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# Development of Water Quality Test Kit Based on Substrate Utilization and Toxicity Resistance in River Microbial Communities

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**ABSTRACT:** Methods for measuring toxicity or respiratory activity of microbial cultures can be used as tools for assessing the presence of chemicals and their impact on the streams. The proposed toxicity test is based on the respirometric characteristics of the bacteria according to the principals of Biolog's microplate system. As the microorganism are utilizing the carbon source (peptone), the reduction of the tetrazolium dye as the redox indicator is taking place, leading to a developing change in the well's color The tests provided us with information in regard to the toxicity range of the chemicals with activated sludge and Alcaligenes.

**Key words:** Toxicity, Bioindicators, Monitoring, Microorganisms, Biodegradation, Substrate

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## INTRODUCTION

Traditionally, toxicant levels in water effluents and other sources have been estimated by bioassays employing the micro and macro vertebrates. Lately, there has been an increased tendency to use microbial systems for screening toxicants as an alternative to tests with animals. Bacterial communities represent sensitive yet informative indicators of the presence of toxic substances in the environment. As prime mediators of biogeochemical cycling, bacteria are ubiquitous, diverse, and adapted to exist on dissolved substances that are often present in the environment at very low concentrations. Because of the versatility of bacterial populations, some strains are capable of tolerating or even thriving in the presence of high concentrations of a potentially inhibitory substance, whereas others are eliminated. The consumption of different contaminants present in various industrial and agricultural sectors through biodegradation, or toxicity resistance to these pollutants by the microbial communities can provide information about pollutant exposure, metabolic diversity and the potential source of contamination and the potential for the ecosystem natural attenuation, thus be a practical indicator of the water quality. Microorganism-based assays have been developed as practical and cost-effective methods for water toxicity testing. Studies have shown that each microbial species and test procedure has its own sensitivity pattern to toxicant (Dukta and Kwan 1982) and no single species is able to respond to all chemicals (Toussaint, *et al*. 1995). Emerging the microplate technology by the Biolog in late 1980 has provided the chance to apply a moderately practical method to monitor the pollutants and their toxicity levels in the environment. The 96-well microplate was designed to test the ability of inoculated microorganism suspensions to consume (oxidize) a panel of different carbon sources. Each well contains a redox dye, a specific carbon source, and a buffered nutrient medium that has been developed and optimized for a wide variety of bacteria. (Stumm and Morgan 1996). Development of redox sensitive dyes, such as tetrazolium, and incorporation of these dyes into microtiter plates, has allowed for rapid profiling of sole carbon source consumption by bacterial isolates. Practically, when redox dyes are used as the indicator, the response is indicated by the change in color that is visual and sometimes quit dramatic. The color change can be quantified using common methods such as spectrophotometry or even with naked eyes, and the

resulting data can be used to characterize the environmental system conditions.

Basically, different organic compounds with various concentrations will lead to different growth and hence different biodegradation rates. On the other hand, various strains may have wide range of lag phase before they actually start the growth and degradation process, which is leading to a shorter / longer time of color formation. A lag phase may result from a lack of acclimation of the microbial inoculum to the wastewater. Usually, the more toxic the water is, the longer the lag phase will be. The rate of increase in the color development represents the rate of substrate removal. More over, one should always keep in mind that the inoculum density has a considerable effect on the rate of color development. Normally at higher concentration of the inoculum, the color appears faster (Konopka, *et al.*1998). Moreover, it is due to the different physiochemical properties of various chemicals that they may have different impacts on separate compounds of the biological system. Theoretically, low toxicant concentration may produce no observable effect (NOEC), but as the concentration increase beyond a critical level, an increasing adverse effect can be observed, finally reaching death (Connel, *et al.* 1999). To assess the toxicity of a compound on a biological system, an observable and well defined effect (endpoint) must be defined (Layton, *et al.* 1999). For example, Bacterial toxicity tests measure a wide variety of endpoints including population growth (Nenzda and Seydel 1988), CO2 production (Jardin, *et al.* 1990), mutagenicity (Ames, *et al.* 1973), enzyme biosynthesis (Dutton, *et al*.1990) and glucose mineralization (Reteuna, *et al*.1989).

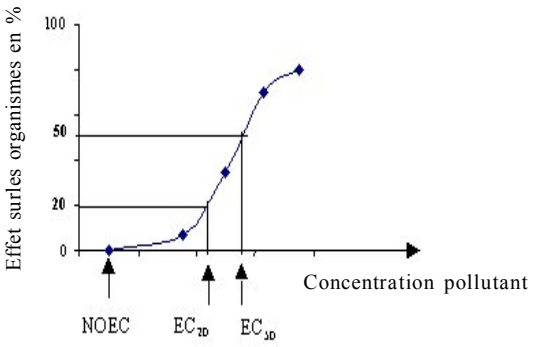



**Fig. 1. Terms related to the toxicity tests; Effective concentrations on 20, 50 to 100 % of the population (EC20, EC50, and EC100) and none observed effective concentration (NOEC)**

Majority of toxicity tests bring an estimate of the amount which affects 50% of the population. It can be for example the average lethal concentration which kills 50% of the population. It is also possible to consider the maximum concentration which does not cause any effect. If the tests are carried out on "end points" other than mortality then one defines an EC pollutant affecting 50% of the population (Fig. 1). From this point of view, we proposed to develop a rapid analytical water quality test kit to evaluate the microbial utilization of substrates generated by different industrial, domestic and agricultural sectors and also a toxicity resistance test of the microbial communities as potential bioindicators of water quality on these substrates. Thus, being able to find out more about the contaminated sites as well as proposing the possible remediation.

## MATERIALS & METHODS

Activated sludge as the main inoculum was taken from Malmö waste water treatment plant and kept in the fridge in closed bottles, it was aerated before inoculation for two to three days by using a normal pump in order to reactivate the existing bacteria and remove the remaining biodegradable organic matter present in the sample air to the system. An Alcaligenes strain was taken from the agar plates that contain a selective medium of these bacteria containing nutrient agar, mineral salt medium and 120 mg/L phenol. Then some colonies were taken and introduced to a new medium, cultivated in the flasks of 30 ml, in a shaker, at room temperature in the dark place. The growth medium was further supplied with 0.5 g/L phenol and 0.5 g/L peptone, in order to reduce the phenol content and at the same time help with the growth rate. Following the cultivation period of three to four days, bacterial growth was observed in the flasks. Before the inoculation of the Alcaligenes to the system, 15 to 20 mL (depending on the number of the tests) of the cell suspension was introduced to the sterile tubes and washed three times. Each washing steps, started with the centrifugation of the medium at 10000 rpm for 15 minutes, the discarding the supernatant and then, the mixing of the cell pellets with a 15 to 20 ml fresh mineral salts medium, without any addition of phenol or peptone.

The toxicity tests were first conducted in glass tubes with plastic caps (5 mL). In each set of the experiment for every single contaminant, the tests are done at least duplicate. The order of introducing

different component to tubes was always been the same. Starting from the mineral salt medium, it was prepared according to the OECD 301D (Organization for Economic Co-operation and Development) guidelines for testing of chemicals. The volume of the MSM was dependent on the other component in the test and was defined mainly based on the variable concentrations of the substrates- total volume of the test (5 mL) minus the sum of the volume of all the other components. Following the addition of 500 µl of carbon source (peptone / glucose) is added. The peptone used was the bacteriological peptone (total nitrogen 14.0; amino nitrogen 2.6; sodium chloride 1.6 % w/w and pH of 6.3 at 25 ºC). The initial concentration of peptone was 12 g/l (The concentration of peptone inside the medium was 1.2 g/L). The glucose solution was made at concentration of 10 g/L, both carbon sources were prepared under sterile condition in sterile bottles and kept in the fridge. Then 50 µl of the redox dye (Table1) was pipetted to the tubes. Then toxic chemicals, the substrate (Table 2), in various range of concentrations was added to the tubes. Finally the last step was the inoculation of the 300 µl inoculum to tubes. Inoculation of activated sludge was done under normal conditions while with the Alcaligenes; inoculation of the bacteria was under sterile condition. Then tubes were closed and shaken to let all the components to be mixed. They were incubated in room temperature in the dark. Although the appropriate incubation time was between 24 to 48 hours to allow the color to form, the tubes were checked every 12 hours for any color transformation during the first 2 days and then every 24 hours up to one week. The exact test was run in microplate, under the same conditions and concentrations, parallel to the test tubes. Any considerable change was recorded as pictures, providing the chance for comparison. Besides, the necessity of the presence of control tubes for the time of comparison, were always taken into consideration.

Control tubes, were prepared and incubated under same conditions as the test tubes except the fact that they did not contain any pollutant. Toxicity tests were also done in 96-wells microplates using the same protocol as described above but all the volumes were adjusted to reach a final volume of 250µL in each well.

**Table 1. Concentration of the stock solution of the colors tested as indicators in the toxicity tests**

| Dye | Tested concentration range (g/L) |
|---|---|
| Aniline blue | 1 – 0.1 -0.01 |
| Coomassine brilliant blue | 0.1-0.01 |
| Rhoda mine 6G | 0,1 |
| Fast green FCF | 0.1-0.01 |
| Phenol phethaleine | 0.05 |
| Methylen blue | 0.1 |
| Resazurin | 0.1- 0.01 |
| Triphenyl Tetrazolium chloride (TTC) | 1-0.1 |

**Table 2. The concentrations range of the toxicants in the test**

| Chemical | Initial concentration (g/L) as stock solution | Concentration range, tested in the experiment (mg/L) |
|---|---|---|
| Cadmium Nitrate trihydrate | 20 | 0-200 |
| Copper Nitrate | 2 | 0-100 |
| Potassium Ferro Cyanide | 10 | 0-500 |
| Toluene | 0,78 | 0-300 |
| 2,4-Dichlorophenol (DCP) | 3,9 | 0-200 |
| 4-Nitrophenol (PNP) | 16 | 0-1000 |
| Pentachlorophenol (PCP) | 0,5 | 0-200 |
| Phenol | 10 | 0-1800 |
| Naphthalene | 0.03 | 0-20 |

## RESULTS & DISCUSSIONS

The results reveal that when peptone was used as the carbon source, the change in the color happened faster rather than with glucose. Besides, when peptone is used, the color formation (resazurin as indicator) is much closer to the expected results (transparent and light pink). As a result peptone was chosen as the carbon source to the medium. For the redox selection, the first sets of tests (peptone, activated sludge, mineral medium and dye but no inoculum) show that TTC, resazurin, methylen blue, aniline blue, fast green and rhodamine 6G respectively provided noticeable changes. In the three latter ones, the changes were around 50% of the initial color which may not be satisfactory when it comes to application of the method in small volumes. Moreover in the case of aniline blue the change in color may come from the fact that it is a more easily biodegradable dye compared to others. So TTC (1 g/ L), resazurin and methylen blue (0.1 g/L) were tested again (actual toxicity test) Methylen blue, hardly changed color in 72 hours and was not considered faster. With resazurin not only the experiments took longer (at least three days), but also the color

formation was not clear enough in some of the tests. Accordingly, as the change in the color occurred were fast (in average 48 h.) and clear, TTC was selected as the redox indicator in the toxicity tests.Following the choice of the indicator (TTC) and the carbon source (peptone) the toxicity tests were run on different toxicants chosen among the priority pollutants list (EPA 2000) and after some optimization, toxicity of each substance considering the formed color, which represents the effective concentration (EC), is discussed. In the discussion part, Tables 3, 4 and the related graphs (Figs. 2 and 3) are two examples of the evaluation of the test results for PCP and DCP as representatives of the tests with other substrates on activate sludge and Alcaligenes.

Figure 2 shows the toxicity of DCP at different concentration on the activated sludge in relation to the formed color. Both the tables and the graph show that the EC100 was somewhere between 50 and 80 mg/L. Besides, NOEC (no observed effective concentration) for DCP is up to the 20 mg/L and LOEC (lowest observed effective concentration) is between 20 and 30 mg/L. For PCP, EC100 (the concentration which has 100% effect on the test organisms) was located at the concentration of 50 mg/L where no change in color and possibly no growth were reported, however, LOEC was below 10 mg/L. Based on the same practice, for naphthalene and toluene it is impossible to come up with the values of EC100 or even LOEC since. At all the concentrations which have been tested, even after optimization, still the color formation is more or less 100% (pink). This means that none of the tested concentrations inhibited the microorganisms. According to the tests, EC100 for the PNP was around 20 mg/L. Besides, as growth only recorded in the control, the NOEC and LOEC were below 10 mg/L. For cyanide and phenol, the case was almost like the toluene and naphthalene, because although the optimization is done, still the extreme change in color and growth was clearly observable. The case of phenol and cyanide, as the results were very far beyond the estimated range, new stock solutions were made for them. Consequently, the toxicity range of phenol has changed and it is narrowed down to 1000 mg/L which is absolutely normal but no change is observed with cyanide meaning that the toxicity range is still above 1000 mg/L, further investigation and optimization are needed. Cadmium's EC100 was in a range of 80 to 100 mg/L, at the same time NOEC and LOEC were respectively around 50 and 55 mg/L Finally for Copper the EC100 laid between 20 and 50 mg/L and LOEC and NOEC were below 5 mg/L. Knowing that the Alcaligenes are phenol-resistant, provide us with the necessity of a cell-washing step in advance to the inoculation. However if the intervals between the cultivation of the bacteria and inoculation is long, around a week, there is no need to do the washing as probably the majority of phenol is consumed by the bacteria. Test results show that for PNP, DCP and PCP, the values for EC100 were respectively between 10-20, 50 - 80 and 50- 80 mg/ L, while the LOEC was below 5 mg/l for PNP, between 30-50 mg/l for DCP and below 10 mg/L for PCP. At the same time with PNP and PCP, NOEC were respectively below 5 and below 10 mg/L while for DCP it is up to the 30 mg/L. The EC100, LOEC and NOEC values are correspondingly; between 50-100 mg/L, below 10 mg/L for cadmium and between 20-50 mg/L, in range of 5 and 10 mg/L, and below 5 mg/L for copper. The outcome of the toxicity test with acclimated strain (Alcaligenes) on phenol, toluene and cyanide was similar to the very same case with the activated sludge. At all of tested concentrations, the microbial growth was observed. Therefore it was very difficult to vary the different EC values. Still EC100 of cyanide on Alcaligenes was recorded between 700 and 1000 mg/L, which were lower than the activated sludge test.

According to the outcome of the experiments, DCP (dichlorophenol) had toxic effects on thegrowth of bacteria above the concentration of 50 mg/L for both Alcaligenes and activated sludge. WithPCP and PNP also, no differences in the intensity have been observed, though in the case of PNP it is not easy to limit the range due to the strong yellow color of the own medium. Although it seems to be strange to have the same range of toxicity for both inoculums, it indicates that probably the dominant consortium of bacteria in sludge functioning the same as what Alcaligenes are doing in regard to toxicity.

**Table 3.Toxicity test results on different concentrations of DCP and PCP with Activated sludge**

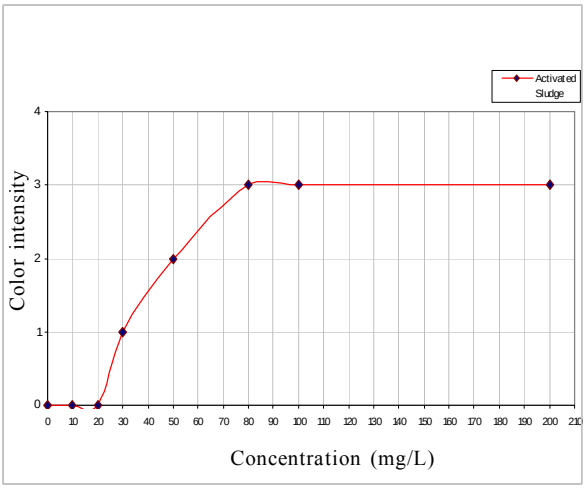
| DCP, Concentration (mg/L) | 0 | 10 | 20 | 30 | 50 | 80* | 100* | 200* |
|---|---|---|---|---|---|---|---|---|
| Color Intensity | xxx | xxx | xxx | xx | - | - | - | - |
| PCP, Concentration (mg/L) | 0 | 10 | 20 | 30 | 50* | 80* | 100* | 200* |
| color Intensity | xxx | x | x | x | - | - | - | - |

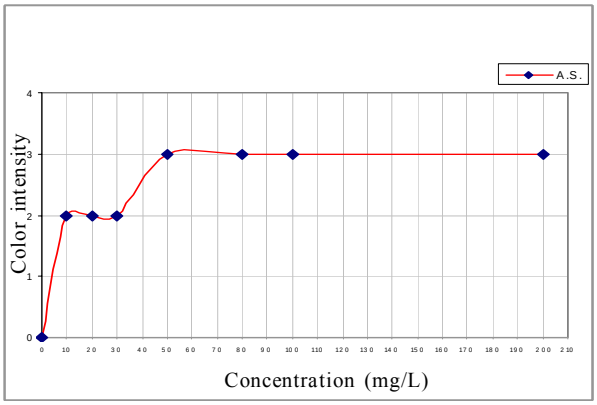
((X), (XX), (XXX) and (-) are respectively representing; light pink, medium dark pink, dark pink and transparent colors observed when TTC is used as indicator).

**Table 4. Toxicity test for different concentrations of DCP and PCP with Alcaligenes**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| DCP, Concentration (mg/L) | 0 | 10 | 20 | 30 | 50 | 80* | 100* |
| Color Intensity | xxx | xxx | xxx | xxx | xx | - | - |
| PCP, Concentration (mg/L) | 0 | 10 | 20 | 30 | 50 | 80* | 100* |
| Color Intensity | xxx | x | x | x | x | - | - |

((X), (XX), (XXX) and (-) are respectively representing; light pink, medium dark pink, dark pink and transparent colors observed when TTC is used as indicator).




**Figs. 2 and 3. The toxicity range of the DCP and PCP on activated sludge regarding to the intensity of the formed color.**

The values at the Y axis, 3, 2, 1 and 0 are respectively representatives of the (-), (x), (xx) and(xxx) in relation to the color intensity

It can also be explained as that the medium in total may contain the chemicals that favor the growth of Alcaligenes on the substrate. With toluene and naphthalene, the toxicity resistance level will go beyond the test limit, 800 mg/L and 20 mg/L respectively. Although according to databases they are toxic materials, it seems the bacteria had high resistance to these toxicants. The case of heavy metals seems a little bit higher than our expectations, specially the cadmium, the EC100 is above 80 mg/L and it's far too high (12 mg/L in databases). As the tests are done in glass tubes, that might cause an accumulation of the metals on the glass walls. So following the low concentration of the compound soluble inside the medium, the bacteria could tolerate higher levels of the metals; this might expand the toxicity range and create error in the test. In order to avoid this problem we can use specific plastic tubes or microplates made of plastic material. But then it is necessary to rinse the plastic tubes with the nitric acid solution before starting the experiment. Furthermore, activated sludge has the ability to adsorb the heavy metals, which will decrease the availability of the metals for the bacteria, thus leading to higher resistance to the toxic metals. Phenol's toxicity was also higher than what was expected. Since the toxicity range of phenol normally is around 1 g/L. Both experiments presented concentration even above of 1.8 to 2 g/L, which seems almost impossible. Of course, one possible explanation with Alcaligenes is that they are acclimatized to the phenol at high concentrations, so they are able to resist elevated levels of it compared to activated sludge. The other explanation might be some inaccuracy with the initial concentrations or with the stock solution. The same case is more or less with cyanide, as it is also very toxic but within the tests the toxicity boundaries which are achieved shows higher values than normal. Moreover, the toxicity of cyanide to aquatic organisms is highly variable depending on environmental conditions and physicochemical and microbial influences. Activated sludge was chosen not only for its high cell density, but also as it is a representative of acclimatized stains. Hence it is normal to have higher EC100 values with activated sludge than those achieved with more sensitive test organism like Alcaligenes. The acclimated stains are adapted to specific types of pollutants. Hence their implementation in toxicity test with the other types of toxicants, may not be working the way it is expected. Acclimated microorganisms, are adapted to survive high levels of the pollutant, therefore if they had grown in the medium containing the pollutant, there is a due need to have cell-washing steps before inoculation, to remove the dominant oriented pollutant from the medium and provide the bacteria with a fresh medium. This reduces the risk of taking phenol through the cell suspension to the test. If phenol goes into the system, it can cause some

errors in the process. It is because when phenol enters the cultivation medium, then the toxicity of the substrate towards the bacteria may change, as both phenol and the substrate are toxic to bacteria.

Following the assessment of the toxicity test for harmful compounds, the results were compared with values with the values available in toxicity databases. Although this comparison appears to be the most reasonable approach to assess our results, the concentration ranges and accordingly the EC values are not necessarily the same for a specific compound in different databases. Usually toxicity testing in the laboratories follows a stepwise related approach, progressing from simple short-term tests to more complex and sophisticated long-term test based on the results of previous tests. While the criteria of each particular test plan are not exactly similar, therefore the details of the protocol for each test may differ but the general test design is similar. Below, the EC values for different compound are compared in Table 5 and the results are discussed. Table 5 indicates that even though the figures seem to be very different, the results of our experiment are more or less in the range and it is hopefully according to our expectations. Deviations, seems to be quit normal and hopefully it seems that the test procedure has fulfilled the criteria of a standard toxicity test.

**Table 5. Comparison of the achieved EC100 values with values taken from the databases on bacteria**

| The toxic compound | EC100 achieved in experiment (mg/L) | EC100 achieved from databases(mg/l) |
|---|---|---|
| DCP | 50 < EC100 < 80 | 1.83 (Biolog)<br>1 (Toxalert)<br>100 (Manistö *et al*,1999) |
| PCP | 80 | 53.3 (Manistö *et al*,1999)<br>10 (Manistö *et al*,1999) |
| PNP | 20 | 2.08 |
| Toluene | EC100 > 800 | 276.5 (Evans, *et al.*, 1991) |
| Phenol | 1000 | 1000 (Biorem LTH) |
| Naphthalene | EC100 > 20 | ------ |
| Copper | 50 | 6.5 (Madoni *et al*, 1992) |
| Cadmium | 100 | 6 (Madoni *et al*, 1992)<br>12 (Madoni *et al*, 1996) |
| Cyanide | 700 < EC100 < 1000 | ------- |

## CONCLUSION

The pivotal role of microorganisms in coordinating ecosystem services, such as the cycling of nutrients, means that microbial communities reflect fundamental environmental processes. The rapid growth rate of many microorganisms means that microbial communities respond rapidly to a wide range of environmental disturbance. Thus, microbial indicators could provide early warning of potential problems. In this study the microplates have been used for toxicity testing and the results obtained are encouraging. The main advantages are that the process is simple to carry out (the experimental equipment can easily be provided with the ordinary lab equipment). Besides that, according to the comparisons that are done among the achievements of our toxicity test with the other toxicity test methods, the test results are reliable. On the other hand this technique is very inexpensive, not only the method is based on the visual observation which eliminate the need for costly automatic readers , but also it is labor saving. While measurement time is quite short, normally within couple of days, the method is specific since the toxicity effects are evaluated directly on the system. However, the visual method for analyzing the toxicity of the water samples are dependent upon somewhat subjective judgment and this may lead to not very satisfactory results according to the reproducibility between different analyses.

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