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A Cost/Effective Screening Method for Assessing the Toxicity of Nutrient Rich Effluents to Algae

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Abstract Screening whole effluent toxicity tests are cost/effective methods for detecting the presence of toxic concentrations of unknown pollutants, but the application must solve the problem associated with the effect of high and variable concentrations of nutrients in the effluent on the results of algal toxicity tests. This work proposes a cost/effective test, based on three dilution levels measured at a single point time and a discriminant model for establishing if this kind of complex samples, with difficult interpretation of dilution-response curves, should be considered toxic to algae. This procedure identified properly around 85% of the samples considered toxic by expert judgement.

Keywords WET · Effluents · Nutrients · Algae · Toxicity

Whole effluent toxicity (WET) tests were proposed in the 80s as a proper tool for the assessment and control of industrial effluents and have a relatively widespread use in the regulatory arena. Nevertheless, efforts for developing and selecting cost/effective test batteries still receive a significant attention (e.g. Ra et al. 2007; Picado et al. 2008). The capacity of whole effluent toxicity tests for predicting the environmental effects on the receiving aquatic communities is limited (Diamond et al. 2008; Hall et al. 2009); but they are an excellent complement to chemical assessment, and therefore, guidance and monitoring programmes have been implemented in different countries (USEPA 2002; Sponza 2002; Tinsley et al. 2004).

The need of a test battery covering different relevant taxonomic groups has been highlighted in most proposals. Primary producers are usually covered through the use of standardised tests on unicellular algae (Lanciotti et al. 2004; Silva et al. 2009). The use of aquatic plants has been suggested by some authors, in particular when the effluents characteristics, e.g. turbidity or colour, may interfere with algae growth measurements. As the algae test measure inhibition in algae growth versus a control, the presence/absence of nutrients in the effluent affects the results and must be considered (USEPA 2002). Originally, WET tests were designed for assessing industrial effluents, and the influence of nutrients was initially considered as related to false positives for effluents with low nutrient content, a problem that can be solved through the addition of nutrients from stock solutions (USEPA 2002).

Obviously, there are also potential effects associated at high nutrient levels in the effluent; but the solution in this particular case is not so simple. Ideally, the effluent should be analysed for all relevant nutrients and the nutrient concentration corrected for each tested concentration/dilution. In practice, this is not feasible for screening purposes, where rapid and low cost methods are required.

In several parts of the world, including Spain, industrial effluents from small and medium enterprises are frequently connected to the municipal sewer systems and, diluted with urban wastes, are treated at municipal wastewater treatment plants. The presence of nutrients in the final effluents is usually high and largely variable, while the toxicity is expected to be generally low. Emission limits are established for the industrial discharges into the municipal sewer. Under these circumstances, screening whole effluent

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toxicity tests are cost/effective methods for detecting the presence of toxic concentrations of unknown pollutants, but its application must solve the problem associated to the high and variable concentration of nutrients in the effluent.

This work proposes a cost/effective test, based on three dilution levels measured at a single point time and a discriminant model for establishing if this kind of complex samples, with difficult interpretation of dilution-response curves, should be considered toxic to algae.

Materials and Methods

Sixty-two samples of effluents from four Spanish wastewater treatment plants (WWTP) were used. Samples were collected from municipal WWTPs located in the north (Barcelona), centre (Madrid), and southeast of Spain (Almería). Sub-samples were taken at 1 h intervals, and integrated for providing a sample representative of a 24-h period. The collecting period comprised from February 2007 to January 2009.

The selected WWTPs represented typical municipal treatment plants, receiving urban wastewater as well as effluents from different activities (industrial and agro-industrial). The treatment facilities included a primary treatment to eliminate suspended material, an activated sludge biological treatment and a final clarification. Samples were collected after this final step, representing the treated effluent to be released or re-used. Effluent samples were collected by using pre-rinsed (3 times with the same sample) amber glass bottles and sent to the laboratory for toxicity tests.

To assess ecotoxicity of collected samples, *Daphnia* acute immobilisation test (OECD 202 method) and alga growth inhibition test (OECD 201 modified method) were performed. *Daphnia magna* immobilisation test was conducted for each sample at three sample dilutions (v/v) 10%, 1% and 0.1%, respectively in duplicate, in a temperature-controlled chamber (20 ± 1°C) with 16-h light/8-h dark photoperiod; test medium was carried out in parallel as control. 48 h after test initiation, daphnids unable to swim for 15 s after gentle stirring were considered as immobile.

The unicellular green algae (*Chlorella vulgaris*) growth inhibition tests were conducted following the adaptation of the OECD 201 method using 96-well microplates (Bischoff and Bold 1963; Blaise et al. 1986; Thellen et al. 1989; St-Lauren et al. 1992; Ramos et al. 1996). This method evaluate the algal growth using 96-well microplates by absorbance (450 nm) and/or fluorescence (430 Ex/680 Em) multiwell plate reader (TECAN-Genius spectrofluorometer); when interference of effluent samples with absorbance and/or fluorescence determination was detected, microscopic counts on aliquots of each treatment were performed by manually enumerating whole algal cells in the 25-square field of a hemacytometer (Neubauer camera).

The screening test, using three dilutions: 10%, 1% and 0.1%, was conducted for all samples. Additional dose response assays, using progressive dilutions in a geometric distribution with a factor of 2, were conducted on selected effluent samples.

Briefly, the testing procedure is described below:

Sample dilutions were prepared in Bold Basal Medium (BBM, Bischoff and Bold 1963) test containing 10×10^4 algal cells/ml. Each well was filled with 200 µl of diluted sample, in six replicates. Microplates were incubated at 20 ± 1°C, 8,000 lux and 100 rpm for 72 h. At that time, the number of algal cells in each well was measured and test dilutions compared with controls in order to calculate the percentage of inhibition or increment.

Total N and P Determinations

Nitrogen and Phosphorous were selected as indicators of the nutrient content and measured in the undiluted samples.

Total Nitrogen (N) was determined by persulfate oxidation at 121°C and 15 psi according to the procedure described by Bachmann and Canfield (1996). Total N was quantified using the second derivative UV/visible spectroscopy technique as described by Ferree and Shannon (2001). Urea was used as standard.

Total Phosphorus (P) was determined according to APHA 1992. Samples were oxided with persulfate, determining the phosphate spectrometrically (820 nm) before ammonium molibdate reaction.

The percentages of reduction or increment versus control calculated for each sample and dilution level were analyzed by a multivariate method using a discriminant model. All samples were classified in a categorical variable as toxic, non-toxic and uncertain, based on dose/response behaviour; total N and P content was used as supporting evidence. This categorical variable was used as classification to develop the correspondent discrimination functions to be used with unknown samples. Statistical significant level of $p < 0.05$ and PC software Statgraphics Plus 5.1 (Statistical Graphics Corp.) were used.

The software gives the results as a table showing the two highest scores among the functions of the classification for each of the 62 observations used to adjust the model, and for any new observation. Comparing the classification gave for the 62 cases used to build the model with the classification supplied by the fitted model, we can assess the percentage of cases classified properly and improperly.

The model can predict additional observations by adding new rows to the current data file, or using the equation algorithms.

Results and Discussion

At a maximum dilution of 10% v/v, no sample showed acute toxicity to *D. magna*. On the other hand toxicity test with *Clorella vulgaris* yield results difficult to explain. In some samples the percentage of inhibition correlate with the percentage of dilution, but in other samples this "dose–response" is very confuse (see Fig. 1). This behaviour could be related to the presence of nutrients in the effluents and or synergic/antagonic effect changes with dilution.

Analysis on content of total N and P in effluents samples reveals a great variability: 0.8–20 mg P/L and 2–5 mg N/L. The basic problem for the interpretation of the results, as comparison of algae growth versus a control with a fix nutrients level, is that the effluent dilution provokes two opposite independent actions, resulting in complex and even apparently contradictory results. The dilution reduces the influence of the additional nutrients provided by the nutrient-rich effluent, and therefore, a reduction in the algal growth with dilution is expected; on the other hand, in effluents containing pollutants at toxic levels for algae, the dilution reduces the concentration and therefore, an increase in algal growth with dilution is expected. Both processes have non-linear concentration responses, which could be theoretically described by "S" shape curves if there is a single nutrient and a single pollutant limiting the algal growth. As the algal growth medium already contains sufficient nutrients level to increase at least 16 folds the initial cells number, the results of the addition of the different effluent dilutions may affect the ratio and relative relevance of the different nutrients, producing more complex curves.

Some samples present a significant toxicity, with clear toxic responses even at dilution factors of 0.1% (effluent: medium, 1:999); when the effect of effluent nutrients is negligible. However, at lower dilution ratios, the toxicity is

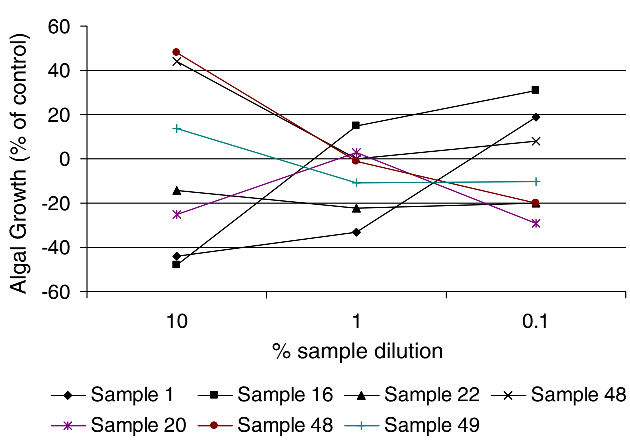

Fig. 1 Concentration/response curves for some selected municipal effluents on *Chlorella vulgaris* growth

partially compensated by the beneficial effect of the high nutrient addition, and maybe, by reductions in the toxicant bioavailability due to adsorption/complexation to organic matter and other major effluent constituents. In some samples the toxicity to algae is not so evident; although theoretically the possibility of a chemical producing "hormesis" and or synergism – antagonism effects could not be fully disregarded, the most likely explanation for these results is the role of the nutrients present in the effluent. The immediate consequence of assuming this explanation is that the slope of the dilution/response curve is reduced due to the nutrient effects; thus, the real toxicity of the less diluted levels should be higher than that observed (see sample 1 and sample 22 in Fig. 1).

The cost/effective screening method is based on testing three dilution levels at orders of magnitude (1:9; 1:99, and 1:999, representing 10%, 1% and 0.1% dilution). If clear toxic effects are observed at any concentration the presence of toxic chemicals in the effluent must be assumed. However, if this criterium is employed alone, false negative results, were nutrient effects at the selected levels confound the toxicity observations, may be significant.

Figure 2 summarised the results obtained for a set of measurements in four Spanish municipal wastewater effluents. The facilities receive urban, industrial and rainfall influents at ratios that varies with time. The presence of pollutants at toxic levels for algae was detected in all four facilities, also with large temporal variability. The summary statistics presented in the Fig. 2 suggests the relevant effects on nutrients in all four treatment plants: the algal growth tend to be higher than in the control at the less diluted level and the expected dose response, with growth inhibition being reduced with the additional dilution, is not observed.

An expert judgement, supported by testing at additional concentrations for same samples and total N and P content, was conducted on the observed algae growth results for each facility and sampling time. Three qualitative levels, toxic effects observed (−1), toxic levels non-observed (1), and uncertain results not allowing a final decision (0) were assigned to each sample.

Then, a discriminant analysis, based on the observed algae growth at each dilution level was applied. The discriminate analysis is a multivariable statistical procedure designed to develop a series of discriminant functions that can help predict classification based on the values of other quantitative variables. 62 cases were used to develop a model to differentiate among 3 classification levels; 3 predicted variables were introduced. The statistical software supplies discriminant functions with p-values less than 0.05; these functions are then used to classify additional cases.

The model was able to classify properly about 90% of the cases where toxicity was considered by the expert

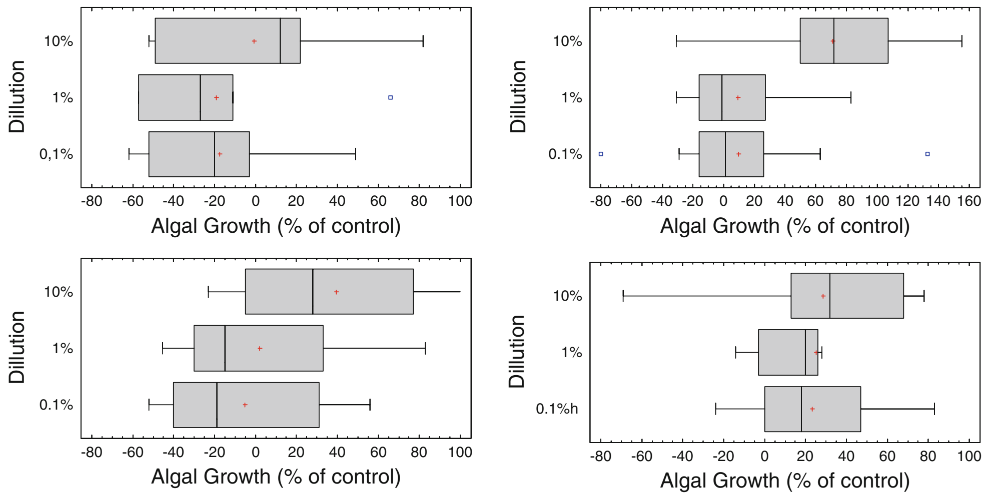

Fig. 2 Box and Whiskers plots of the distribution of algal toxicity at three dilution levels in four Spanish municipal wastewater treatment plants receiving different rates of urban and industrial effluents

assessment. The percentage of false positives and false negatives was about 5%. The larger difficulty was for the uncertain level (0), which obviously is the one showing the larger uncertainty also for the expert assessment.

The discriminant model is built on three complementary linear equations. Each equation provides a value associated to each classification category (−1, 0 or 1), the sample is assigned to the category for which the highest value is obtained. The three equations are:

$$\text{Category : Toxic}(-1)\mathrm{Y_T} = 0.00262527\mathrm{G10} - 0.0237124\mathrm{G1} - 0.0171692\mathrm{G01} - 1.60565$$

$$\text{Category Uncertain } (0)\mathrm{Y_U} = 0.011131\mathrm{G10} + 0.0199687\mathrm{G1} + 0.0318165\mathrm{G01} - 2.20627$$

$$\text{Category Non-toxic}(1)\mathrm{Y_N} = 0.0235764\mathrm{G10} + 0.02143\mathrm{G1} + 0.0177773\mathrm{G01} - 2.45828$$

where G10, G1 and G01 are the differences in the algal growth versus the control, at the dilution rates of 0.1 (10%), 0.01 (1%) and 0.001 (0.1%) respectively, expressed as percentages.

Using the fitted discriminant functions, and the G10, G1 and G01 values, representing the growth difference versus control in percentage at 10%, 1% and 0.1% effluent dilution respectively, we can built a three dimensions scatter plot and scatter matrices of the measured samples indicating the assigned category (−1 toxic, 0 uncertain or 1 non-toxic). Figure 3 presents the relationship between the differences in algal growth at each dilution rate and the assigned category. Figure 4 summarised the results of the model validation, comparing the data with right and wrong predictions for each dilution level. Figure 4 shows, sorted by classification category and algal inhibition response, the number of cases classified properly and improperly by the model.

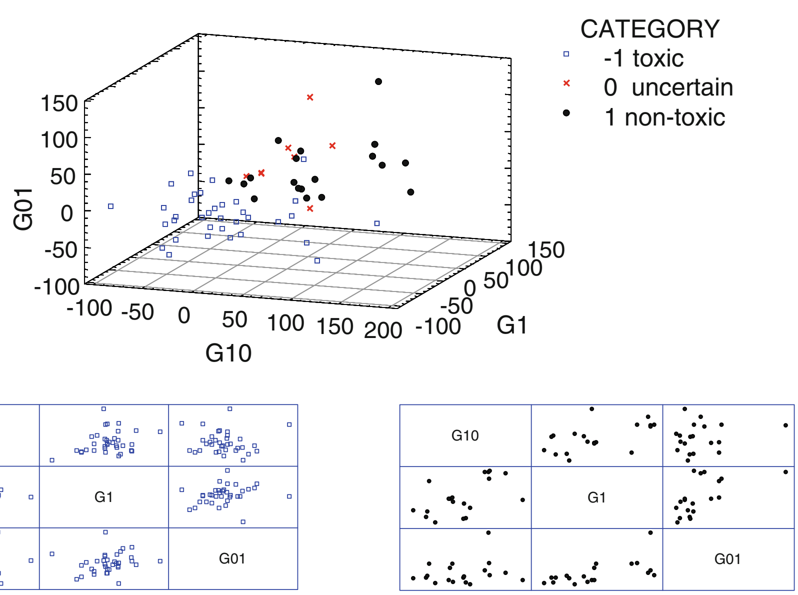

Fig. 3 Three dimensions scatter plot and scatter matrices of the measured samples indicating the assigned category (−1 toxic, 0 uncertain or 1 non-toxic). G10, G1 and G01, represent the growth difference versus control in percentage at 10%, 1% and 0.1% effluent dilution respectively

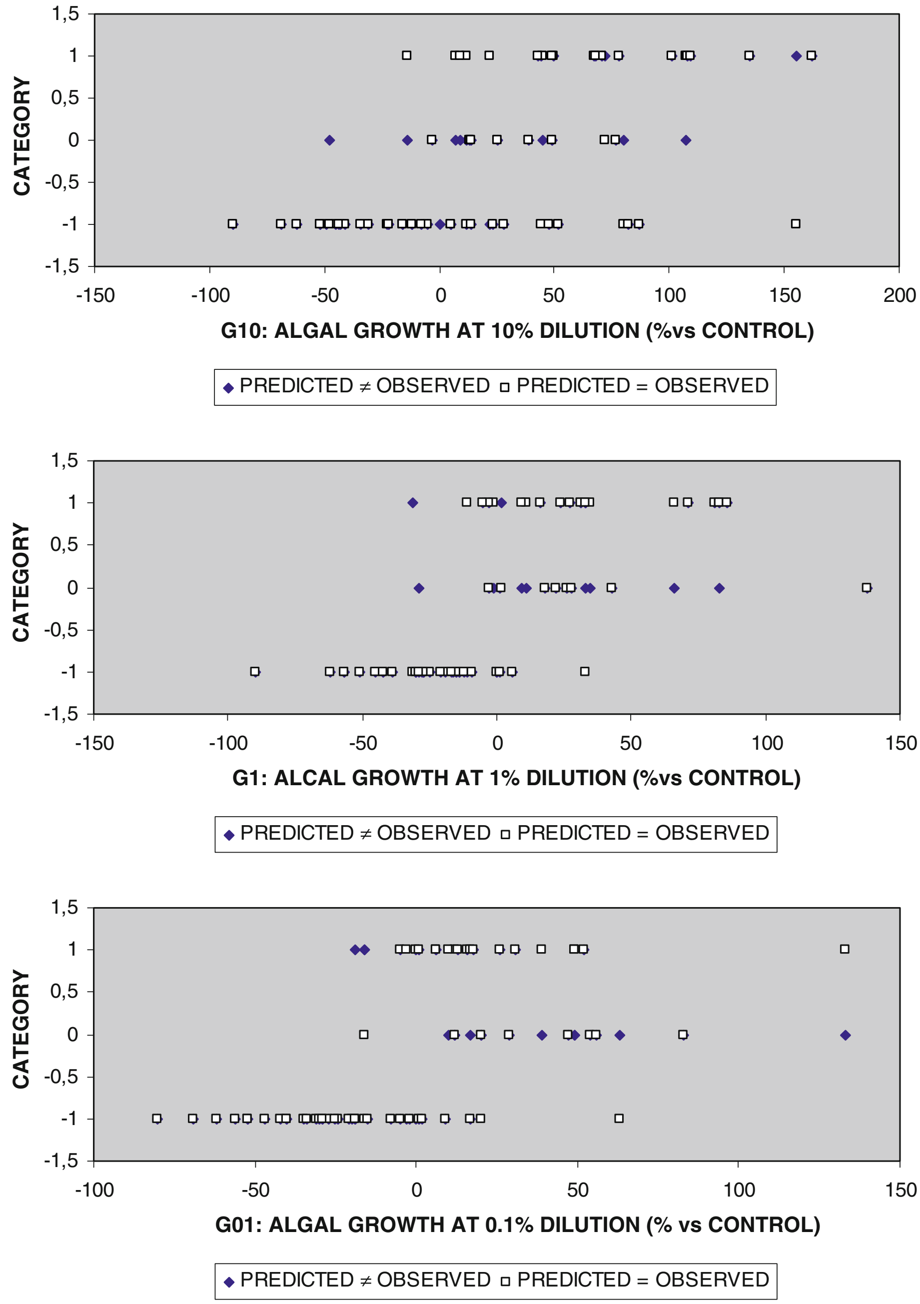

Fig. 4 Effluent samples with correct and false prediction sorted by classification category and algal inhibition response

Although algal tests have been used for decades in whole effluent toxicity testing (USEPA 2002; Daniel et al. 2004; Ra et al. 2007), its application to nutrient rich effluents with limited toxicity presents practical problems which require additional efforts for measuring and correcting the effects of effluent addition in the final nutrient concentration at each dilution level. Although most technical problems are solved for industrial effluents (USEPA 2002; Tinsley et al. 2004), the nutrient/toxicity contradictory effects create difficulties for the development of screening methods for municipal effluents with large seasonal variability in nutrient concentrations. Whole effluent toxicity testing is particularly useful as a screening technique for assessing the sporadic presence of toxic concentrations of pollutants in wastewater effluents (Tinsley et al. 2004). These programmes may require testing a large number of samples, collected at short time periods, for detecting episodic toxic emissions; nutrient corrections and testing large numbers of dilutions may result impossible, and, therefore, cost-effective alternatives are required.

The screening method proposed here only requires measurements at three dilutions, ranged in orders of magnitude, and at a single time point. With a proper protocol, hundred of samples can be handled weekly by a single routine laboratory at very low cost and person-power. As test design and quality control assurance is an essential element for the application of whole effluent toxicity testing in real situations (Johnson et al. 2004), a pilot project for assessing the capacity of the proposed method was conducted. The study focused on municipal facilities, receiving also industrial effluents and rainfall wastewaters. The inclusion of an algal growth inhibition assay in the test battery was considered essential for covering pollutants with potential effects on primary producers (Blinova 2004; Ra et al. 2007; Picado et al. 2008); therefore, the potential interference of the changes in nutrient concentrations at the different dilution rates was studied. Other tests were included in the pilot project, although the results are not presented in this paper.

The counteracting response in toxicity-and nutrient-related effects was clearly observed in the four studied municipal wastewater treatment plants. The dilution-response assays presented in Fig. 1 confirmed the complexity of this interaction in representative Spanish municipal effluents, resulting in very different dilution/response shapes for different effluent samples.

From a total of 62 samples, expert judgement was able to assign a clear category (toxic or non-toxic) to over 85% of cases on the basis of three dilutions measured at a single time point. For 11 samples, the analysis was completed with a full dilution/response curve, using a geometric dilution rate of 2 as recommended by the USEPA (USEPA 2002); in all cases, the expert assessment based on the full curve was in agreement with that initially considered based on three dilution levels. For facilitating the implementation of the proposed method, a discriminant model, has been developed and mathematically implemented in a data sheet. The model identified properly around 85% of the samples considered toxic by expert judgement; the percentage of false positives and false negatives was around 5%. The emission of industrial effluents, in particular from small and medium enterprises, to the municipal sewer system is common practice in large areas of the world including Spain. The emission is controlled by imposing emission limits for those substances expected to posse the highest risk. Whole effluent toxicity testing appears as a proper screening tool for setting additional controls, allowing the treatment facility to confirm, on a regular basis, the absence of toxic levels of pollutants in the inflow and in the effluent (Sponza 2002, Pessala et al. 2004; Barata et al. 2008). The method proposed in this study avoids the need of complementary nutrient analysis and corrections, and largely reduces the number of samples to be testing by selecting three dilution levels in order of magnitude. Although the quality control/assurance requirements are lower for discharge screening than for monitoring compliance programmes (Johnson et al. 2004), it is recommend calibrating periodically the discriminant model at each treatment facility, using random samples tested for the whole dilution/response curve.

The presence of high and variable levels of nutrients in municipal effluents influences dramatically the results of algal growth toxicity tests. Measuring and correcting nutrient levels is impractical for screening purposes.

Based on a wide monitoring programme, a screening method has been developed. A cost/effective test, measuring effects at just three dilution levels and a single time point, coupled to a decision tool based on a discriminant analysis is proposed.

The screening method identifies correctly over 85% of the cases, with a percentage of false negatives of about 5%.

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