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Applicability of Miniscale Algal Growth Inhibition Bioassay using Microtitration in the Central Moroccan Laboratory (ONEE)

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Abstract

The standard algal growth inhibition bioassay using Erlenmeyer flask is one of most chronic toxicity tests demanding infrastructure. Thus, limiting monitoring efficiency, especially with large number of samples. The miniaturized algal bioassay using 96-well microplates may be used as substitute to flask bioassay. For validation, this study reported on the comparison of two algal growth inhibition bioassays performed in conventional 250 mL Erlenmeyer flasks and miniaturized 96-well microplates using eight chemical substances. The results of regression analysis revealed a significant concordance with good coefficient of determination (r^2 = 0.99, p <0.05). The repeatability criteria of microplate bioassay using ($ZnSO_4 \cdot 7H_2O$), and ($K_2Cr_2O_7$) reflected a better precision with lower variation coefficients (V.C: 3.3% and 10.13%) as compared to flask bioassay, where V.C were 10.13% and 17.7%, respectively. The application of such mini-format as a valid alternative to conventional algal growth inhibition bioassay is promising tool for pollution control programs.

1. Introduction

Office National de l'Electricité et de l'Eau Potable (ONEE) is the producer and/or distributor of potable water in Morocco. The negative impact of urban and industrial rejects on aquatic resources in Morocco pushes ONEE to try and control the levels of such pollution in water [1]. To be able to do so, this instance has implemented all analytical methods for pollution controls (e.g., physicochemical method). However, this classical assessment has some limits residing in their immediate screening. In fact, such a tool is insufficient to explain the global potential toxicity in complex mixtures [2]. The conventional physicochemical approach presents a lack of information about the identification of all pollutants in complex mixtures, and detection of bioavailability, synergistic, additive or antagonistic of their combined effects on living organisms in aquatic environments [3]. The use of ecotoxicological endpoint is a supplementary approach that completes the shortcoming of conventional methods. Currently, many bioassays are used in the central laboratory of ONEE. Some of these tools have recently been adopted by the Institut Marocain de Normalisation (IMANOR), including algal growth inhibition test using *Pseudokirchneriella subcapitata*, *Daphnia magn*a acute test, *Daphnia magna* long term test and Zebrafish acute test [4-7].

Given the importance of phytoplankton in aquatic ecosystems as dominant primary producers [8], algal growth inhibition bioassay was adapted for a widespread use in routine aquatic ecotoxicity testing as early as 1910 [9]. The benefit of using the common freshwater green microalgae *Pseudokirchneriella subcapitata* is its structural simplicity, ubiquity, ease of culture, short response time, high levels of standardization and sensitivity to spectrum of contaminants [10-14]. Furthermore, the algal toxicity test is rapid, simple, and relatively inexpensive as compared to other bioassays using fish or invertebrates [15].

However, the algal growth inhibition bioassay using Erlenmeyer flasks in ONEE laboratory is logistically intensive, space and time consuming, and demands large volume sampling. The classical algal growth inhibition bioassay needs professional skill and expertise. Additionally, the manual quantification leads

to some uncertainty due to user errors as cited by Davey [16]. The efficiency of screening is seriously minimized, especially when considering application in routine.

Recognizing these disadvantages, several increasing scientific attempts were carried out to simplify and minimize the traditional algal growth inhibition bioassay in mini-versions using cuvettes, scintillation tubes, or immunological microplates [17-21]. By analogy with other testing platforms, the use of a miniaturized microplate provides a simple alternate to algal standard test. The significant benefits of using microplates include: small sample volume, incubator space economy, good replication, and high sample throughout [22], which makes of it a standard biological test adopted by Environment Canada [23]. Algal growth inhibition bioassay using microplate is widely used for different applications, such as species sensibility distribution analyses (SSD), herbicides risk assessments as well as the good performance for pharmaceutical compounds and their binary mixtures [24-26].

The aims of this work were to establish a quick and economic substitute to conventional algal growth inhibition bioassay for routine screening programs in the ONEE laboratory. To achieve this goal, a comparative study against of the common standard Erlenmeyer flasks and the miniaturized algal growth inhibition bioassay using microtitration was carried out using seven heavy reference metals and one organic substance (phenol). The verification of the applicability of microplate algal growth inhibition bioassay was evaluated by an exercise of repeatability with potassium dichromate ($K_2Cr_2O_7$), and zinc sulfate ($ZnSO_4 \bullet 7H_2O$).

2. Material and Methods

2.1. Tested compounds

Seven metal references: zinc sulfate ($ZnSO_4 \bullet 7H_2O$), potassium dichromate ($K_2Cr_2O_7$), copper sulfate ($CuSO_4 \bullet 5H_2O$), cobalt chloride ($CoCl_2 \bullet 6H_2O$), manganese chloride ($MnCl_2 \bullet 4H_2O$), iron chloride ($FeCl_3 \bullet 6H_2O$), mercury chloride ($HgCl_2$), and one organic substance (phenol) were used in the comparative study of algal growth inhibition bioassays using flasks and microplates. The stock solutions were prepared with distilled water for a final concentration of 100 mg / L and stored in darkness at 4°C [4].

2.2. Test organism and culture preparation

The green microalgae *Pseudokirchneriella subcapitata* was isolated in 1959 from the Nitelva River (Akershus county, Norway) and preserved at the Norwegian Institute of Water Research (NIVA). This green microalgae was initially obtained from University of Toronto Culture Collection (UTCC) and subsequently pre-cultured in aseptic conditions into 50 mL of Environment Canada medium [11]. The cultures were conducted in controlled room conditions under continuous fluorescent illumination "cool white" having a standard intensity of 4500-6000 lux at the surface, at constant temperature (24 ± 2°C), and manual swirling (twice a day).). The pH value, light intensity and temperature degree were monitored at the beginning and at the end.

The cultures used for biotesting were prepared according to recommendation of Environnemental Canada protocol [11]. An inocula of an initial cell concentration of 10 000 (cells/mL) and 100 mL of culture medium were combinated in 250 mL Erlenmeyer flasks. The cells were incubated under the conditions described above after approximately four days (exponential phase).

2.3. Conventional flask bioassay

The conventional algal growth inhibition bioassay was carried out in accordance of United States Environmental Protection Agency [11]. Inoculum of *Pseudokirchneriella subcapitata* taken from exponential phase was exposed to serial concentrations of chemical substances in 250 mL glass Erlenmeyer flasks with 10 000 (cells/mL) of algal concentrations and a final volume of 100 mL. The used concentrations were : (0.01, 0.02, 0.04, 0.06, 0.08, 0.16 mg/L) for zinc sulfate and copper sulfate, (0.1, 0.2, 0.4, 0.6, 0.8, 1.6 mg/L) for potassium dichromate, mercury chloride, and cobalt chloride, (1, 2, 4, 6, 8, 10 mg/L) for iron chloride and manganese chloride, and (10, 20, 40, 60, 80 mg/L) for phenol. The tests were performed in triplicates for each treatment and control. The inoculated flask was incubated under 24-h uninterrupted fluorescent illumination (cool-white) having 4500-6000 Lux at the surface and manual shaking (twice daily) at (24 ± 2 °C) for a period of three days (72 h). The average of growth inhibition rate was quantified by direct counting using an optical microscope and a Hemocytometer (cell of Malassez). pH value, light intensity and temperature degree were monitored at the beginning and at the end of tests in all control groups.

2.4. Microplate Bioassay

Algal growth inhibition bioassay using microplate was carried in accordance to Environnemental Canada protocol [11]. Ranges of serial dilutions were prepared by combining an appropriate volume of chemical components and distilled water using test tubes with final volume of 10 mL. For each substance, the

concentrations were the same used in classical Erlenmeyer flasks. The algal growth inhibition bioassay was performed in polystyrene 96-well microplates. Per tip, a 200 µL of test solution, 10 µL of nutrient and 10µL of algal suspension were introduced by multichannel pipet. The configuration of microplate was kept according to Saint Laurent et al. [27] with five replicates for each treatment (rows B, C, E, F, G…) and ten control groups (row D). After filing, the microplates were sealed by parafilm to minimize the evaporation of well contents. The tests were run under the same experimental conditions of the classical tests. After 72 hours, algal biomass was firstly suspended by rapid drawing using a micropipet. The growth inhibition was recorded by measuring absorbance at 450 nm using microplate reader (ELx800).

2.5. Validity criteria

According to Environment Canada standard [11], variation coefficient (V.C) in control groups must be generally less than 20%. The algal cell density in control groups must increase by a factor of more than 16 after 72 h.

3. Statistics

The EC_{50}-72h values (Effective Median Concentration) and their confidence limits were determined by the Hill model using REGTOX, a macro software for Microsoft Excel [28].

Chronic toxicity values (EC_{50}-72h) obtained from both algal growth inhibition bioassays were compared through linear regression analyses. Statistical significance was established at $p < 0.05$ using software statistical program, (STATISTICA version 6 for Windows, Statsoft, Tulsa, OK, USA).

4. Results and discussion

4.1. Applicability of miniaturized algal growth inhibition bioassay using microtitration

Toxicity results (EC_{50}-72h) obtained from the biotesting of seven reference substances and one organic compound using flask and microplate algal growth inhibition bioassays are presented in Table 1. In summary, the toxicity responses followed the same profile and supported the decreasing order: Zn> Cu> Hg> Co> Cr> Fe> Mn> phenol, where zinc was the most toxicant compound against microalgae. Additionally, analyzing of EC_{50}-72h data derived from two approaches showed apparent consistency (the average ratio of microplates EC50 to flasks EC50 values was about 1.25) with lesser microplates sensitivity reported by weakly higher EC_{50}-72h values as compared with those resulted from conventional flask bioassays. Regarding linear regression analysis of EC_{50}-72h of all compounds tested (Figure 1), a good inter-method correlation between the microplates and common flask bioassays with significant coefficient of determination ($r^2 = 0.99$, $p < 0.05$) was found. Overall, these results demonstrated the applicability of microplate growth inhibition bioassay.

The slightly lesser sensitivity detected in microplate algal growth inhibition bioassay met the findings of previous researchers [27]. For example, Pavlic and coworkers [29] proved lower toxicity responses in microplate than flask bioassays in comparative sensitivity study of three green algae species to herbicides [29]. Many explications were offered for this phenomenon. Blaise et al [18] linked this sensitivity to metal adhesion on polystyrene walls of microplate wells. Furthermore, Rojickova et al [19] attributed this to the total ratio volume to surface area of microplate test solution as compared to flask and tube assays using chemicals and real samples. In addition, Paixao et al [20] explained the EC_{50} deviation in microplate photometric method at the beginning that allows sensitive measurement of algal growth. Finally, Stratton and Giles [30] demonstrated the importance of availability of total atrazine to interact with individual algal cells and invertebrates in higher bioassay volumes. In our study, the total less biodisponibility of toxicants due to ratio volume to surface area seems to as the most adequate explication of this slight loss of sensitivity in algal microplate bioassay.

Table 1: EC_{50}-72h and their 95% confidence intervals (CIs) for flask standard and microplate growth inhibition bioassays

Chemicals	Flask bioassays		Microplate bioassays	
	EC_{50} (mg/L)	CI (95%)	EC_{50} (mg/L)	CI (95%)
$ZnSO_4•7H_2O$	0.067	0.039- 0.095	0.062	0.049-0.083
$K_2Cr_2O_7$	1.011	0.952-1.194	1.191	0.736-1.321
$CoCl_2•6H_2O$	0.441	0.293- 0.631	0.530	0.389-0.691
$CuSO_4•5H_2O$	0.105	0.086- 0.141	0.143	0.121-0.167
$MnCl_2•4H_2O$	6.447	5.688- 6.950	10.595	9.031-15.391
$FeCl_3•6H_2O$	5.662	5.061- 6.226	8.611	3.916-10.593
$HgCl_2$	0.426	0.378- 0.921	0.698	0.571-0.846
phenol	37.263	22.306- 42.252	61.816	50.267-73.213

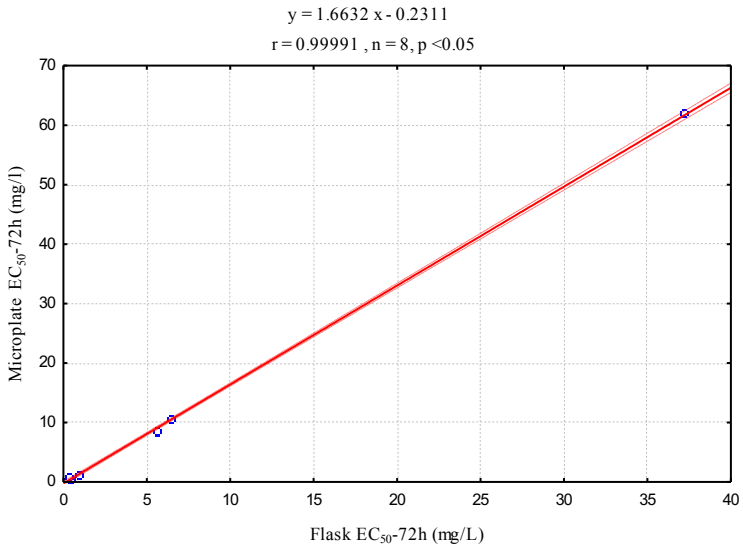

Figure 1: Comparison of the microplate and standard flask bioassays based on linear regression of the EC50-72h values for eight substances

The outcome of comparison based on regression analyses rejoined other worldwide inter-procedural investigations [19, 20, 27, 29, 31-34]. According to Paixao et al [20], comparison of EC_{50}-72h data pairs from 96-well microplate and standard flask bioassays with *Pseudokirchneriella subcapitata* using five reference chemicals and six wastewater samples illustrated good correlation. The coefficient of determinations in their study were (r^2 = 0.975, p <0.0017) and (r^2 = 0.984, p <0.0001), respectively. The performance of microtiter plates was also demonstrated by Rojickova et al [19] using eleven chemicals with coefficient of correlation (r_s = 0.991, p <0.0017). Therefore, accordance between conventional flask and microplate algal growth inhibition bioassays was showed by Pavlic et al [29] (correlation coefficient, r = 0.9956, p <0.05). The applicability of algal growth inhibition microplate bioassay was proved using other additional riverine periphyton algal species including diatoms and cyanobacteria [24].

4.2. Repeatability criteria

For further validation, an exercise of repeatability was evaluated by testing zinc sulfate ($ZnSO_4.7H_2O$), and potassium dichromate ($K_2Cr_2O_7$). Overall, the EC_{50}-72h of three experiments with three replicates demonstrated better tests repeatability precision tests in microplates (Table 2). The percentage of coefficient variation (C.V) reached only 3.3% and 10.13% for zinc and chromium, respectively, while were inferior as observed with standard flask bioassays (8.2% and 17.7%, respectively). The significant test precision in microplate chambers may be linked to small volume requirements, uniform illumination, and automated reading system. These results were in agreement within those obtained by Paixao et al [19] that also detected algal microplate bioassay repeatability criteria. In their study, the percentage of (C.V) in microplate bioassays was 9.2% and still lower than the coefficient of variation in algal flask bioassays (25.4%) using *Pseudokirchneriella subcapitata* and potassium dichromate ($K_2Cr_2O_7$). The precision of a microplate test was evaluated by other taxonomical algae groups. As mentioned by Eisentraeger et al [33], the repeatability characteristic of microplate was (9%) against flask bioassays (16.8%) using *Desmodesmus subspicatus*. For comparative purposes, Table 2 lists the results of repeatability of microplate algal growth inhibition bioassays derived from different studies in term of mean of EC_{50} and their coefficients of variation (C.V).

The data provided in (Table 2) showed that our results and their confidence limits overlap the results mentioned by many authors [20]. Nevertheless, a deviation against other literature data was observed. Our results of testing $CuSO4•5H_2O$ in standard bioassays (EC_{50}-72h: 0.10 mg/L) came out twice inferior as compared to results outlined in Rojickova et al [19] (EC_{50}-72h: 0.36 mg/L). This difference was probably imputed to specific parameters, such as photoperiod, light irradiance, temperature, culture composition, and lower inoculum algal density [35].

The miniaturized algal growth inhibition bioassay using microtitration conferred other benefits, especially in reducing laboratory resources, handling rapidity, economy and space-saving, flexibility of reading, and extensive replication with small variation and good replicability. However, the polystyrene composition of microplate can trend to favor adsorption of toxicants by wells. In response to this drawback, variable solutions were suggested by different authors.

Table 2: Repeatability criteria reported in literature in term of EC_{50} and their variation coefficients (C.V)

Chemicals	Flask bioassays			Microplate bioassays			References
	n	EC_{50}(mg/L)	CV (%)	EC_{50} (mg/L)	CV (%)	n	
$K_2Cr_2O_7$	3	1.26	25.4	1.06	9	3	[20]
	3	0.73 [b]	16.8	0.73	9	3	[33]
	3	0.84	17.7	0.92	10.13	3	Our study
$ZnSO_4•7H_2O$	3	0.061	8.2	0.076	3.3	3	Our study
Dichlorophenol	-	-	-	3.38	38	9	[24]
	2	10.85	1.95	11.4	3.72	2	[33]
phenol	-	-	-	69.7 [b]	34.9	-	[31]
$CdCl_2$	-	-	-	56 [ab]	24.3	-	[31]
$AlCl_3$	2	7.75	20	14.05	3.5	2	[33]
Formaaldehyde	2	5.75	21	7.66	4.6	2	[33]
Metribuzin	-	22.5 [a]	10	37[a]	5.4	-	[29]
Alachlor		12[a]	16	15[a]	16		
Isoproturon		41[a]	16.8	47[a]	6.4		

[a] EC_{50} values are given in (µg / L).
[b] Median Effective Concentration for 96 hours (EC_{50}-96h).
n: numbers of experiments.

Radetski et al [36] proposed a semistatic microplate bioassay with daily renewal solution to require constant toxicants and experimental conditions. Therefore, Arensberg et al [37] demonstrated the toxicity effect of microplate tissues on algal culture. For this end, a harmonization of test protocol such as sterilization by UV radiation has been suggested [19, 20]. In contrast, microscopical enumeration elaborated by Eisentraeger et al [33] gave comparable growth curves in microplate and confirmed no effect of growth which warned with results observed by Arensberg et al [37] and discussed by Rojickova et al [19]. The volatility criterion of organic substances is another principal concern that became more important in smaller surface ratio like plate formats. In our study, biotesting of phenol in microplate gave a lower sensitivity as comapared with flask bioassay. The 72h-EC_{50} (61.81 mg/L) in microplate was twice superior than the 72h-EC_{50} (37.26 mg/L) in flask assay. This may be due to the hight volatility of phenol. Eisentraeger et al, demonstrated that the volatility characteristics of organic substances generate an increase of adjacent well variation effects [33], thus conducting to significant difference in control wells [31]. The tube assays may be suitable for testing samples containing volatile substances as recommended by Rojickova et al [34].
Generally, algal growth inhibition bioassay using microtitration showed a high acceptance and recognition by international organizations and scientific communities. This approach appears to be an attractive choice to address an extensive scale of screening programs. However, this chronic bioassay is time consuming (three days). For this reason, more endpoints were explored to optimize the time factor. Recently, algal growth inhibition bioassay using microplate was optimized to shorter temporal exposure (few hours) based on the inhibition of esterase activity of green alga *Pseudokirchneriella subcapitata* [38]. These biomarkers provide early diagnostic tools especially for a high sample throughout ecotoxicity screening.

Conclusion

The results of EC_{50}-72h obtained from ecotoxicity biotesting of eight reference substances using microplate algal growth inhibition tests were generally comparable with those obtained from standard algal growth inhibition bioassays using Erlenmeyer flasks with lesser sensitivity in microplates reported by weakly EC_{50}-72h. Moreover, the good repeatability that was demonstrated in microplate tests suggested the feasibility of this miniaturized method as a tool in routine screenings in the central ONEE laboratory. This alternate approach offers some advantages over classical growth inhibition test in reduction of volume handling (53%), minimization of laboratory resources (21 Erlenmeyer's/ 96 wells), economization of incubator space (94%), and to prohibit an extensive replication (five replicates/ concentrations) with potential reading automation (73%).

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