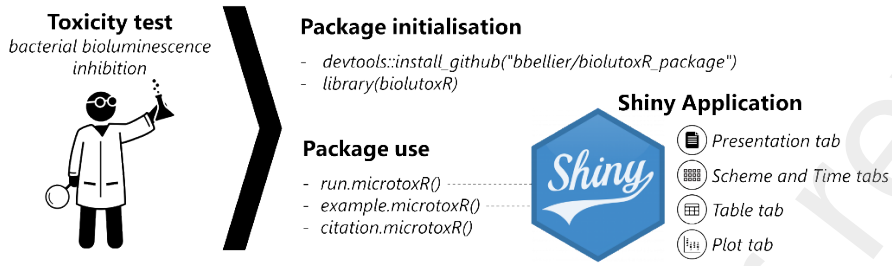

Figure 2. *biolutoxR package structure with its main functionalities.*

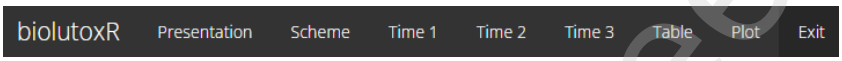

Figure 3. *biolutoxR Shiny app navigation bar.*

Figure 4. *biolutoxR app Shiny "Scheme" tab for an 5x6 matrix.*

Figure 5. *biolutoxR app Shiny "Time" tab for an 5x6 matrix.*

3. Illustrative example

The example used is not based on actual data. This example can be found in the function: *example.biolutoxR()*.

In a geographical area, new agricultural and industrial practices were introduced close to a river (Fig. 6). Ecological disasters followed rapidly afterwards, with fish species seeing their populations collapse.

Thus, local authorities have called on the scientific community to study and understand this phenomenon. To understand the toxic effect of new molecules used in agriculture and industry close to the river, scientists proposed the use of a bioassay: the toxicity test based on bacterial bioluminescence inhibition; using samples of the new agrochemicals and industrial effluent at various relevant concentrations (Fig. 6).

Following the test, the bacterial bioluminescence values were recovered and entered into the Shiny application offered by the *biolutoxR* package. A cleaned final table was exported with the test results (Fig. 7) and a graph was obtained to analyse the global situation of this locality (Fig. 8). The graph showed that even at low dilution, the percentage inhibition of bioluminescence indicates high toxicity for both effluents. The scientists then focused on substance B (i.e. industrial effluent), which appears to be more toxic, to obtain its EC_{50} value from its dose-response curve. An EC_{50} of 12.33% (with a standard deviation of 0.86 and a 95% confidence interval of 9.58-15.08) was obtained for the industrial effluent. In other words, the concentration required to obtain a 50% response in exposed organisms compared with the negative control was 12.33% (Fig. 9). Furthermore, the value of the R^2 (0.99) and the significance of the EC_{50} parameter (< 0.05) can be verified to ensure the robustness of the EC_X value obtained.

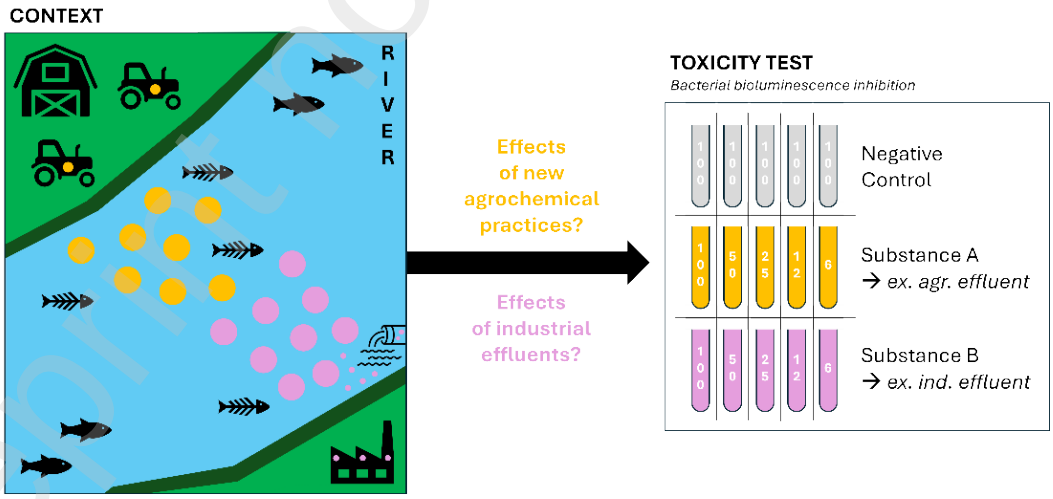

Figure 6. *Context of the example study.*

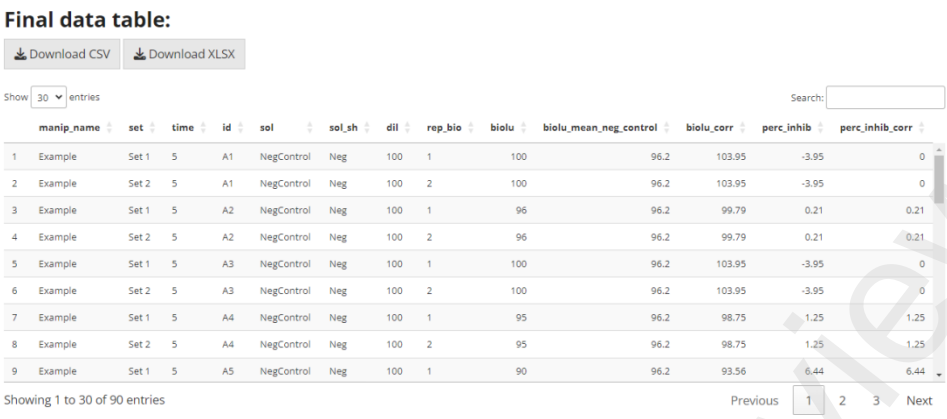

Figure 7. *Screenshot of the final data table.*

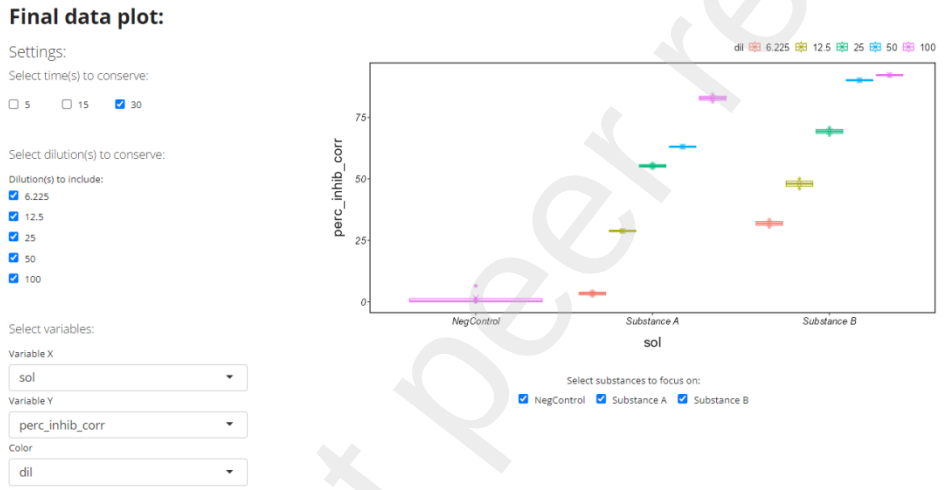

Figure 8. *Screenshot of the simple and dynamic plot proposed by the application.*

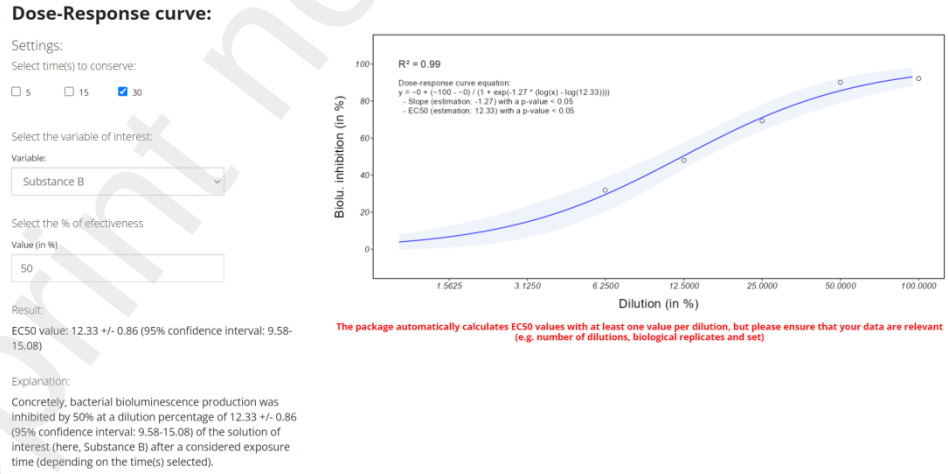

Figure 9. *Screenshot of the dose-response curve and EC_X results proposed by the application.*

Further information is available on the website dedicated to the *biolutoxR* package (available at https://bbellier.github.io/biolutoxR_website/).

4. Impact

The *biolutoxR* package simplify data analysis of toxicity tests based on bacterial bioluminescence inhibition for beginners and experimented users. Indeed, an introduction to this type of test is included. Furthermore, this package providing an automated, simple and dynamic tool to enter, recover and visualize results. In addition to providing an example, an introduction to the application enables users to quickly understand its functioning. Widespread use of this tool, particularly through the construction of a dose-response curve and EC_X calculations, will reduce the time allocated to data cleaning and calculations, increase the reproducibility and comparability of data and facilitate access to ecotoxicological data.

The *biolutoxR* package can be used for all environmental compartments (air, sediment, freshwater and marine water) and applications can vary from the assessment of the toxicity of a single molecule (e.g. N-(1,3-dimethylbutyl)-N'-phenyl-p-phenylenediamine), of a mixture of molecules (e.g. tire and road wear particles) or of a matrix (e.g. stormwater runoff). In this way, this tool can contribute to improving our knowledge of toxicity mechanisms, particularly by supplementing the ecotoxicity databases available with toxicity values (EC_X), which are currently still very limited in terms of quality and quantity [21].

5. Conclusion

Overall, the *biolutoxR* package facilitates data analysis (data cleaning, results plotting, toxicity data accessing) while retaining the use of the classic matrix format for a toxicity test based on bacterial bioluminescence inhibition. More concretely, the advantages of this toxicity test (speed, reproducibility and low cost) ensure the relevance of this tool for improving ecotoxicological knowledge for a wide range of applications. To our knowledge, this R-Shiny application represents the first initiative to generalize flexible data analysis for these bioassays, improving the reproducibility and comparability of the data recovered following these tests.

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Credit authorship contribution statement: *Coralie Le Picard*: Conceptualization, Writing & Editing. *Benjamin Bellier*: Conceptualization, Writing & Editing.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability. The package is accessible on the github of Bellier Benjamin (https://github.com/bbellier/biolutoxR_package) and on the website dedicated to the *biolutoxR* package (https://bbellier.github.io/biolutoxR_website/).

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Supplementary materials.

Supplementary materials n°1.

General reaction equation of bacterial bioluminescence:

- $CH_3(CH_2)_nCHO + FMNH_2 + O_2$ — luciferase → $CH_3(CH_2)_nCOOH + FMN + H_2O + hv$

In other terms:

- Long-chain aldehydes + reduced flavin mononucleotide + molecular oxygen — luciferase → long-chain acids + oxidized flavin mononucleotide + water + light emission