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Microbial Population Inhibition Method Through Spectrophotometry Absorption of Visible Light Applied to Ecotoxicological Analyses

Pedro Henrique Mainardi (Corresponding author)

São Paulo State University Júlio de Mesquita Filho (UNESP)

Inst. de Biociências/Dept. de Biologia Geral e Aplicada, Rio Claro, São Paulo, Brasil

Tel: 55-193-526-4192 E-mail: pedro.h.mainardi@unesp.br

Ederio Dino Bidoia

São Paulo State University Júlio de Mesquita Filho (UNESP)

Inst. de Biociências/Dept. de Biologia Geral e Aplicada, Rio Claro, São Paulo, Brasil

Tel: 55-193-526-4191 E-mail: ederio.bidoia@unesp.br

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Abstract

Ecotoxicology is a science that studies the effects of pollutants and forecast their transformations on the environment. Ecotoxicological studies have been used in soil and water quality assessment, development and implementation of new techniques of water and effluent treatment, tools for better industrial management, bioremediation techniques and sustainable agriculture approaches. Microorganisms, which were seen to detain a fundamental importance in nutrient cycling and energy flow, have been increasingly used as bioindicators in ecotoxicological analyses. The populational inhibition of microbiological strains may be measured through the absorbance of visible light, an efficient, fast, low-cost and reliable method that has been widely used in qualitative and quantitative analysis. In this manuscript, a real textile effluent sample was analyzed regarding its electric conductivity, pH, turbidity, solids, alkalinity, biochemical oxygen demand (BOD_5), chemical oxygen demand (COD) and a spectrophotometry microbial population inhibition (MPI) method using the *Bacillus subtilis* bacteria and the *Saccharomyces cerevisiae* yeast. The EC_{20}, EC_{50} and acute

toxicity indexes were satisfactory in relation to the widely used method of light reduction of the *Vibrio fischeri* luminescence bacteria. The MPI was shown to be a feasible method to determine the hazardous effects caused by the textile effluent sample towards the microbial populations.

Keywords: Environmental assessment, Ecotoxicological method, Ecotoxicological index, Microbial ecotoxicology, Biosensors, Residue management

1. Introduction

Ecotoxicology is a science that studies the effects of pollutants and predicts their routes on the environment through the use of bioindicators and biomarkers that informs the status of one or more species in the ecosystems (Moriarty, 1988; Levin et al., 1989; Hoffman et al., 2002). Ecotoxicological analyses, as well as physicochemical and microbiological ones, were seen to be extremely important in the assessment of soil and water quality (Brettschneider et al., 2019; Kim et al., 2019; González-Pleiter et al., 2019). They were seen to be crucial in the development and implementation of new techniques of water and effluent treatment, and also in the development of tools for better industrial management practices (Correia, 1994; Wang, 2002; Zhang et al., 2012; Liang et al., 2018; Slaveykova et al., 2019). Ecotoxicological analysis have also been used in the development of new bioremediation techniques, detoxification methods and sustainable agriculture approaches (Pascoli et al., 2019; Phulpoto et al., 2018; Aparicio et al., 2019; Kumar et al., 2020).

Microorganisms, which were seen to detain a fundamental importance in nutrient cycling and energy flow, have been increasingly used as bioindicators in ecotoxicological analyzes (Babich et al., 1980; Gu, 2019). Methods that use microorganisms as indicators were seen to be generally simple and rapid to be performed (la Farré et al., 2001). Reports indicated that ecotoxicological analysis that use microorganisms also have required a modest sample volume (Blaise, 1991). Moreover, the use of microbial indicators has not faced ethical dilemmas and provided reproducible responses (Parvez et al., 2006).

The luminescent bacteria *V. fisheri* method, e.g., has been successfully applied in the determination of the ecotoxicity of different compounds, including industrial effluents (Al-Mutairi, 2006; Abbas et al., 2018). The method has been done through the measurement of the reduction of the light emitted by the microorganism in the presence of a sample for a certain exposure time. Microorganisms of the genus *Bacillus*, which are gram-positive bacteria, and yeasts of the genus *Saccharomyces*, have also been successfully applied in ecotoxicological analyses of different compounds. According to reports, a *Bacillus* specie was successfully used to evaluate the environmental toxicity of different sediments extracts (Dutka and Gorrie, 1989). *B. subtilis* strains were also effectively used to investigate contaminated soil and sediments samples (Horváth et al., 1997; Kahru et al., 2005). Studies also indicated *S. cerevisiae* strains that were successfully applied in the assessment of the toxic effect of nanoparticles and industrial wastewaters (Kasemets et al., 2009; Giorgetti et al., 2011).

The populational variation of microbial strains, indeed, may be measured through the use of

the spectrophotometric method of UV-VIS absorbance (Reller et al., 2009). Spectrophotometric methods of the UV-VIS region have been commonly used in qualitative and quantitative analysis. Reports have indicated that spectrophotometric techniques were, in general, reliable, economics and simple (Rojas and Ojeda, 2009; Østergaard, 2016). The technique was successfully applied in the determination of metal ions on industrial wastewater and traces of hydrogen peroxide in aqueous solution (Zhou et al., 2019; Zou et al., 2019). The UV-VIS Spectrophotometry method was also successfully used to classify salts and cocrystals (Kiguchiya et al., 2019).

In this manuscript, a real textile effluent sample was analyzed regarding its electric conductivity, pH, turbidity, solids, alkalinity, BOD_5, COD and a spectrophotometry microbial population inhibition (MPI) method using the *Bacillus subtilis* bacteria and the *Saccharomyces. cerevisiae* yeast. The EC_{20}, EC_{50} and acute toxicity indexes obtained from the bioindicators were compared to the reduction of light of the *V. fischeri* luminescence bacteria.

2. Material and Methods

2.1 Microorganisms and Maintenance Conditions

The *B. subtilis* strain was obtained from the ATCC culture collection (6633) and the *S. cerevisiae* yeast (0758) from the CCT collection from the Instituto de Insetos Sociais da Universidade Estadual Paulista/Campus de Rio Claro (CEIS/IB/UNESP). The bacteriological Peptone (Himedia) was obtained at the Multidisciplinary Laboratory, from the Department of General and Applied Biology of the Biosciences Institute (UNESP/Campus de Rio Claro-SP). The microorganisms were maintained in inclined sterile tubes of medium size that contained 2.0 mL of Plate Count Agar (Scharlau) medium in a refrigerated place.

2.2. Sampling and Physicochemical Parameters

The raw effluent was collected from a textile industry located in the state of São Paulo, Brazil. The material was collected before mixing with any traditional sewage or treatment system, transported and maintained refrigerated until the analysis. The pH value was measured in the pH meter DMPH-2 (Digimed) and the electrical conductivity in the CA150 conductivity meter (Comitec), both calibrated in their respective standard solutions. The turbidity, settleable solids, suspended solids, dissolved solids, total solids alkalinity, BOD_5 and COD were analyzed by the standard methods 2130b, 2540f, 2540d, 2450c, 2320b, 5210b and 5220d respectively (APHA, 2017).

2.3 Microbial Population Inhibition Test

The populational inhibitions of the *B. subtilis* and *S. cerevisiae* strains were calculated through the absorbance of their respective cellular suspensions at the 600nm region. The experiment was done, firstly, by cultivating the bacteria and yeast separately in 125 mL sterilized Erlenmeyer flasks that contained 50 mL of nutrient broth for 24 hours under constant stirring at 220 rpm at 35°C. Then, 10 µL of the cultivation broths were added into sterile test tubes of standard size that contained 5.0 mL of different concentrations of effluent

diluted in a sterile solution with microbiological peptone (0.1% w/v). The effluent sample was previously centrifugated at 4000 rpm for 30 min and autoclaved for 20 minutes at 120°C and 1 atm. The testing tubes were prepared in quadruplicate, whereas, two of the tubes were inoculated by the testing microorganisms and the other two maintained sterile and used as control. After the inoculation process, both inoculated and sterilized tubes were kept on a rotary shaker at 220 rpm, 35°C for 24 h. The populational inhibitions were calculated between the difference of the absorbance (600 nm) of the cellular suspensions between the sterilized and non-sterilized tubes after the exposure period. The result was compared to the variation of a non-toxic sample, tubes that contained only a solution with microbiological peptone (0.1% w/v) (Equation 1).

Equation 1

$$MPI = \left[1 - \frac{(sAbs - esAbs)}{(cAbs - ecAbs)}\right] x100$$

where *MPI* referred to the microbial population inhibition (%), *sAbs* to the cellular suspension absorbance of the sample, *esAbs* to the cellular suspension absorbance of the sterile sample (non-inoculated sample), *cAbs* to the cellular suspension absorbance of the control (peptone solution), *ecAbs* to the cellular suspension absorbance of the sterile control (non-inoculated peptone solution).

2.4 Reduction of Light Emission by the V. fischeri Bacteria Test

The luminescent bacterium *V. fischeri* test was performed according to CETESB (2001). The experiment was done by the measurement of the reduction of light emitted by the bacteria over 20 min of exposure period. The reduction of the emission of light by the microorganism was estimated according to equation 2 (Hoffman & Christofi, 2001).

Equation 2

$$LR = \frac{LUsample_{t(x)}}{CF\ x\ LUsample_{t(0)}} x100;\ CF = \frac{LUcontrol_{t(x)}}{LUcontrol_{t(o)}}$$

where *LR* referred to the reduction of light emission by the bacteria (%), *LU* to the luminescence units of the sample and the control before *t(0)* and after the exposure period *t(x)*, *CF* to the correction factor of the light loss by the control.

2.5 Determination of the Effective Concentration

The EC_{20} and EC_{50} indexes were calculated by the linear regression of the valid values of the biological factor as a function of effluent concentration (Moriarty, 1988). The acute toxicity index (ATU) was calculated using the mean effective concentration ($ATU = 100/EC_{50}$) and classified according to Sanchez et al. (1988) toxicity criteria.

3. Results and Discussion

3.1 Physicochemical Parameters

The physicochemical results of the analyses were described in Table 1. The effluent had an unpleasant odor, probably due to the presence of volatile components such as nitrous, sulfuric gases and aromatic molecules. According to reports, volatile compounds in textile effluent may represent a major environmental problem. Volatile toxic substances may be carried for kilometers and capable to modify the composition of the atmospheric air (Pereira, 2002). Reports have shown that those compounds may be extremely harmful to the environment and humans (Müezzinoğlu, 1998; Gavrilescu, 2010; Dey & Islam, 2015; Sivaram, 2019).

The high value of electrical conductivity value of 7260 µS/cm was due to the use of high quantities of salts for dyeing fixing, such as sodium chloride, sodium carbonate, sodium bicarbonate and sodium hydroxide (Correia et al., 1994; Rodrigues et al., 2013; Khatri et al., 2015). Although indispensable for the living organisms, the excess of salts was seen to decrease the osmotic potential of the soil, decline the water absorption of seeds and cause inhibitory effect in plants (Flowers et al., 2014; Geilfus et al., 2018; Roy et al., 2018). Studies also indicated that the excess of salts was responsible to suppress the seed germination, inhibit the root growth and cause significant effects on the development of plants (Foy, 1992; Joutti et al., 2003).

The high pH value of 10.31 and the quantity of 4200 mg L^{-1} of $CaCO_3$ in the effluent were due to the use of caustic reagents in several stages of the textile processing, such as sodium hydroxide, sodium carbonate and sodium bicarbonate (Beltrame, 2000; Rodrigues et al., 2013; Khatri et al., 2015; Tchamango et al., 2017). The pH value, which is known to influence chemical and biochemical reactions, was seen to strongly affect the metabolism of living organisms and cause great influence in aquatics and terrestrial ecosystems (Goodwin et al., 1988; ŠImek & Cooper, 2002; Lacoul & Freedman, 2006; Hartman et al., 2008; Wootton and Forester, 2008). According to reports, high alkalinity conditions were also seen to increase the ecotoxicological degree of samples and cause significant variations on the metabolism of roots (Clément & Merlin, 1995; de la Torre-González et al., 2018).

The value of 650 NTU of turbidity indicated the presence of high quantity of dyes, pigments and colloidal substances (Hongve & Åkesson, 1996; Guaratini & Zanoni, 2000; Carmen & Daniela, 2012; Ghaly et al., 2014; Chandran, 2016). High colored effluents were seen to affect the visual appearance of water bodies, influence the rate of photosynthesis and, in extreme cases, cause the eutrophication of aquatic ecosystems (Pierce, 1994; Beltrame, 2000; Sarayu & Sandhya, 2012; Favas et al., 2016; Gómez et al., 2017). The quantity of 1.0 mg L^{-1} of settleable, 395 mg L^{-1} of suspend, 6395 mg L^{-1} of dissolved and 6790 mg L^{-1} of total solids, indicated the presence of high quantities of yarns and lint that were released from the fibers, as much as the presence of substances like salts, alkalis and acids that were used in the textile processing (Correia et al., 1994; Ghaly et al., 2014; Chandran, 2016). According to reports, if released in excess, those solids may affect the oxygenation of water bodies, decrease the photosynthesis rate and cause negative effects on aquatic ecosystems (Dey & Islam, 2015; Favas et al., 2016). The BOD_5 value of 1747 mg L^{-1}, COD a value of 3595 m L^{-1} and

BOD/COD ratio of 0.49 indicated a mean presence of non-biodegradable organic matter. The non-biodegradable organic matter content in this type of effluent may have included molecules with high molecular weight and complex chemical structure, such as dyes and fibers residues (Samudro & Mangkoedihardjo, 2010; Akpor & Muchie, 2011; Dey & Islam, 2015).

Table 1. Physicochemical parameters of the textile wastewater

Parameter	Result	Unit
Electric conductivity	7260	µS/cm
pH	10.31	-
Turbidity	650	NTU
Settleable solids	1.0	mL L^{-1}
Suspended solids	395	mg L^{-1}
Dissolved solids	6395	mg L^{-1}
Total solids	6790	mg L^{-1}
Alkalinity	4200	mg$CaCO_3$ L^{-1}
BOD_5	1747	mg L^{-1}
COD	3595	mg L^{-1}

3.2 Ecotoxicological Parameters

According to the ecotoxicological results, the *V. fischeri* bacteria was the microorganism that exhibited the highest sensitivity towards the effluent sample, followed by *S. cerevisiae* and *B. subtilis* respectively. The original effluent concentration caused a full reduction of the light emission of the *V. fischeri* luminescent bacteria (Table 2). According to equation 3, the luminescent bacteria indicated an EC_{50} of 10.72% and an acute toxicity index of 9.3 ATU, which classified the effluent as very toxic. In studies conducted by Rosa et al. (2001), Scheers et al. (2002), Somensi et al. (2010) and Bedoui et al. (2015), the luminescent bacteria, widely used in ecotoxicological experiments, also indicated high levels of toxicity of textile effluents, with EC_{50} values of 5.4, 13.0, 3.5 and 3.0% respectably.

The UV-VIS spectrophotometry technique was capable to successfully detect the population variations of the microbiological strains. The original effluent concentration caused a MPI of the *B. subtilis* bacteria of 91.4% and Table 3. According to Equations 4, the *B. subtilis* bacteria indicated a moderate degree of acute toxicity, with an EC_{50} of 30.72% and 3.3 ATU. The gram-positive bacteria, which was reported as highly sensitive towards dyes and heavy metals, obtained the third sensibility rank (Horváth et al., 1997; Kahru et al., 2005). Reports indicated that toxic compounds were seen to influence the genetic regulation of *Bacillus* species, and cause morphological and growth variations even at low concentrations (Ogawa et al., 1988; Dutka & Gorrie, 1989; Sharma & Sobti, 2000). *B. subtilis* species, however, were seen to be capable to produce spores that allowed their survive for long periods of time in the absence of nutrients and towards the exposure of various types of chemical compounds,

including organic solvents, oxidizing and alkalizing agents (Setlow, 2006).

The yeast displayed the second highest sensitivity according to the acute toxicity index. The analysis indicated that the *S. cerevisiae* suffered a MPI of 84.7% towards the original effluent concentration (Table 4). Through the Equation 5, it was seen that yeast indicated that the effluent was highly toxic, with an EC_{50} of 16.26% and 6.2 ATU. Noteworthy, the yeast exhibited the highest sensitivity towards the effluent sample according to the EC_{20} index. The equations indicated that the EC_{20} value of the yeast was estimated in 2.91%, while the EC_{20} of the luminescent bacterium *V. fischeri* was 3.53% (Table 5, Figure 1). According to studies, the *S. cerevisiae*, which has been an excellent model for eukaryotic cells, was seen to detain genes such as Msn2p and Msn4p that were responsible to transcribe proteins with protective functions (Martinez-Pastor et al., 1996; Kim et al., 2006; Braconi et al., 2016). The activation of those genes, which were seen to be done under stressful physiologic conditions, may be involved in the populational variation seen in the yeast growth.

Table 2. Effluent concentration and the LR effect on the *V. fischeri*

Concentration	Units of luminescence		Gamma (Γ)	LR (%)
	Control	Sample		
0.00 (Control)	99	134	1344	-
2.56	96	119	0.085	8.42
5.12	98	96	0.373	27.63
10.24	99	64	1.071	52.24
20.48	96	36	2.609	72.29
40.95	101	15	8.120	89.03
81.90	101	1	108.8	99.27

Table 3. Effluent concentration and the MPI effect on the *B. subtilis*. The different letters represented significantly differences between the means using Fisher's statistical test (95%)

Effluent concentration (%)	MPI (%)			
	Replicates		Standard deviation	Mean
	i	ii		
0.00 (C)	0.00	0.00	-	0.00 c
12.50	-	3.33	-	3.33 c
25.00	45.74	26.36	13.70	36.05 b
50.00	85.11	97.58	8.82	91.34 a
100.00	90.43	92.42	1.41	91.42 a

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Table 4. Effluent concentration and the MPI effect on the *S. cerevisiae*. The different letters represented significantly differences between the means using Fisher's statistical test (95%)

Effluent concentration (%)	MPI (%)			
	Replicates		Standard deviation	Mean
	i	ii		
0.0 (C)	0.00	0.00	-	0.00 f
0.78	2.38	9.89	5.31	6.13 ef
1.56	11.36	13.81	1.73	12.58 de
3.13	18.68	5.97	8.99	12.33 de
6.25	24.54	21.27	2.31	22.91 d
12.50	-	41.60	-	41.60 c
25.00	58.42	61.75	2.35	60.09 b
50.00	78.02	69.78	5.83	73.90 a
100.00	80.77	88.62	5.55	84.69 a

Table 5. Ecotoxicological parameters of the textile effluent

Parameter	Bioindicator		
	S. cerevisiae	*B. subtilis*	*V. fischeri*
EC_{20}	2.91	15.70	3.53
EC_{50}	16.26	30.72	10.72
ATU	6.2	3.3	9.3
Class	Very toxic	Moderately toxic	Very toxic

Equation 3

$$LR = -14.4091 + 62.5055\ log_{10}(x);\ r^2 = 0.99$$

Equation 4

$$MPIbs = -102.9486 + 102.8821\ log_{10}(x);\ r^2 = 0.84$$

Equation 5

$$MPIsc = 1.335 + 40.3575\ log_{10}(x);\ r^2 = 0.94$$

where LR referred to the luminescence reduction of *V. fischeri* (%), *MPIbs* to the population inhibition of *B. subtilis* (%), *MPIsc* to the population inhibition of *S. cerevisiae* (%) and x to the effluent concentration.

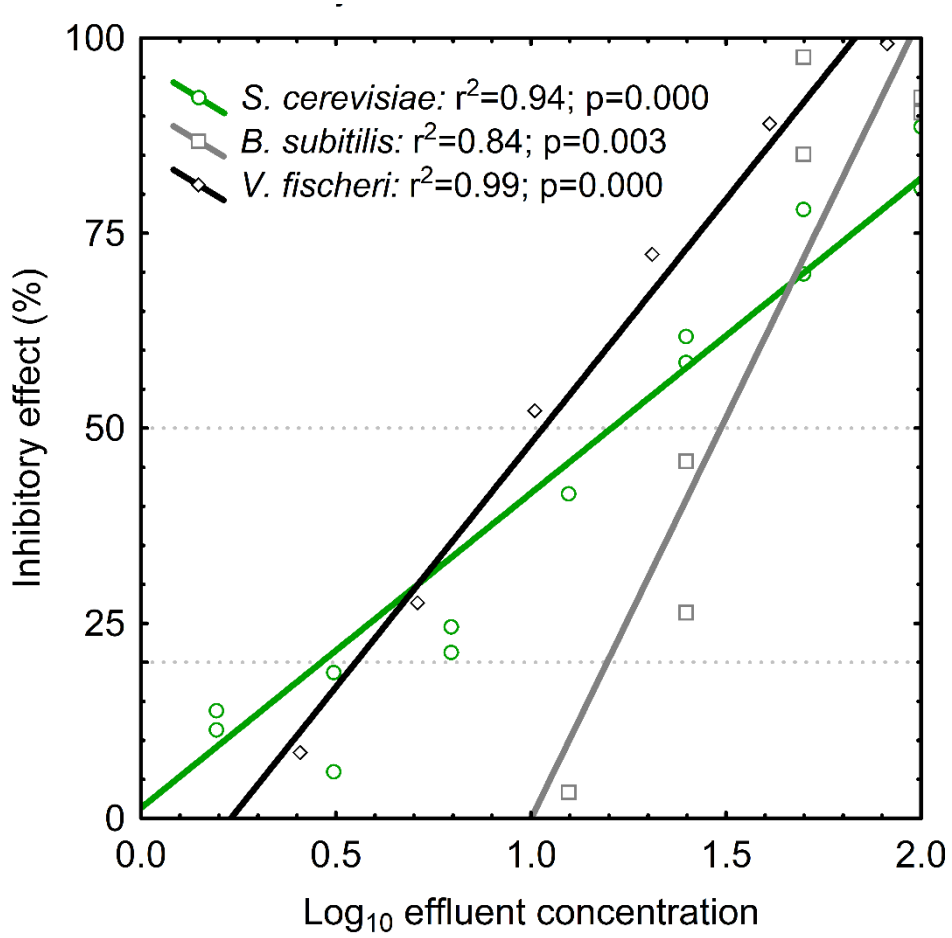

Figure 1. Textile effluent concentration as a function of the inhibitory effect on the bioindicators

4. Conclusion

It was concluded that the physicochemical parameters of textile effluents were highly variable in comparison to other studies in the literature, mostly due to the use of different types of chemical reagents, dyes and raw material in the textile processing. Indeed, textile effluents were seen to be generally highly colored, alkaline, with high amounts of organic matter, solids, heavy metals and harmful organic compounds. The ecotoxicological tests indicated that the *V. fischeri* luminescent bacterium detained the highest sensitivity towards the textile effluent, with EC_{50} value of 10.7% and a high degree of acute toxicity with 9.3 ATU. The yeast *S. cerevisiae* obtained the second highest sensitivity, with EC_{50} value of 16.3% and also a high level of acute toxicity, with 6.2 ATU. The *B. subtilis* obtained an EC_{50} value of 30.7% and a moderate acute toxicity index of 3.3 ATU. It was seen that, in the case of EC_{20} index, the *S. cerevisiae* was the most sensitive microorganism, with a value of 2.9%, followed by *V. fischeri* and *B. subtilis*, with EC_{20} values of 3.5 and 15.7% respectably. The UV-VIS spectrophotometry MPI technique was seen to be a feasible method to obtain conclusive results regarding the harming effect of the textile effluent sample. The method was shown to be simple, quick to be proceed, and required a modest sampling volume. Ecotoxicological bioassays that use microorganisms were seen to be a great tool to obtain

conclusive answers concerning the hazardousness effects of industrial effluents and various organic and inorganic compounds. From this study, it is expected the use of microorganisms and spectrophotometry methods to determine the ecotoxicity of environmental samples and pollutants.

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