CHLORINATED AROMATIC COMPOUNDS: MICROTOX TEST RESULTS AND STRUCTURE-TOXICITY RELATIONSHIPS

Juan M. Ribo

Environmental Contaminants Division
National Water Research Institute
Burlington, Ontario, L7R 4A6, CANADA

NWRI Unpublished manuscript report
numbered series ; 84-3

ABSTRACT

The toxic effects of chlorosubstituted benzenes, phenols, anilines, nitrobenzenes and pyridines to the luminescent bacteria Photobacterium phosphoreum have been determined using the Microtox test. Quantitative relationships between these toxicity values and certain compound specific structural parameters make it possible to develop a mathematical model for the prediction of their toxicities to aquatic biota.

The dependance of the test results on variables such as temperature, pH, exposure time, age of the bacteria culture, etc., are discussed as well as the correlations of the Microtox values with the effects of these compounds on other organisms which are commonly used for aquatic toxicity studies.

INTRODUCTION

Over the last few decades the manufacture and consumption of chemicals has increased dramatically to the extent that even the lowest estimates place the number of existing chemicals and mixtures well over 5 million, and new compounds are continously being added to this figure. However, a much smaller number is important for environmental considerations(1), namely the aproximately 60,000 compounds which are in everyday use. Unfortunately, even this number is too large to allow their classification into toxicity categories. Moreover, as the number of potential contaminants is constantly increasing, the huge backlog of untested compounds alone calls for better, simpler and more rapid tests.

The idea of using luminescent bacteria as a test organism for toxicity measurements is not entirely new. More than one specie of light emitting bacteria has been tested and proposed as a biological system for toxicity assays. In such organisms, the production of light is usually the result of the interaction between the enzyme luciferase, oxygen and flavin in its reduced form. This

biochemical reaction pathway is part of the electron-transport system of the respiratory chain and the emission of light depends on this flow of electrons which reflects the metabolism of the organism. Therefore a bioassay can be based on the light production of these luminescent bacteria.(2)

A new method for rapid measurement of air pollution based on the bioluminescence of Photobacterium phosphoreum then called Photobacterium fisheri, was described in 1965 (3,4). Later on, Bulich and coworkers (5,6) proposed a new bioassay for the assessment of the toxicity of aquatic samples using the same organism. An application of this type of bioassay for routine toxicity analysis of water samples was developed by researchers at Beckman Instruments Inc. and the method is now commercialized and standardized (7,8,9) under the trademark MICROTOX.

A different strain of luminescent bacteria, Benekea harveyi has been proposed and evaluated in terms of its ability in predicting aquatic toxicity (10).

MICROTOX TOXICITY BIOASSAY

This bioassay is based on the reduction of light

emitted by the luminescent bacteria Photobacterium phosphoreum when exposed to a toxic sample. This is the overall result of the interactions that occur between a chemical substance and one or more enzymes forming part of the chain of metabolic reactions of which the light is a by-product.

Evaluations of this test describe it as useful and providing good results, which are comparable to those obtained with bioassays on other organisms. Several publications have compared the Microtox Test with toxicity tests based on other microorganisms (11), various fish species (12,13), and other aquatic organisms (14,15).

Other studies show its applicability for the determination of the toxicity of single chemicals as well as complex water effluents. Due to its speed and simplicity, long and costly fish tests can be avoided, in particular for prescreening purposes (16,17,18,19). It is therefore of value as an alternative to some bioassays which are compulsory for premanufacturing or preimportation notices under some environmental protection laws, particularly in respect to the effects of toxic chemicals to the aquatic biota.

As part of our current research on structure-activity correlations for the evaluation and

prediction of the toxicity of potential organic contaminants to the aquatic environment, we report here on test results for chloro-substituted aromatic compounds, and quantitative structure-activity relationships (QSAR's) with molecular parameters. In addition, a review on the dependance of the Microtox test on test conditions such as exposure time, pH, temperature, concentration etc. is presented.

MATERIALS AND METHODS

-Instrument

The Microtox Toxicity Analyzer^{TM} consists on a light-tight chamber where the light emitted by the bacteria is received by a photomultiplier, transformed into an electrical signal, and either displayed on a digital meter, graphically recorded, or both, as intensity relative to the control.

Since the light emitted by the luminescent bacteria depends on the temperature, three sites of the instrument are temperature controlled: a pre-cooling well where a stock of the reconstituted bacteria is kept at 3^{o}C, the turret, light measuring chamber, and 15

incubation wells in which temperature can be selected between 10 and 25°C within a 0.1°C accuracy (FIG, 1,2).

-Reagent

The test organism is the marine luminescent bacteria (Photobacterium phosphoreum NRRL* B-11177) provided by the manufacturer in a lyophilized form. Every vacuum-sealed vial contains 1mL of freeze-dried suspension of the bacteria culture in 2% NaCl solution, containing an average of 10^8 cells. All the necessary solutions and diluents are also available from the instrument manufacturer.

The lyophilized organisms are reconstituted by adding 1mL of Microtox Reconstitution Solution™. This is especially purified water free of organic compounds. After quickly stirring to reach complete homogeneization of the resulting suspension, this bacteria stock is kept in a standard disposable cuvette of non-toxic borosilicate glass at 3°C in the pre-cooling well provided in the instrument. This stock is ready for use as control and for the preparation of the test samples up to a maximum of 5 hours.

---

* Northern Regional Research Lab. California. USA.

-Sample Preparation

Since the bacteria come from the marine environment, the sample has to be osmotically adjusted by adding NaCl to reach a 2% saline concentration. For this purposes the proper amount of Microtox Osmotic Adjusting Solution™, 22% NaCl in organic free water, is added to the test sample to obtain the Primary Sample Solution.

Four dilutions of the Primary Sample Solution are prepared using the Microtox Diluent™ (2% NaCl in organic free water) to result in 5 test samples of decreasing concentrations, ranging from 100% to 12.5% of the original solution; a 2:1 dilution is the standard procedure, but this can be varied at will. Identical volumes of each solution (1.5mL for duplicate measurements) are kept in the incubation wells, at the selected test temperature (usually 15°C) along with a fifth cuvette containing the same volume of Microtox Diluent™ for control measurements.

-Test Procedure

All chemicals tested are dissolved in distilled

water except those whose solubilities are below the desired concentrations. For such compounds, methanol is used as co-solvent at up to 5% concentration in the test sample. From our experience, this concentration does not interfere with the toxicity determination. A temperature of 15°C was selected for the test and 1 mL of each of 4 dilutions of the Primary Sample Solution was used per test with no duplicate measurements. To prepare the Final Test Solution, 0.5 mL of of these sample solutions and of a blank solution (pure Diluent) are added to each one of 5 cuvettes. For duplicate tests, two series of 5 cuvettes are filled at this stage each containing 0.1 mL of Reagent in 0.5 mL of Microtox Diluent at 15°C (temperature equilibration time: ~15 minutes). The light output is recorded before the test and again at 5, 15, and 30 min. after the sample addition. Each batch of bacteria was used for an average of 5 hrs., after which the light output decreases dramatically and the bacteria have to be discarded. The cuvette containing only luminescent bacteria and diluent is used as a blank to compensate for the natural loss of light emission. (FIG. 3).

-Data Reduction

The concentration of a particular toxic substance causing a 50% reduction in the light output is the value calculated from the actual light intensity readings and referred to as Effective Concentration 50% (i.e. EC50).

The readings are taken at three different exposure times therefore resulting in three different toxicity values, namely 5 min-EC50, 15 min-EC50, and 30 min-EC50. (5-EC50, 15-EC50 and 30-EC50) (TABLE 1).

The data reduction scheme, following the recommendations published by the manufacturer, takes into account the time-dependance of the response by introducing the Blank-Ratio (BR) as a correction factor of the actual percentage of light reduction. (FIG. 4).

Instead of using the simple percent of reduction in light emission, the gamma function (Γ) is computed, which is the ratio of light emitted to the light remaining:

$$\Gamma[c]_t = \frac{BR_t \cdot [c]_t - I[c]_o}{I[c]_t} = BR_t \cdot \frac{I[c]_o}{I[c]_t} - 1 \qquad BR_t = \frac{I[0]_t}{I[0]_o}$$

- $I[0]_o$ and $I[0]_t$ refer to the Blank reading (control cuvette) at Time 0 and at Time t
- $I[c]_o$ and $I[c]_t$ refer to the reading of a cuvette containing sample concentration c at Time 0 and at Time t
- BR_t refers to the Blank Ratio for the readings at Time t

The dose/response curve (Γ vs. c) is a classical sigmoid-shaped curve from which the concentration for $\Gamma = 1$ can be determined. Logarithmic transformation of the values, plotting log gamma versus log C results, in the ideal case, in a straight line from which it is easier to read the EC50 values. It also allows to extrapolate for the EC10 values ($\Gamma = 0.111$) which are frequently used as threshold values for that particular compound. (Fig. 4). Linear regression analysis of these data allows to calculate the most suitable straight line through the four pairs of values, and the correlation coefficient will show the quality of fit. This operation results in a calculated value for the EC50.

All our tests results (EC50 values) are expressed as negative logarithms of the effective concentration causing 50% reduction of the light output after 30 minutes exposure with the concentration expressed in $mmol.L^{-1}$ of toxic chemical in the test solution and are is given as p(30-EC50).

PHYSIOLOGY OF THE LUMINESCENT BACTERIA

The specific biochemical processes from which this

luminescent bacteria emits light is only partially known. It is thought, however, that their metabolism is not too different from other species described. It seems appropriate now to consider a few remarks on the dependance of the light emission on several variables that will be encountered when running the test (20), in order to project a clearer picture of the whole mechanism of this bioassay.

-Emission spectra

The wavelength of the light emitted, spans from 420nm to 630nm with a maximal emission intensity at 490nm (Fig.5). The comparison between fresh bacteria and those rehydrated from freeze-dried cultures does not show significant loss in light intensity.

Although the bacteria, also emit light in the freeze-dried form, the emission has a very different spectrum probably due to differences in the light production reaction in this state. This energy-liberating process follows the known relationship between reaction rate and temperature. Therefore, to reduce the intensity of light emission and consequently to increase the life of the reagent, it is advisable to

store the freeze-dried bacteria at low temperature. The lyophilized reagent can be used for at least six months if the bacteria are stored at 2°C to 8°C, without significant loss in its typical light emission characteristics.

-Dependance on the saline concentration

Photobacterium phosphoreum is a marine organism and therefore genetically adapted to the saline environment. Consequently, the NaCl salinity of the test solution will influence the intensity of the light emitted (Fig. 6).

It is recommended to use a concentration of NaCl 20g/L, even if the curve shows an optimum light output at a 30g/L of NaCl. Nevertheless, this difference in light intensity is not significant for the purpose of the test and using this lower NaCl concentration presents obvious advantages in the case of hard water samples.

A NaCl concentration of 10 g/L in the Final Test Solution means that no Osmotic Adjusting Solution has been added to the Primary Sample Solution. In this case a reduction of 70% in light intensity is observed. The

cell membranes begin to break down at a salinity of less than 5 g/L. At the upper end, concentrations of more than ~35 g/L (50g/L in the Primary Sample Solution) will give untrue results due to a light reduction resulting from the abnormally high osmotic pressure and the toxic activity of the compound being tested.

In summary, this curve shows that the bacteria response is good for the purpose of the test using a NaCl concentration range between 20 and 50g/l in the Primary Sample Solution i.e. NaCl concentration range of 20-35 g/l in the Final Test Solution.

-pH dependance

The pH range for the optimal living conditions and therefore light emission by the Photobacterium phosphoreum, is between pH 5 and 9. Figure 7 shows the change in light intensity of the emission versus the pH for the Primary Sample Solution and Final Test Solution. For pH values outside these limits, the light intensity decreases sharply. (Fig. 7).

This allows to study the differences in toxicity between ionized and non-ionized forms of one compound within this pH range without any loss of sensitivity in

the meaurements. Consequently, the pH dependance is important for the determination of toxicity values for classes of ionizable compounds such as phenols, amines, pyridines, etc. Care must be taken, to keep the sample solution of such ionizable compounds in this pH range in order to get accurate and reliable toxicity values using this test.

-Temperature dependance

Like any other biochemical reaction, the metabolism of the luminescent bacteria is affected by the temperature. It has been found that the light intensity varies approximately 10% for every $1^{o}C$ change in temperature (8).

However the effect of temperature on Photobacterium phosphoreum as for other organisms (21) is influenced by the nature of the toxic substance substance to which the bacteria are exposed.

Figure 8 shows the relative light intensity dependance on the test temperature for the blank control and for a test solution of sulfanilamide (0.0025 mol/L); (From reference 22). It is evident that with increasing temperature, shorter exposure times are needed to get

the same light output, but at the same time the decay in light output is smaller at lower temperatures.

The Microtox Toxicity Analyzer has a system of regulating the test temperature to an accuracy of 0.1°C in the 10°C to 25°C range. For the purpose of standardization it is recommended to run all tests at the same temperature. The most commonly selected temperature, 15°C, is a compromise in getting enough light intensity and good response in terms of minimal drift of the light emission during the exposure time.

-Time dependance

The intensity of the light emitted by Photobacterium phosphoreum, changes with the time elapsed since the beginning of the test. Furthermore, this dependance is also a function of the age of the reconstituted bacteria suspension.

During the first 5 to 10 minutes, the light intensity increases as a result of the temperature equilibration from 3°C to 15°C and a maximum in intensity is usually observed varying its position from one bacteria culture to another. In other cases only a steady declining trend can be observed (FIG. 9 and 10).

After the recommended equilibration period of 15 minutes, the drift of light intensity becomes small and it is even smaller for older reconstituted bacteria suspensions. To correct for this drifting in light output, due merely to the time elapsed, the Blank Ratio (BR) is used.

In most cases, this BR is smaller than 1, but with older bacteria suspensions, this value may reach 1 as the slope of the plot of light intensity vs. time approaches zero. Figures 9 and 10 show the relation between light intensity and time for two different bacteria batches in relation to the age of the reconstituted suspension for the control solution (no toxic substance added).

-Dependance on the age of the culture and the age of the reconstituted bacteria

The light intensity of the fresh reconstituted bacteria fluctuates with the age of the freeze-dried culture and its storage conditions. Those variations however, are minimal and although differences in light intensity can result in distinct sensitivity to toxic substances, normally these effects are too small to

cause a substantial deviation in the final value. No mention of such effects on the final toxicity values due to differences in the lyophilized bacteria is made in the literature, and this is confirmed by our own experience. Only in those cases (about 1% of the vials used) where no vacuum is found in the vial of freeze-dried bacteria, the stock solution has to be discarded.

Literature reports discuss this dependance on the age of the reconstituted bacteria and it has been recommended to start the test within a few minutes after the reconstitution (10), and use the suspension for a few hours. Some authors use this reagent for only one hour (16) while some others find no relation between the age of the suspension and the response obtained (15). Our own findings confirm this conclusion and from our experience we conclude that the suspension of the reconstituted bacteria can be used for an average of 4 to 5 hours if it has been properly prepared. After 5 to 6 hours the light output is too low to perform any measurements and after 24 hours the remaining light intensity, if any, is not detectable by the instrument. Storing the suspension at low temperature does not make any difference in this context.

-Relation between response and exposure time

The time of exposure of Photobacterium phosphoreum to any substance has great influence on the type of response in the Microtox test. This effect is entirely dependant on the nature of the compound being tested.

There are some chemicals which require a longer exposure time to show their full toxic activity while other compounds are fully active within the first seconds of contact, and a third class of compounds for which an asymptotic approach to its maximum toxicity value is shown and longer exposure times have no influence or at least very small effect on the final value. Compounds with similar hydrophobicity, however, behave similarly in this respect, likely because of the similarity in transport across the cell membrane.

There is also a concentration effect on this dependance. The same substance can behave differently if solutions of different concentration are used (FIG. 11,12). On this basis, it is difficult to standardize this test in terms of exposure times, yet some normalization has to be achieved in order to be able to compare the results for different classes of toxic

substances. It is advisable, therefore, to run the test at 5, 15, and 30 min. exposure time if very different classes of compounds are to be tested.

The hydrophobic properties of most organic contaminants, as reflected by the octanol/water partition coefficient, are very comparable, i.e. within a range of 3 to 4 log P units, and therefore the test can be normalized in terms of exposure times for consistency of the toxicity values obtained and for the ranking of the chemicals according to the toxicity. In order to compare toxicities of different classes of compounds or individual contaminants, all Microtox values must be based on the same exposure time. In our experiments we record the 5, 15 and 30 min. Effective Concentrations causing a 50% reduction of light emission and we use consistently the 30-EC50 values for further calculations.

-Reaction types according to the test response

An aproximate classification of toxic substances can be achieved by looking at the response of Photobacterium phosphoreum after its exposure to the compound. In terms of response, as mentioned above,

three different reaction types can be distinguished (FIG. 13):

The first class of compounds will give an immediate response (FIG. 14-type I) with a distinct end point at the 50% reduction of light output.

Type II, in FIG. 14, shows a slow response with the result that longer exposure time will give increasing toxicity values. Finally, Type III shows an intermediate example with an asymptotic approach of the final value.

-Dependance of the light intensity on the bacteria population

The differences in response due to the number of bacteria present are of little consequence for the final value, as the response is much more dependant on the substance tested. It has been shown that using 20 µl of Reagent suspension instead of the standard recommended 10 µL., i.e., increasing the number of test organisms from 10^6 to 2×10^6 has no detectable effect on the final value for penicillic acid (15).

From our experience, even if the actual reading of the light output increases proportionally using when

higher concentration of bacteria, the final EC50 value remains unchanged.

RESULTS AND DISCUSION

-Comparison of the Microtox Test with other tests

The Microtox values for several classes of potential aquatic contaminants were determined (TABLE 2) according to the procedure described above and the toxicity values obtained are correlated with literature values on acute and sublethal effects of these chemicals on several species of fish, bacteria, and other biota. Prior to correlation analysis, the 30-EC50 and other toxicity values were converted to the negative logarithms [-log(mmol/L)]. (TABLES 3,4 and 5). Each set of compounds was chosen for its significance as water contaminants. TABLES 6,7 and 8 show the correlations found between the toxicity values obtained using the Microtox test and values reported in the literature from toxicity tests which use other aquatic organisms. Plots of these correlations are given in FIG. 14, 15 and 16, where the Microtox values are

plotted versus those of alternative tests.

-Structure-Activity correlations

The utility of the Microtox test is most evident where large numbers of toxicity data are needed. This is the case of our investigations on (QSAR) (Quantitative Structure-Activity Relationships), which is, as its name implies, the study of the relationships between a chemical's biological activity and its molecular structure. In this context, our goal is the modelling and prediction of the toxic effects of potential water contaminants on living organisms based on typical structural parameters of the compound.

A flow scheme for this type of study is given in FIG. 17. It indicates the various steps, such as selection of the set of chemicals to be studied, literature searches on toxicity data and structural parameters: experimental, physico-chemical and computed quantum mechanical data, and simple mathematical or topological indices. These values are fed into a computerized data bank along with our own Microtox values for the training set of compounds. An important aspect of the selection of the chemicals is the quality

of commercially available compounds and the need for purification by recrystalization or redistillation. If necessary, even high quality compounds have to be refined to prevent false results due to the presence of impurities, which could lead to erroneus toxicity determinations. The question of the chemicals' purity frequently is not addressed in the biological literature and therefore is always a potential problem with such data.

From all these data, a mathematical equation is then computed to model the toxicity of the compounds as a function of some of the structural parameters. The quality of the estimation is optimized and quantitated by means of a regression analysis program. The equation will have power to predict the toxicity of chemical compounds similar to those on the the training set.

New series of compounds are being tested in order to increase the number of equations and to be able to combine such results in favour of multidimensional QSAR equations with enhanced prediction power.

Our results regarding the Microtox Toxicity values for five series of polychlorinated aromatic compounds are summarized in TABLE 2.

The partition coefficient is a physico-chemical parameter closely related to the structure of a chemical

compound (34) as a quantitative measurement of its tendency to solubilize in two separable phases.

The octanol/water system has been chosen to simulate the biochemical transport mechanism, i.e the ability of a chemical compound to trespass the lipid membrane of a living organism thus being able to interfere actively in its metabolism. In this context, biological activity of chemical compounds has been found to correlate well with the logarithm of its octanol/water partition coefficient (35).

We have found that these theories apply as well to our results on the toxic effects of those compounds to the Photobacterium phosphoreum. The Microtox toxicity values correlate fairly well with the logarithm of the octanol/water partition coefficient. These and other correlations are summarized in TABLE 9.

The slope of all these regression lines is quite similar (Fig. 18). This suggests a fairly similar effect of these clases of compounds to the Photobacterium phosphoreum in terms of transport of the chemicals into the cell through its membrane. A preliminary conclusion can be drawn regarding the predominant effect of the chlorine substitution over the hetero substitution in the aromatic ring. Some facts, however, can be taken into consideration, from the study

of these equations:

(a) The addition of $(\log P)^2$ as a new independent variable increases the correlation coefficient in all cases except for the phenols, and the change is particularly noticeable in the case of pyridines. That can be only attributed to some electronic or steric property of the OH group which differs form the other fragments, and the contribution of the lone pair of electrons of the aromatic nitrogen. Differences in the interaction of these groups and water molecules are also suggested by this discrepancies.

(b) The position of the chlorine substitution plays an important role in the case of chlorobenzenes, -phenols and -nitrobenzenes as shown by the increase on the correlation coefficient in these cases after the inclusion of a new independent variable, S = number of symmetry planes for the molecules of chlorobenzenes, and N = number (0, 1 or 2) of chlorine atoms in ortho positions in the other series.

(c) The unchanging correlation in the case of the anilines has no inmediate explanation. However, it may be assumed that their toxic effects depend more strongly

on some other electronic property more typically related to the $-NH_2$ group, and less significant in the case of $-OH$ and $-NO_2$ groups.

The most common structural parameters reflecting substituent effects of these fragments, usually employed in Structure-Activity correlations, are shown in TABLE 10 being π, MR, R and F the most relevant in biomedicinal QSAR studies.

The values of the field effect F for the fragments considered, suggest that the contribution of the NH_2 substituent will differ from the contribution of the NO_2 and OH groups for which the field effects are more similar. However, the inclusion of this effect, as a variable in the correlations shown above presents some difficulties, as this value would have to be the same along the same family, disregarding differences in chlorine substitution. This is the subject of future investigations.

(d) Finally, the addition of pKa as a second independent variable gives good results in terms of increasing the correlation coefficient only in the case of the chlorophenol congeners. This may suggest stronger pH dependance of the mechanism of action for this series than for both the anilines and pyridines.

CONCLUSIONS

The high correlations found for the toxicities of several sets of contaminants to the Photobacterium phosphoreum in the Microtox test let us conclude that this test is very useful as a preliminary screening test. It is capable of providing toxicity values for a large number of potential toxicants in a short time at a low cost and with good accuracy, precision and reproducibility.

For compounds not yet tested the test gives a quick toxicity measurement that allows the ranking of that compound with others in the data bank. For compounds already knownn to be contaminants, the determination of Microtox values is an useful and characteristic property which is part of the fundamental data used for the prediction of toxicity and hazard assessment of new toxicants on the basis of structural parameters of the compounds.

One of the major problems in QSAR investigations is the consictency and comparability of the toxicity values on which the model is based. For many compounds the literature sources do not provide information to

determine whether or not the data are reliable. The ideal situation would be that each QSAR research group made its own toxicity measurements to minimize inter-laboratory discrepancies of experimental conditions and hence absolute toxicity values. The Microtox system has provided us with a reliable yet quick source of toxicity measurements which should be virtually free of such inter-laboratory data normalization problems.

The QSAR equations presented show a strong dependance of the toxic effects of the five series of chlorine substituted aromatic compounds on the number of chlorine atoms and consequently on the partition coefficient and this dependance seems to be stronger than the effect that the hetero group may have on the toxicity of the compound.

The nearly parallel slope of the regression lines for the various sets, suggests similar transport dynamics through the cell membrane of Photobacterium phosphoreum, hence similar mechanisms of the toxic effects of these compounds on that species.

This correlation seems to be broken for compounds with higher partition coefficients, especially when a Nitrogen atom is present. This can be the result of the low solubility involved, giving as a consequence, a

toxicity value lower than expected if a linear relationship is considered. Studies are currently under way in order to develop a solvent system that will provide sample concentrations high enough to find toxicity values of chemical compounds with no side effects due to the toxicity of the solvent.

This and the search for new parameters providing better description of the structure of the chemical compounds tested will contribute to an improved use of QSAR in the assessment and prediction of the toxicity of organic contaminants to the aquatic environment.

REFERENCES

1 - Maugh, T.H. II: "Chemicals: How Many Are There?". Science 199, 162 (1978).

2 - Serat W.F., Budinger F.E.,Jr., and Mueller P.K.: "Evaluation of Biological Effects of Air Pollutants by Use of Luminescent Bacteria". J. Bact. 90, 832-833 (1965).

3 - Chang, J.C., Taylor P.B., and Leach F.R.: "Use of the Microtox Assay System for Environmental Samples". Bull. Environm. Contam. Toxicol. 26, 150-156 (1981), (and references therein).

4 - Serat W.F., Kyono J., and Mueller P.K.: "Measuring the Effect of Air Pollutants on Bacterial Luminescence: A Simplified Procedure". Atmos. Environ. 3, 303-309 (1969).

5 - Bulich A.A. and Greene M.W.: "The Use of Luminescent Bacteria for Biological Monitoring of Water Quality". Proc. Int. Symp. Analyt. Appl. Bioluminescence Chemiluminescence. E. Schram and P. Stanley (Eds.) pp. 193-211. (1978).

6 - Bulich A.A.: "Use of Luminescent Bacteria for Determining Toxicity in Aquatic Environments". in Aquatic Toxicology: 2nd Conf. ASTM STP STP 667, 98-106 (1979).

7 - Bulich A.A. and Isenberg D.L.: "Use of the Luminescent Bacterial System for the Rapid Rapid Assessment of Aquatic Toxicity". ISA Transactions 20(1), 29-33 (1981).

8 - Bulich A.A.: "A Practical and Reliable Method for Monitoring the Toxicity of Aquatic Samples". Process Biochem. 45-47 (1982).

9 - Beckman Instruments Inc.: "Microtox System: Operation Manual". (1982).

10 - Shiotsuka R.N., Hegyel A.F., Gibbs P.H., and Siggins B.A.: "A Short-Term Toxicity Screening Using Photobacteria. A Feasibility Study". Report 1980 U.S. Army Med. Bioeng. Res. Lab. USAMBRDL-TR-8002; Gov. Rep. Announc. Index (U.S.) 80(23), 4924-4987 (1980).

11 - Dutka B.J. and Kwan K.K.: "Comparison of Three Microbial Toxicity Screening Tests with the Microtox Test". Bull. Environm. Contam. Toxicol 27, 753-757 (1981).

12 - Lebsack M.E., Anderson A.D., DeGraeve G.M., and Bergman H.L.:"Comparison of Bacterial Luminescence and Fish Bioassay Results for Fossil-Fuel Process Waters and Phenolic Constituents". Aquatic Toxicol. and Hazard Assess.: 4th.Conf. ASTM STP 737, 348-356 (1981)

13 - Curtis C., Lima A., Lozano S.J., and Veith G.D.: "Evaluation of a Bacterial Bioluminescence Bioassay as a Method for Predicting Acute Toxicity of Organic Chemicals to Fish". Aquatic Toxicol. and Hazard Assess.: 5th Conf. ASTM STP 766, 170-178 (1982).

14 - Qureshi A.A.,Flood K.W.,Thompson S.R.,Janhurst S.M. Inniss C.S., and Rokosh D.A.: "Comparison of a Luminescent Bacterial Test with Other Bioassays for Determining Toxicity of Pure Compounds and Complex Effluents". Aquatic Toxicol.and Hazard Assess., 5th. Conf. ASTM STP 766, 179-195 (1982).

15 - Yates I. T. and Porter J. K. : "Bacterial Bioluminescence as a Bioassay for Mycotoxins". Appl. Environ. Microbiol. 44, 1072-1075 (1982).

16 - Bulich A.A., Greene M.W., and Isenberg D.L: "Reliability of the Bacterial Luminescence Bioassay for the Determination of the Toxicity of Pure Compounds and Complex Effluents." Aquatic Toxicol. and Hazard Assess., 4th. Conf. ASTM STP 737, 338-347 (1981).

17 - Ribo J.M. and Kaiser K.L.E.: "Effects of Selected Chemicals to Photoluminescent Bacteria and Their Correlations with Acute and Sublethal Effects on Other Organisms". Chemosphere 12,1421-1442 (1983).

18 - Ribo J.M. and Kaiser K.L.E.:"Toxicities of Aniline Derivatives to Photobacterium phosphoreum and Their Correlation with Effects on Other Organisms and Structural Parameters". Proc. Workshop QSAR on Environmental Toxicology, Hamilton, Ont.,1983. K.L.E. Kaiser (Ed.) D. Reidel Publishing Co., Dordrecht, Holland, in press.

19 - DeZwart D. and Slooff W.: "The Microtox as an Alternative Assay in the Acute Toxicity Assessment of Water Pollutants". Aquatic Toxicology 4, 129-138 (1983).

20 - Krebs F.: "Toxicity Test Using Freeze-Dried Luminescent Bacteria". Gewasserschutz, Wasser, Abwasser 63, 173-230 (1983), (and refs. therein).

21 - Cairns J. Jr., Bukema A.L. Jr., Heath A.G. and Parker B.C.: "Effects of Temperature on Aquatic Organism Sensitivity to Selected Chemicals". Virginia Water Resources Research Center. Bulletin 106. (1978).

22 - Johnson F.H., Brown D., and Marshland D.: "A Basic Mechanism in the Biological Effects of Temperature, Pressure and Narcotics." Science 95, 200-203 (1942).

23 - Kaiser K.L. E., Hodson P.V., and Dixon D.G.: "Quantitative Structure-Activity Relations of Lethal and Sublethal Effects of Benzene and Phenol Derivatives on Rainbow Trout (Salmo gairdneri)". Proc. 9th. Aquatic Toxicology Workshop. Edmonton, 1982 (in press).

24 - Buccafusco R.J., Ells S.J., and LeBlanc G.A.: "Acute Toxicity of Priority Pollutants to Bluegill (Lepomis macrochirus)". Bull. Environm. Contam. Toxicol. 26, 684-691 (1980).

25 - Heitmuller P.T., Hollister T.A., and Parrish P.R.: "Acute Toxicity of 54 Industrial Chemicals to Sheepshead Minnows (Cyprinodon variegatus)". Bull. Environm. Contam. Toxicol. 27, 596-604 (1981).

26 - Koenemann H.: "Quantitative Structure-Activity Relationships in Fish Toxicity Studies. Part I: Relationship for 50 Industrial Pollutants". Toxicology 19, 209-221 (1981).

27 - LeBlanc G. A.: "Acute Toxicity of Priority Pollutants to Water Flea (Daphnia magna)". Bull. Environm. Contam. Toxicol. 24, 684-691 (1980).

28 - Liu D., Thomson K.,and Kaiser K.L.E.: "Quantitative Structure-Toxicity Relationship of Halogenated Phenols on Bacteria". Bull. Environm. Contam. Toxicol. 29, 130-136 (1982).

29 - Yasuda Y., Tochikubo K., Hachisuka Y., Tomida H., and Ikeda K.: "Quantitative Structure-Inhibitory Activity Relationships of Phenols and Fatty Acids for Bacillus subtilis Spore Germination". J. Med. Chem. 25, 315-320 (1982).

30 - Hattula M.L., Wasenius V.M., Reunanen H., and Arstila A.U.: "Acute Toxicity of Some Chlorinated Phenols, Catechols and Cresols to Trout". Bull. Environm. Contam. Toxicol. 26, 295-298 (1981).

31 - McLeese D.W., Zitko V.,and Peterson M.R.:"Structure Lethality Relationships for Phenols, Anilines and Other Aromatic Compounds in Shrimps and Clams". Chemosphere 2, 53-57 (1979).

32 - Kwasniewska K. and Kaiser K.L.E.: "Toxicities of Selected Chloro-Anilines to Four Strains of Yeast". Proc. Workshop QSAR on Environmental Toxicology, Hamilton, Ont., 1983. K.L.E.Kaiser (Ed.), D.Reidel Publishing Co., Dordrecht, Holland, in press.

33 - Hermens J.: "Quantitative Structure-Toxicity Relationships and Mixture Toxicity Studies of Aquatic Pollutants". Ph.D. Thesis Rijksuniversiteit Utrech. The Netherlands. 1983.

34 - Kaiser K.L.E.: "A Non-Linear Function for the Approximation of Octanol / Water Partition Coefficients of Aromatic Compounds with Multiple Chlorine Substitution". Chemosphere 12 1159-1167 (1983).

35 - Chiou C.T.: "Partition Coefficient and Water Solubility" in "Hazard Assessment of Chemicals". J. Saxena and F. Fisher (Eds.), Academic Press, New York 1981.(and references therein).

36 - Hansch C. and Leo A.J.: "Substituent Constants for Correlation Analysis in Chemistry and Biology". Wiley Interscience, New York, (1979).

37 - Perrin D.D., Dempsey B., and Serjeant E.P.: "pKa Prediction for Organic Acids and Bases". Chapman & Hall, New York (1981).

38 - Topsom R.D.: "Contribution of Theoretical Chemistry to an Understanding of Electronic Substituent Effects". Acc. Chem. Res. 16, 292-298 (1983).

AKNOWLEDGMENTS

I thank the National Water Research Institute and the Great Lakes Water Quality and Toxic Chemicals Management Programs for financial support, Dr. R.J. Allan, Chief Environmental Contaminants Division, and Dr. K.L.E. Kaiser, Head, Organics-Properties Section, for helpful discussions, and Mr. H. Hunter and Mr. B.M. Zaruk for their assistance with the Microtox tests.

Table 1.- Microtox Test: Data for 4-Chloroaniline

	Concentration (ppm.)	Final Readings 0 min.	5 min.	15 min.	30 min.	Gamma Function (Effect) 5 min.	15 min.	30 min.
0	0 (blank)	94	81	76	69	--	--	--
1	1.17	86	55	53	49	0.35	0.31	0.29
2	2.33	86	45	44	42	0.65	0.58	0.50
3	4.67	88	33	33	31	1.30	1.16	1.08
4	9.33	87	22	21	20	2.41	2.35	2.19

Table 2. MICROTOX Toxicity Values [p(30-EC50)] for Different Chloro-substituted Aromatic Compounds

Substit.	Benzenes(a)	Phenols(a)	Anilines(b)	Nitrobenzenes	Pyridines
--	0.02	0.42	0.12	0.55	-0.97
2	1.00	0.58	0.91	1.56	0.20
3	--	0.96	0.96	0.90	0.21
4	--	1.19	1.40	0.82	--
2,3	1.56	1.52	1.77	2.12	0.63
2,4	1.46	1.47	1.54	1.78	--
2,5	1.44	1.24	1.62	1.35	0.20
2,6	--	1.09	1.97	--	0.25
3,4	--	2.00	--	1.28	--
3,5	--	1.77	1.14	1.05	0.32
2,3,4	1.76	2.20	1.92	1.90	--
2,3,5	1.66	2.25	--	--	--
2,3,6	--	1.19	--	--	--
2,4,5	--	2.19	2.12	1.63	--
2,4,6	1.11	1.41	1.63	2.41	--
3,4,5	--	2.74	1.67	--	--
2,3,4,5	1.73	3.12	2.28	2.27	--
2,3,4,6	1.94	2.26	--	--	--
2,3,5,6	1.68	2.02	2.11	1.50?	--
2,3,4,5,6	--	2.71	1.35?	1.89	--

(a) From ref. 17. (b) From ref. 18.

Table 3. Toxicities of CHLOROBENZENES to Photobacterium phosphoreum (Microtox test, 30 min EC50), Rainbow trout (Salmo gairdneri, 96 hr LD-50, ref.23), Bluegill (Lepomis macrochirus 96 hr LC50, ref.24), Sheepshead minnows (Cyprinodon variegatus 96 hr LC50, ref.25), Guppy (Poecilia reticulata, ref.26), and Daphnia magna (48 hr LC50, ref.27). All values given as logarithms of the inverse concentrations (conc. in mmol/L).

Substit.	Microtox	Rainbow tr.	Bluegill	Sheepshead min.	Guppy	DAPHNIA
--	0.02	1.59	--	--	0.09	--
mono	1.00	2.02	0.85	1.05	0.77	0.12
1,2	1.56	2.13	1.42	1.18	1.40	1.79
1,3	1.46	2.00	1.47	1.28	1.30	0.72
1,4	1.44	1.99	1.53	1.30	1.57	1.13
1,2,3	1.76	2.05	1.73	--	1.89	--
1,2,4	1.66	2.01	--	0.94	1.88	0.56
1,3,5	1.11	1.52	--	--	1.74	--
1,2,3,4	1.73	2.31	--	--	2.43	--
1,2,3,5	1.94	2.11	1.53	1.77	2.43	1.34
1,2,4,5	<1.68	<1.66	2.13	2.43	2.85	-0.39
1,2,3,4,5	--	2.24	3.00	2.50	3.15	1.67

Table 4. Toxicities of CHLOROPHENOLS to Photobacterium phosphoreum (Microtox test, 30 min.EC50), Bacterial dehydrogenase activity (Bacillus sp., IC50, ref. 28), Spore germination (Bacillus subtilis, I50, ref. 29), Brown trout (Salmo trutta, 24hr-LC50, ref. 30), Bluegill (Lepomis macrochirus, 24hr-LC50, ref. 24), Guppy (Poecilia reticulata, 7-14 days-LC50, ref. 26), Water flea (Daphnia magna, 24hr-LC50, ref. 27), and Shrimp (Crangon septemspinosa, lethal threshold 96hr-LT50, ref. 31). All values are given as negative logarithms of the concentration. (conc. in mmol/L).

Subst.	Microtox	BACILLUS	Spore	Trout	Bluegill	Guppy	DAPHNIA	Shrimp
--	0.42	-1.39	-0.18	--	--	0.50	--	1.10
2	0.58	-0.74	0.25	--	1.25	1.06	<0.77	1.39
3	0.96	-0.54	--	--	--	1.30	--	--
4	1.19	-0.49	0.10	--	1.51	--	1.17	1.45
2,3	1.52	0.10	--	--	--	--	--	--
2,4	1.47	0.34	0.62	1.98	1.54	1.59	<1.21	--
2,5	1.24	0.28	--	--	--	--	--	--
2,6	1.09	-0.53	--	1.61	--	--	--	0.93
3,4	2.00	0.50	--	--	--	--	--	--
3,5	1.77	0.81	--	--	--	1.78	--	2.04
2,3,4	2.20	1.18	--	--	--	--	--	2.00
2,3,5	2.25	1.30	--	2.39	--	2.10	--	--
2,3,6	1.19	0.02	--	--	--	1.59	--	1.86
2,4,5	2.19	1.22	0.89	2.34	2.51	--	1.72	--
2,4,6	1.41	-0.08	0.89	--	2.44	--	1.10	--
3,4,5	2.74	1.60	--	--	--	2.24	--	--
2,3,4,5	3.12	1.76	--	--	--	2.48	--	--
2,3,4,6	2.26	--	0.57	2.60	3.09	--	<2.37	1.29
2,3,5,6	2.02	0.63	--	--	2.76	2.23	1.97	--
2,3,4,5,6	2.71	1.47	0.46	3.13	--	2.85	2.25	1.91

Table 5. Toxicities of CHLOROANILINES to Photobacterium phosphoreum, (Microtox test, 30-EC50), four different yeast strains (Growth inhibition, IC50, ref. 32) and to Guppy (Poecilia reticulata, 14 days-LC50, ref. 33).

Substit.	Microtox p30-EC50	Pichia sp.	Sacch. cerev.	Rhod. rubra	Rhod. sp.	Guppy p LC50
--	0.12	-0.03	-0.03	-0.63	-0.03	-3.13
2	0.91	--	--	--	--	-1.69
3	0.96	--	--	--	--	-2.02
4	1.40	0.20	0.11	0.04	-0.07	-2.31
2,3	1.77	--	--	--	--	--
2,4	1.54	0.49	0.13	0.97	0.91	-1.59
2,5	1.63	--	--	--	--	-1.01
2,6	1.97	0.27	0.40	0.48	0.39	--
3,5	1.19	0.78	0.99	1.10	0.75	-1.38
2,3,4	1.92	1.21	0.98	1.21	1.09	-0.85
2,4,5	2.12	--	--	--	--	-1.00
2,4,6	1.63	--	--	--	--	--
3,4,5	1.77	--	--	--	--	--
2,3,4,5	2.37	--	--	--	--	-0.19
2,3,5,6	2.16	--	1.36	1.36	1.20	--
3,3,4,5,6	1.35	0.16	0.80	0.36	0.36	--

Table 6. Correlation Between Microtox and Other Toxicity Values for Chloro-Substituted Benzenes

Species	Test Type	n	r^2
Rainbow trout (Salmo gairdneri)	Acute Toxicity Intraperitoneal Injection IPLD50	9	0.74
Bluegill (Lepomis marochirus)	Acute Toxicity 96 hr LC50 Static Test	6	0.71
Sheepshead Minnow (Cyprinodon variegatus)	96 hr LC50 Static Test.	5	0.80
Guppy (Poecilia reticulata)	7-14 days LC50 Static Test	9	0.86
Water Flea (Daphnia Magna)	48 hr LC50 Static Test	4	0.83

Table 7.Correlation Between Microtox and Other Toxicity Values for Chloro-Substituted Phenols

Species	Test	n	r^2
Bacillus sp	Dehydrogenase activity 50% inhibition IC50	19	0.93
Bacillus subtilis	Spore germination 50% reduction I50	6	0.73
Brown trout (Salmo Trutta)	Acute toxicity 24 hr LC50 Static Test	6	0.92
Bluegill (Lepomis macrochirus)	Acute toxicity 24 hr LC50 Static Test	7	0.77
Guppy (Poecilia reticulata)	Semichronic toxicity 7-14days LC50 Static Test	11	0.89
Water Flea (Daphnia magna)	Acute toxicity 24 hr LC50 Static Test	8	0.87
Shrimp (Crangon septemspinosa)	Lethal Threshold 96 hr LT50 Static Test	7	0.68

Table 8. Correlation Between Microtox and Other Toxicity values for Chloro-Substituted anilines.

Yeast Species	Test type	n	r^2
Pichia sp.	Growth inhibition	6	0.49
Saccharomyces cervisae	Growth inhibition	5	0.89
Rhodotorula rubra	Growth inhibition	6	0.83
Rhodotorula sp.	Growth inhibition	6	0.86
Guppy (Poecilia reticulata)	Acute toxicity 14 day LC50	9	0.79

Table 9.- QSAR of polychlorinated aromatic compounds.

Compounds:	log (1/30-EC50)=	n	r^2
Benzenes			
-0.979 + 0.631	log P	11	0.76
-4.119 + 2.578	log P - 0.286 $(\log P)^2$	11	0.82
0.146 + 0.412	log P - 0.174 S	11	0.95
Phenols			
-0.679 + 0.678	log P	20	0.79
-0.463 + 0.538	log P + 0.020 $(\log P)^2$	20	0.78
-0.802 + 0.805	log P - 0.325 N	20	0.89
-4.203 + 1.041	log P + 0.307 pKa	20	0.88
Anilines			
-0.041 + 0.570	log P	15	0.80
-0.891 + 1.259	log P - 0.126 $(\log P)^2$	15	0.85
-0.009 + 0.528	log P + 0.095 N	15	0.81
0.300 + 0.480	log P - 0.055 pKa	15	0.81
Nitrobenzenes			
-0.549 + 0.656	log P	15	0.52
-2.437 + 1.931	log P - 0.207 $(\log P)^2$	15	0.55
0.155 + 0.339	log P + 0.351 N	15	0.63
Pyridines			
-0.936 + 0.580	log P	7	0.61
-2.582 + 3.010	log P - 0.749 $(\log P)^2$	7	0.94
-0.931 + 0.543	log P + 0.088 N	7	0.62
-0.645 + 0.429	log P - 0.043 pKa	7	0.64

S = Number of planes of symmetry
N = Number of chlorine atoms in ortho positions
log P (ref. 36)
pKa (ref.37).

Table 10. Electronic and steric fragment constants.

	π	H-Bonding Accept	H-Bonding Donor	MR	R	F	σp	σm	Es	σx
-H	0	0	0	1.03	0	0	0	0	0	0
-OH	-0.67	1	1	2.85	-0.64	0.29	0.12	-0.37	-0.55	0.31
-NH2	-1.23	1	1	5.42	-0.68	0.02	-0.16	-0.66	-0.61	0.43
-NO2	-0.28	1	0	7.36	-0.16	0.67	0.71	0.78	-1.77	0.40

(All values from ref. 36 except σx: ref 38)

π = Hydrophobicity.
MR = Molecular Refractivity.
R = Resonance Effect.
F = Field Effect.
σm, σp = Hammet Constants.
Es = Taft's Steric Parameters.
σx = Substituent Electronegativity Effect.

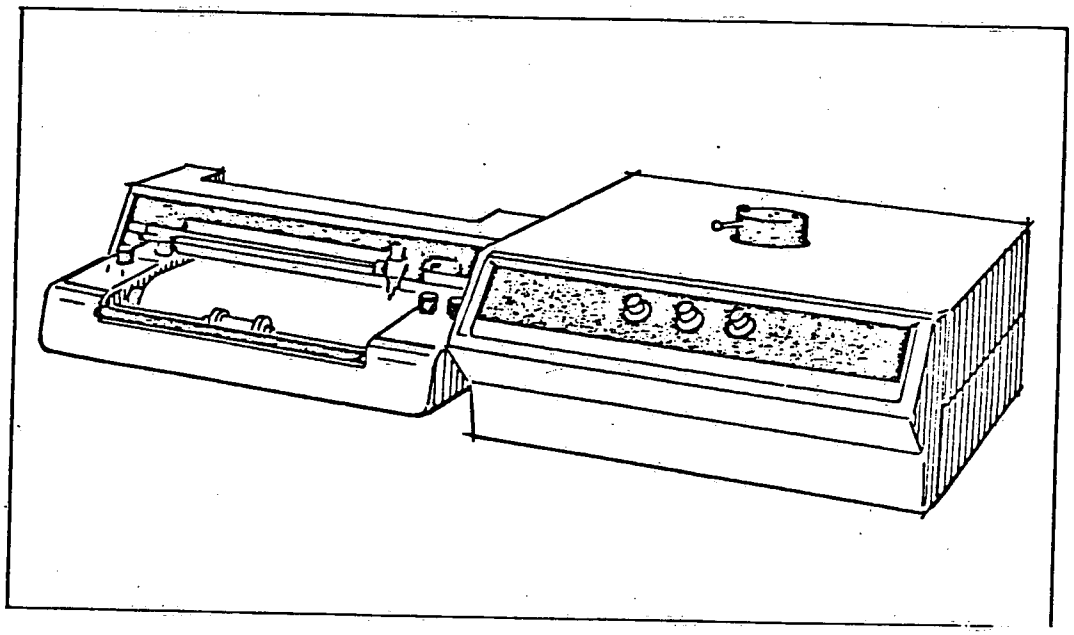


Fig.1.- MICROTOX TEST: Microtox Toxicity Analyzer and Recorder set up.

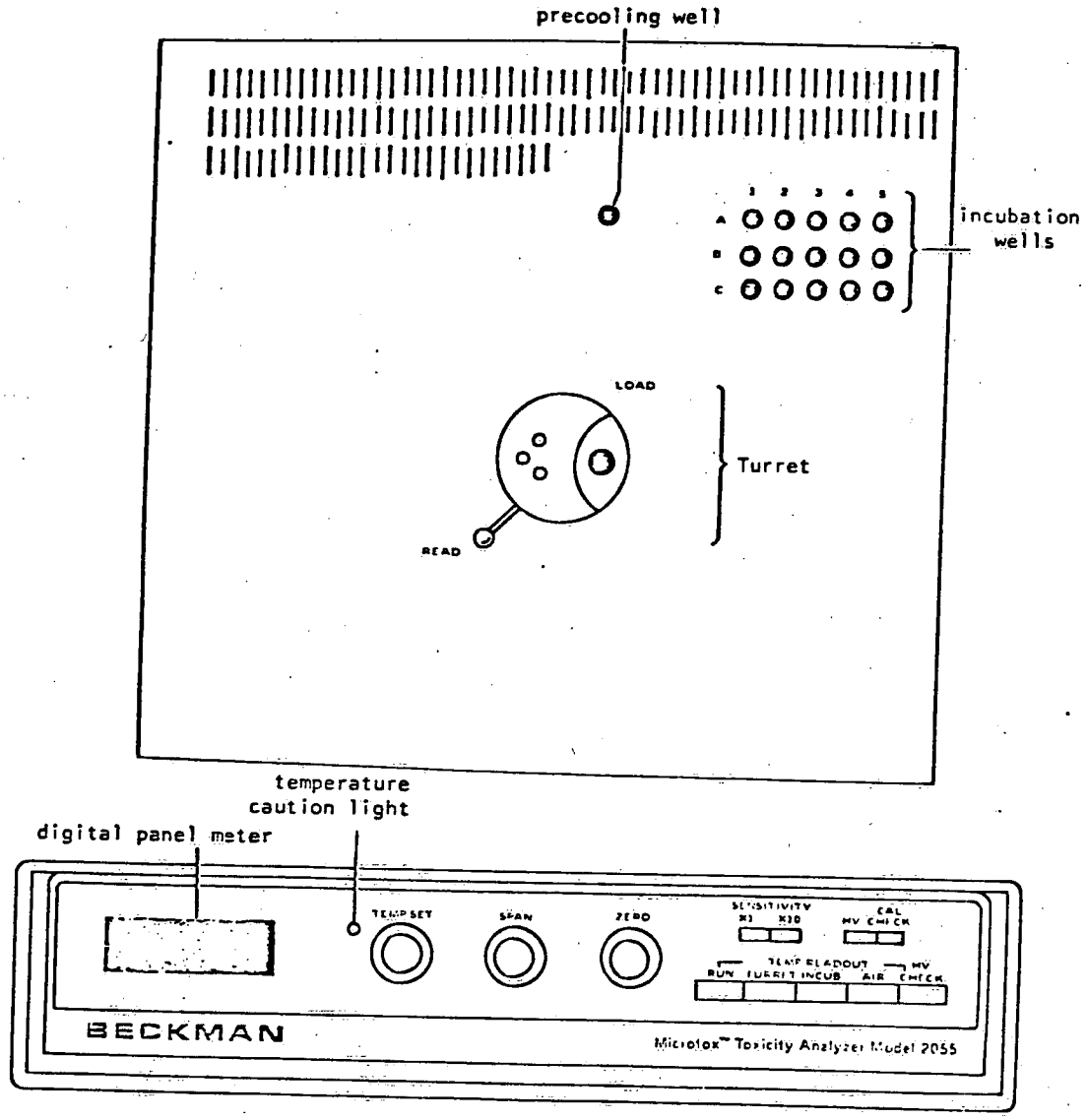


Fig.2.- Microtox Toxicity Analyzer: view of the front panel and the temperature and reding control knobs.

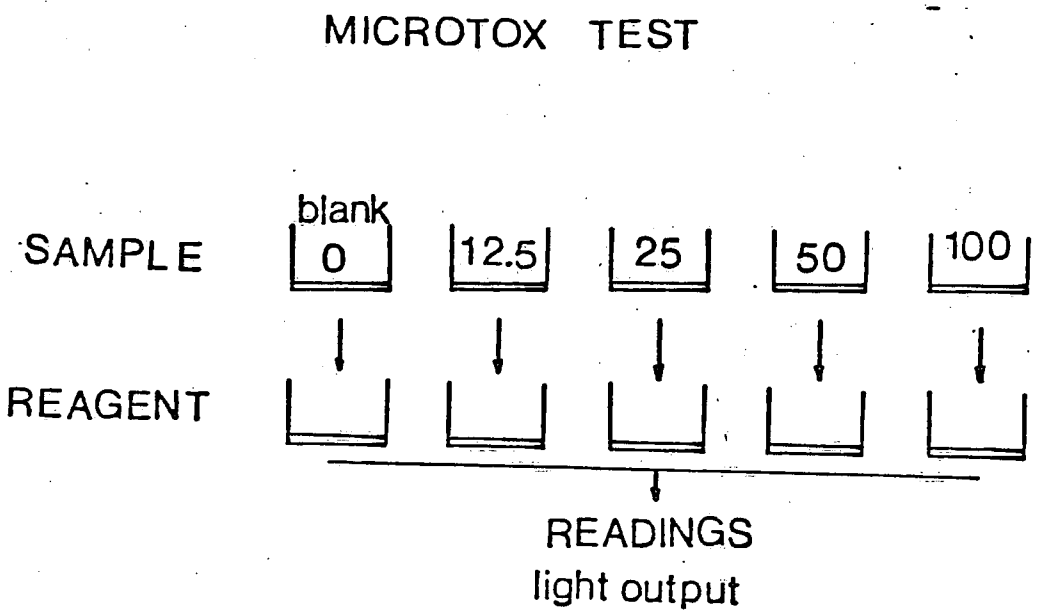


Fig.3.- MICROTOX TEST PROCEDURE: Scheme showing the sample dilutions and its addition to the reagent. [Sample concentrations in %]

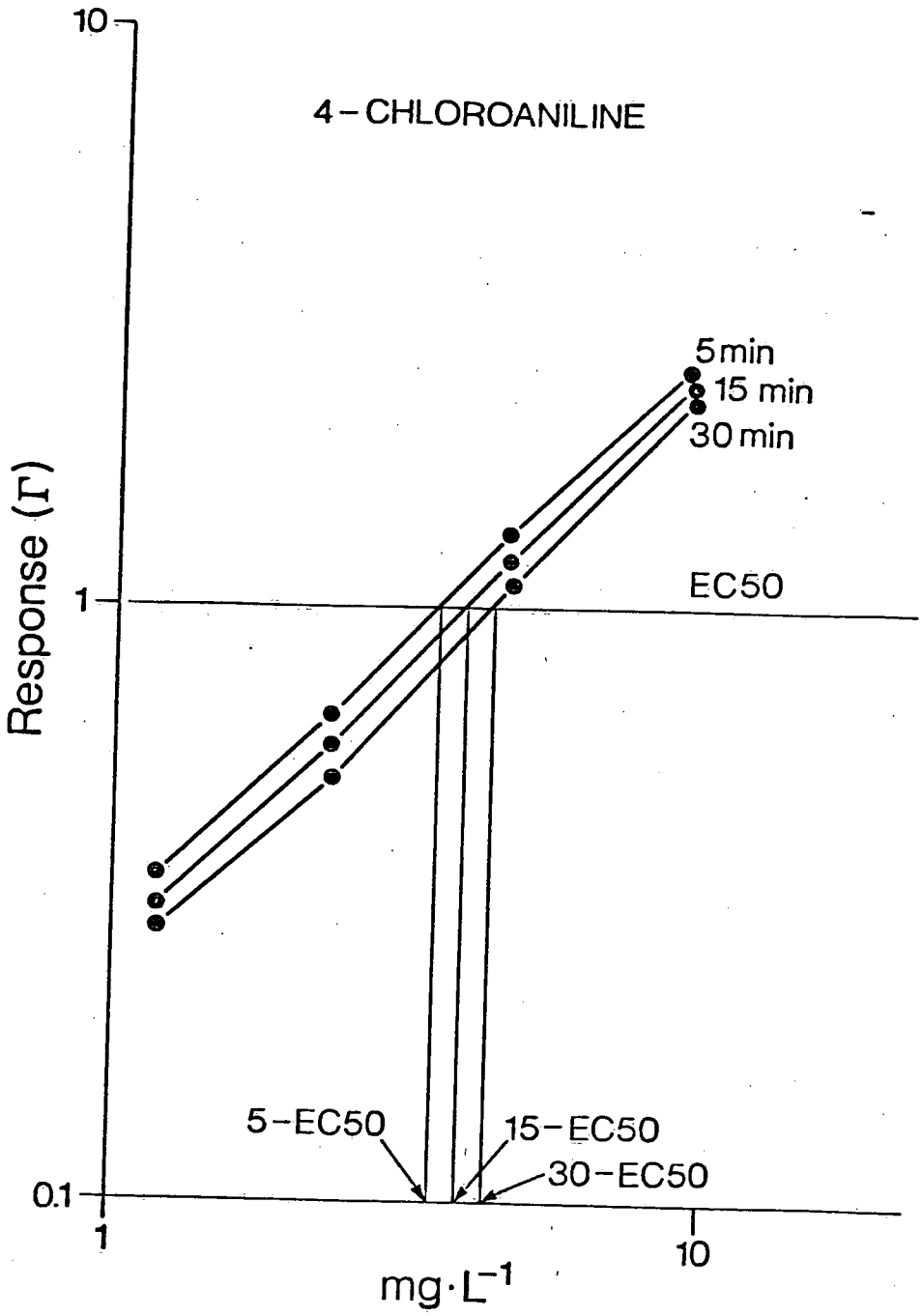


Fig. 4.- DOSE - RESPONSE CURVE FOR 4-CHLORDANILINE FROM THE MICROTOX TOXICITY TEST. (Table 1)

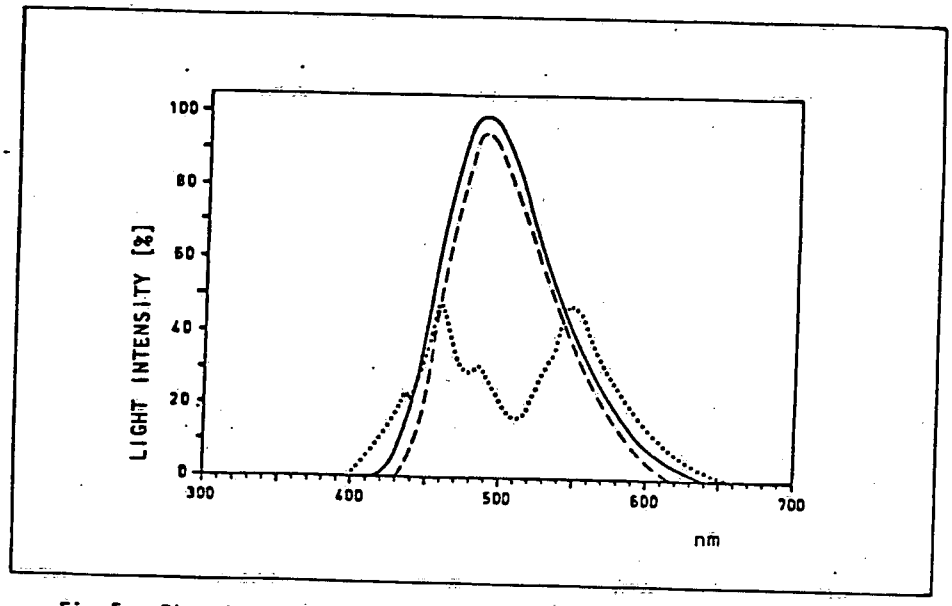


Fig.5.- Photobacterium phosphoreum: Emission spectrum at 15°C

----- Fresh bacteria culture

- - - Rehydrated freeze-dried bacteria

..... Liophilized bacteria (freeze-dried form)

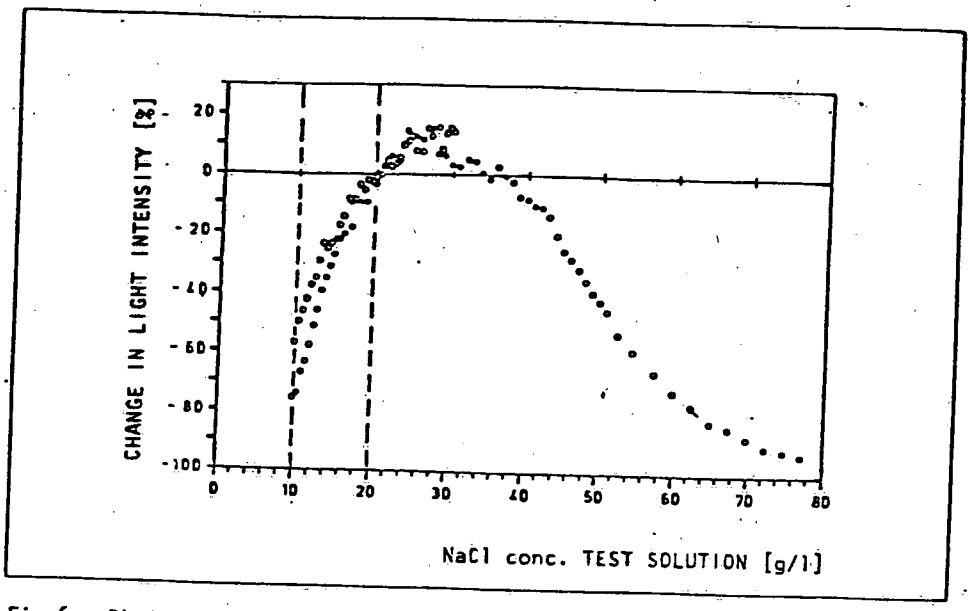


Fig.6.- Photobacterium phosphoreum: Relation between the change in light intensity and the NaCl concentration of the Final Test Solution. o,●: Different bacteria batches. Temp.: 15°C. Exposure time: 15 min.

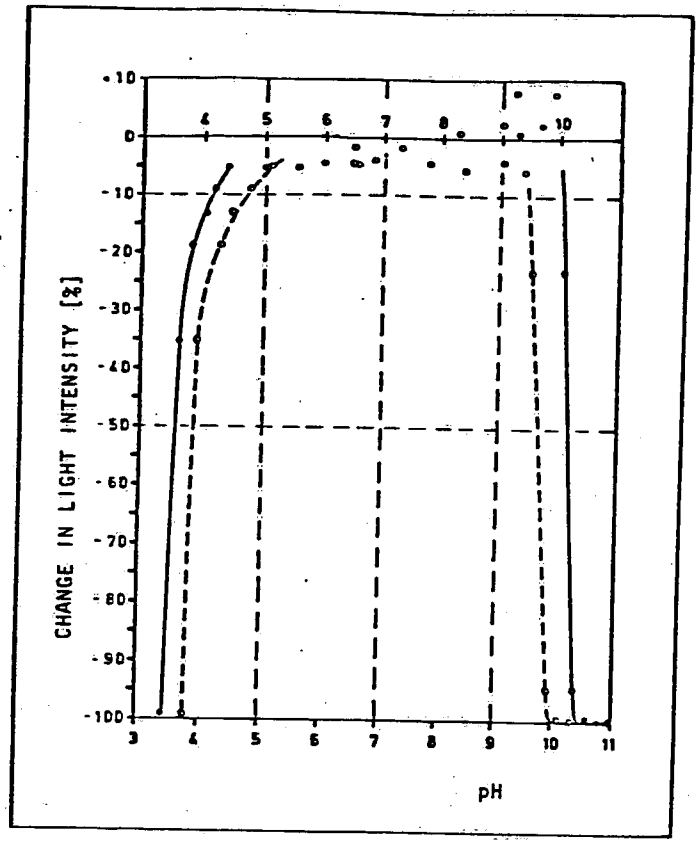


Fig.7.- Photobacterium phosphoreum: Relation between change in light intensity and pH of the Primary Sample Solution (- - -) and the Final Test Solution (----). Temp.: 15°C. Exposure time: 15 min.

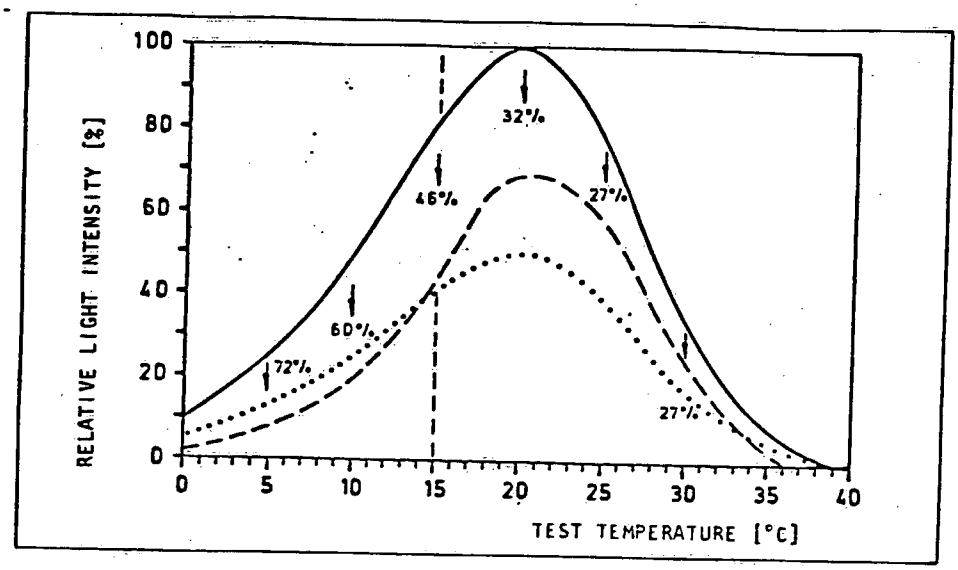


Fig.8.- Photobacterium phosphoreum: Light intensity dependance on the test temperature; Control (----) and Test (- - -) solutions.
(....) Theoretical curve of 50% reduction.
[Sulfanilamide 0.0025 mol/l]

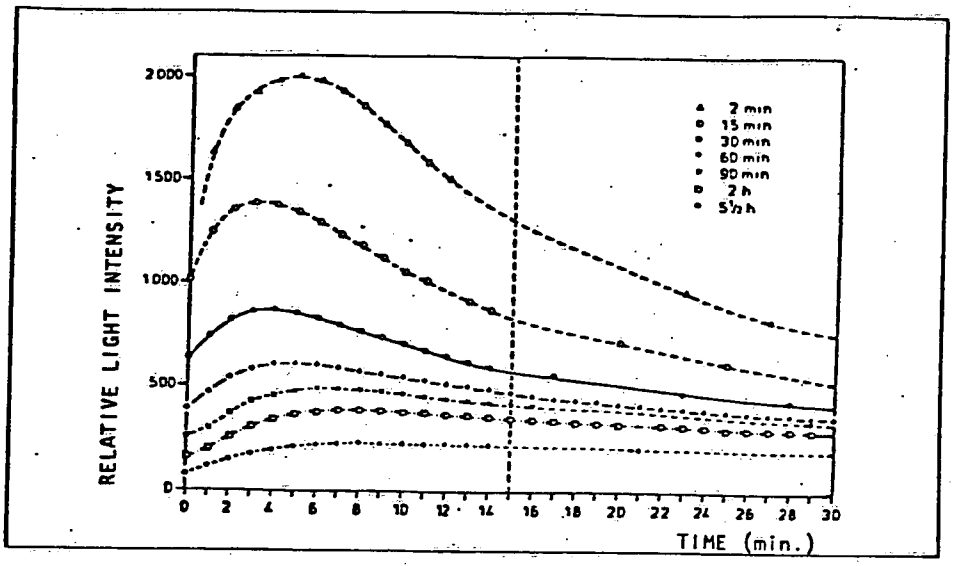


Fig.9.- Photobacterium phosphoreum: Light intensity dependance on the exposure time related to the age of the reconstituted bacteria. [0.5mL 2% NaCl + 0.01mL reagent suspension]

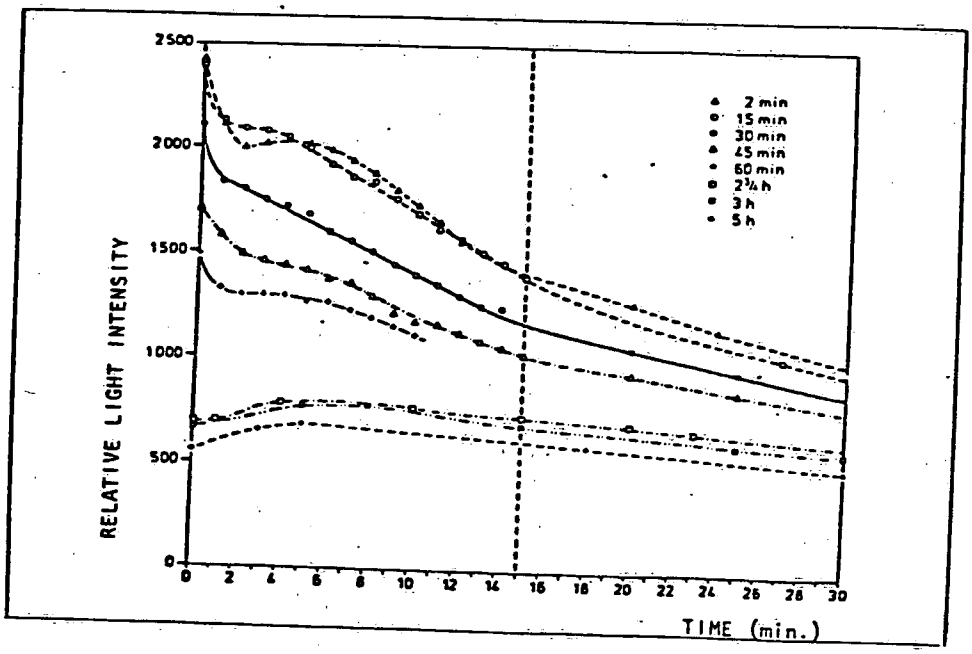


Fig.10.- Photobacterium phosphoreum: Light intensity/time dependance.
[identical Fig.9. Different batch of bacteria]

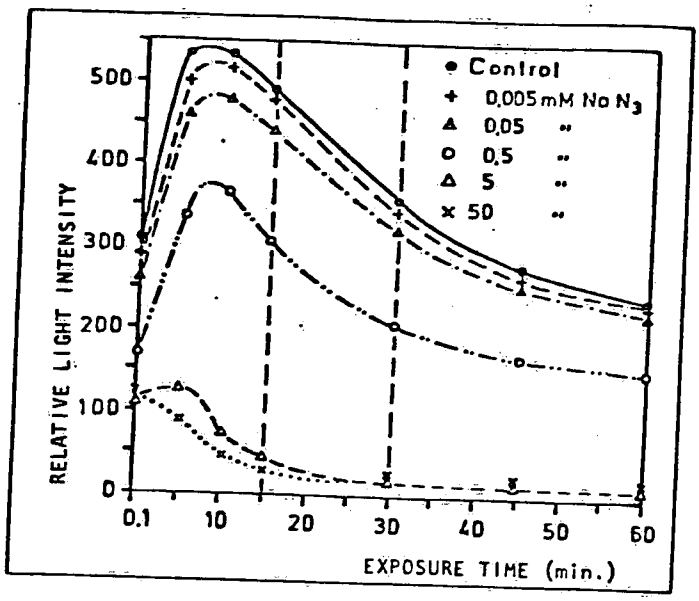


Fig.11.- Photobacterium phosphoreum: Relative light intensity as function of the exposure time for different concentrations of sample.

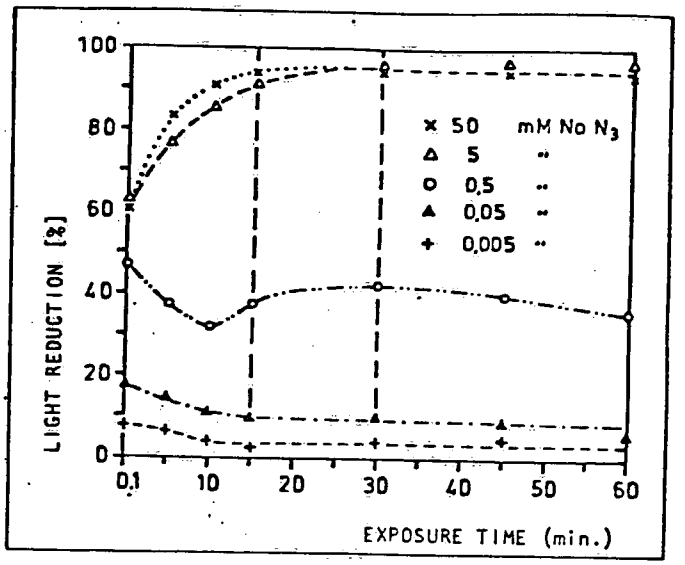


Fig.12.- Photobacterium phosphoreum: Dependance of the light reduction on the exposure time, for different concentrations of sample.

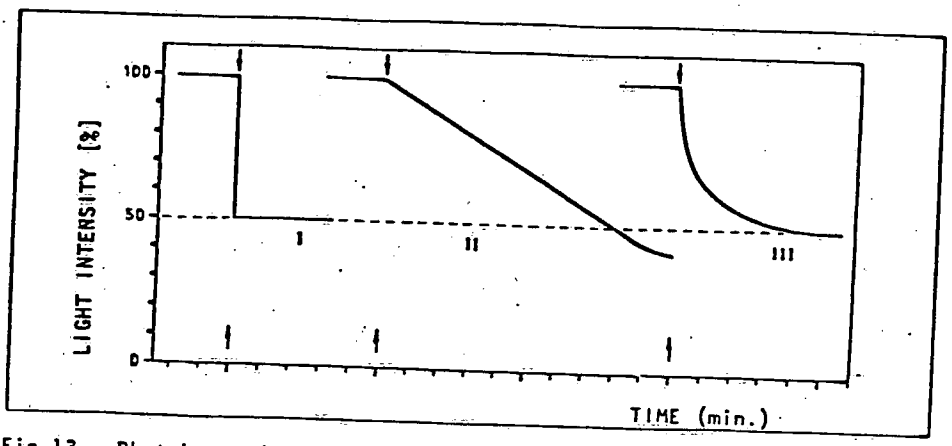


Fig.13.- Photobacterium phosphoreum: Toxic effect-time dependance for three different reaction types:

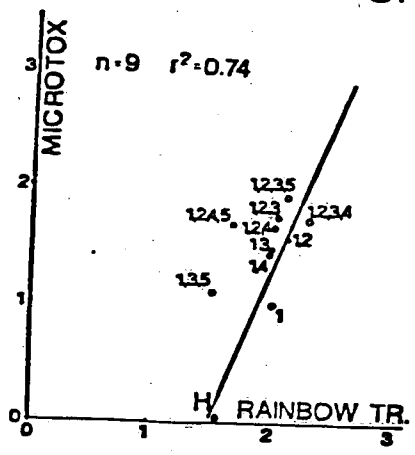
I : Inmediate response. e.g. Phenol

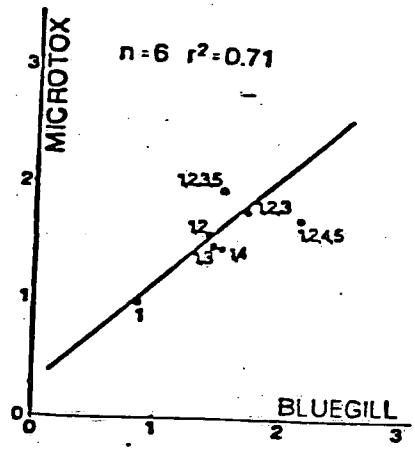
II : Slow and constant increase of the light reduction effect with time. e.g. metals: Hg,Zn,Cr.

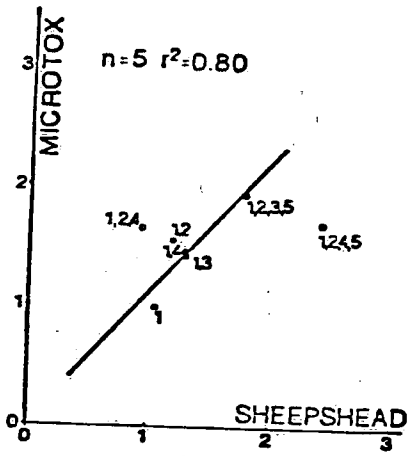
III: Hyperbolic time/response curve with asintotic end point. e.g. mixtures, waste water, Na dodecyl sulfate.

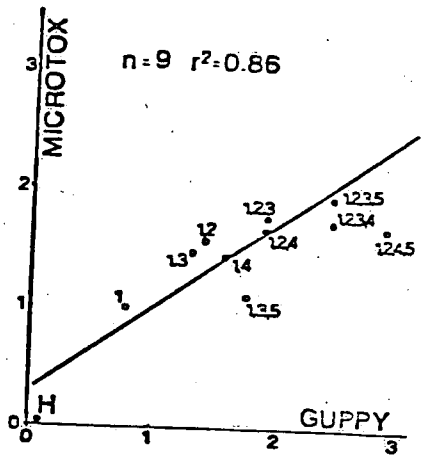
→ Exposure starting time.

CHLOROBENZENES









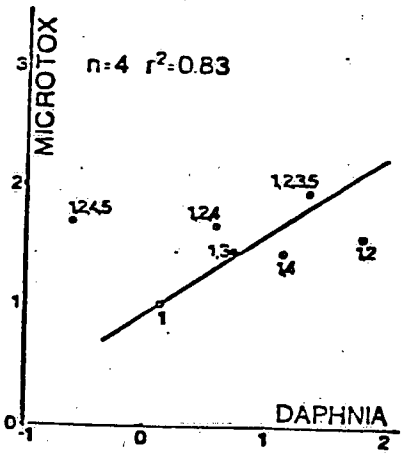
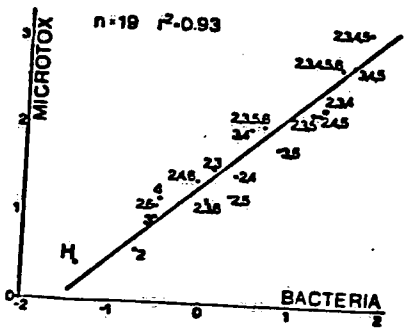
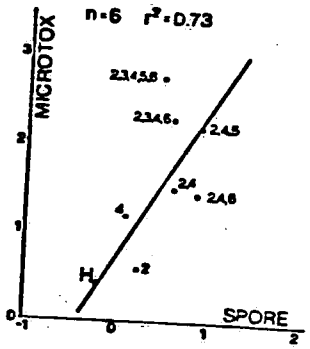


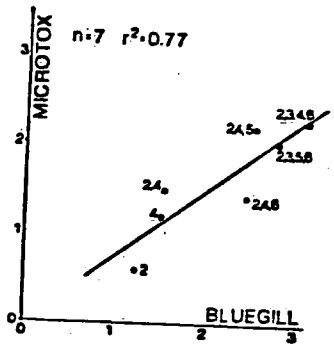
Fig.14

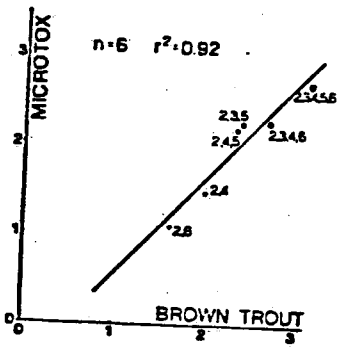
- Correlations between the toxicities of chlorobenzenes to Photobacterium phosphoreum and to other aquatic organisms.

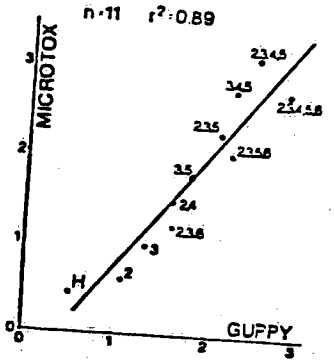
CHLOROPHENOLS

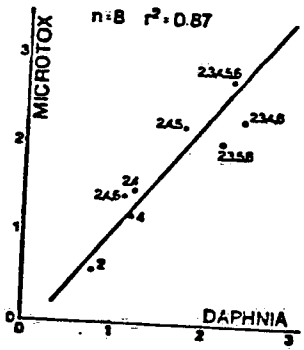












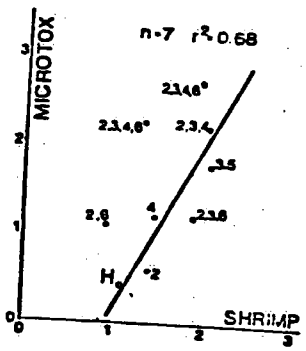


Fig.15

- Correlations between the toxic effects of chlorophenols to Photobacterium phosphoreum and to other aquatic organisms.

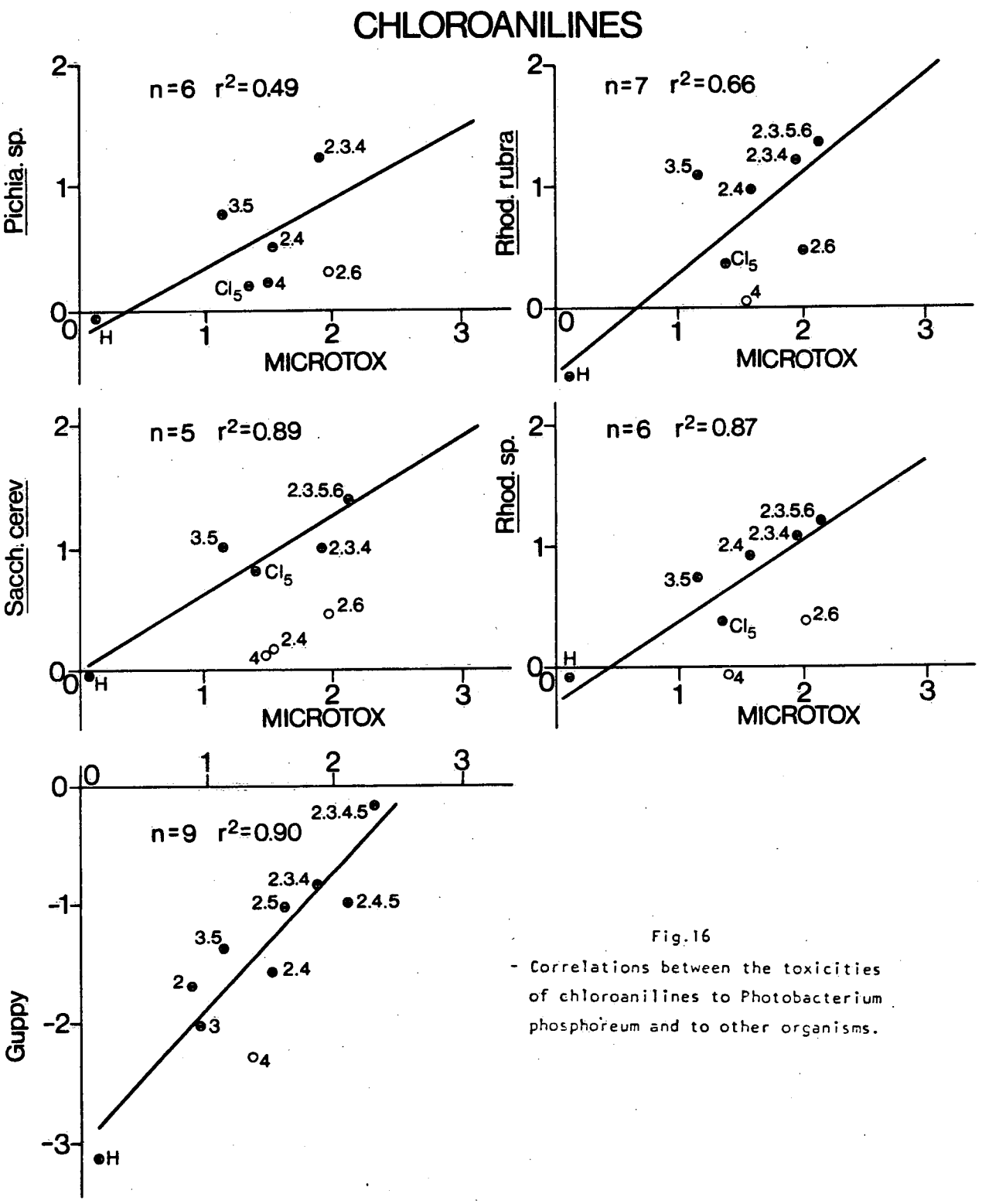


Fig.16

- Correlations between the toxicities of chloroanilines to Photobacterium phosphoreum and to other organisms.

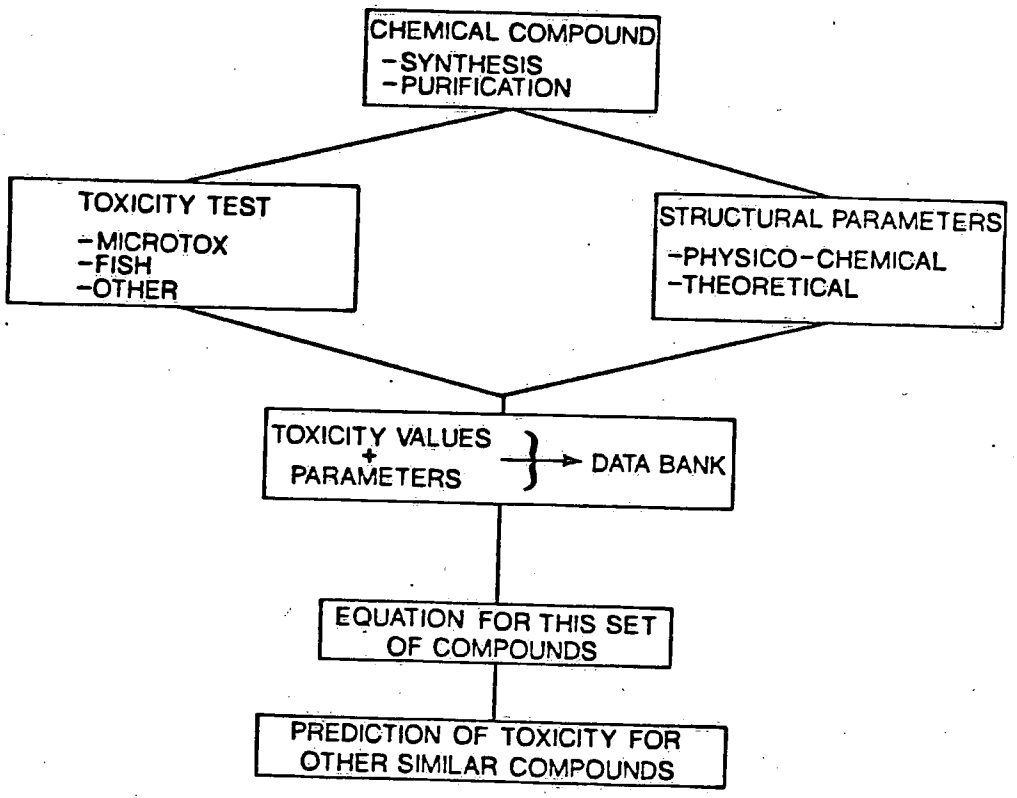


Fig.17.- Flow-chart of the authors' methodology for investigations on QSAR on Environmental Toxicology.

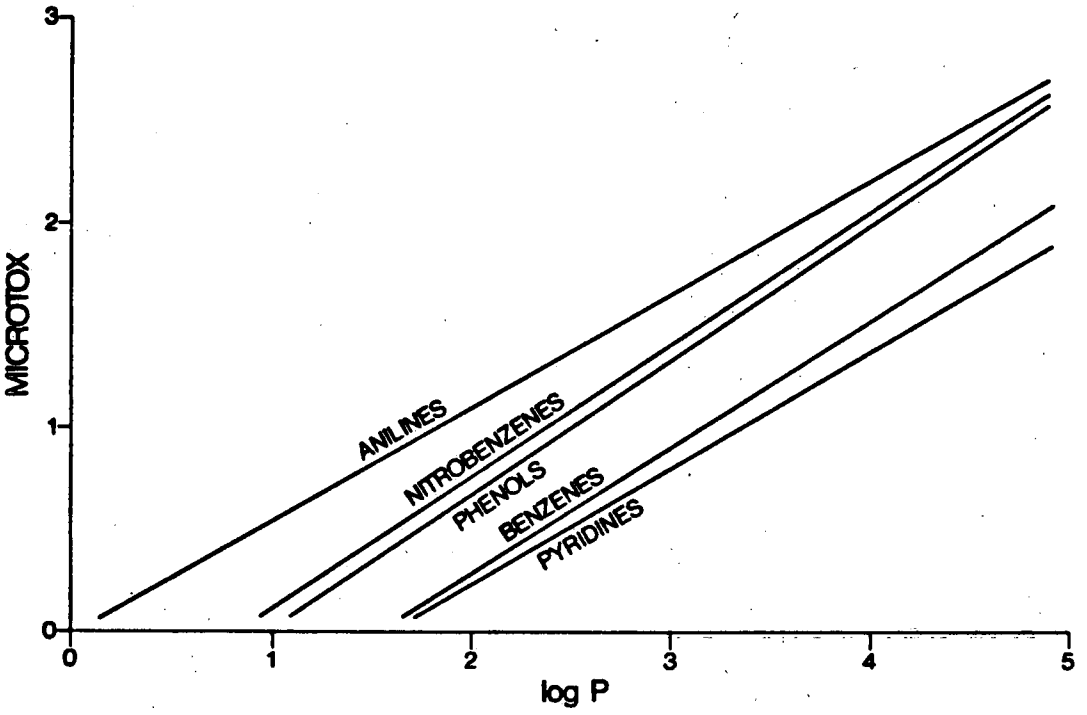


Fig.18.- MICROTOX - log P dependance for chlorinated aromatic compounds.

9764