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9 October 2017

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Protozoan Activated Sludge Inhibition Test

INTRODUCTION

1. This Test Guideline describes a method to assess effects of a test chemical on the phagocytotic activity (consumption of dispersed bacteria) of activated sludge under defined conditions in the presence of different concentrations of the test chemical.

2. The principle of biological sewage-treatment plants (STP) is to transform the organic matter of incoming waste-water in microbial biomass, which in turn is separated from the liquid yielding a purified effluent. The aim of this process is to achieve a maximal reduction of the organic load and a decrease of nutrients such as ammonia, nitrate and phosphate with a minimal bio-sludge production. The phagocytotic activity of activated sludge organisms supports this process. In conventional plants ciliates usually dominate this activity. It is especially their feeding on bacteria which clarifies the waste water, resulting in a higher transparency, i.e. lower organic loads in the output water.

3. The purpose of the test is to provide a means to record effects of test chemicals on consumers in sewage treatment plants, which consist mainly of ciliated protozoa and which – due to their grazing on bacteria - considerably contribute to the functioning of STPs.

4. The test is most easily applied to water soluble test chemicals which, under the test conditions, are likely to remain in the water. In some cases, a solvent may be required in order to produce a suitably concentrated stock solution (see paragraph 22).

5. This guideline proposal is based on an international ring study commissioned by the German Environment Agency and conducted in 2011-2013 (1). The test has been validated in 5 labs with 5 mono-constituent organic test substances with assumed non-polar or polar narcotic mode of action (MoA). The test substances in the validation study had a log Kow range from -1.36 to 6.91. The test substances were 1-octylamine (CAS-no. 111-86-4), 3,5-dichlorophenol (CAS-no. 591-35-5), dimethyl sulfoxide (CAS-no. 67-68-5), phenyl ether (CAS-no. 101-84-8) and hexachlorophene (CAS-no. 70-30-4).

6. Before use of the test guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

This Guideline was adopted by the OECD Council by written procedure on 9 October 2017 [C(2017)97].

PRINCIPLE OF THE TEST

7. The activated sludge sample is exposed to the test chemical in small glass test vials with a culture volume of 2 mL. The vials are closed with oxygen permeable caps and shaken for 22 hrs at 22°C (±1°C). At the start, the sludge samples are fed suspended bacteria. Whereas the turbidity decreases in control samples, in the chemically inhibited samples no elimination of bacteria takes place. To quantify the phagocytotic activity, the decline of the added bacteria with time is followed by two photometric measurements (λ = 440 nm) after 2 hrs (t1) and after 22 hrs (t2) of incubation. Based on the difference between both values the percentage reduction of the activity in relation to an untreated control is

calculated. Linearity between time and optical density has been shown in (8. The test is typically used to determine the EC_X (e.g. EC_{50}) of the test chemical. To compensate for unspecific reactions of the added bacterial substrate with the sludge flocs (e.g. binding) or effects of chemicals on the turbidity of the sludge sample (e.g. by disaggregation) defaunated (and thus phagocytosis-free) parallel samples are included for controls and for each concentration.

INFORMATION ON THE TEST CHEMICAL

8. It is necessary to know the water solubility of the test chemical under the conditions of the test. A reliable analytical method for the quantification of the test chemical in the test solutions should also be available.

9. Useful information includes structural formula, degree of purity, water solubility (2), n-octanol/water partition coefficient (3), vapour pressure (4) and biodegradability (5). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test chemical may occur.

VALIDITY OF THE TEST

10. For a test to be valid the following conditions are to be fulfilled:

- the decrease of the optical density (OD) of controls due to phagocytotic activity must exceed 30% within the testing time between 2 hrs and 22 hrs (cf. para 42) .
- the unspecific decrease of the OD (defaunated controls containing bacterial substrate) must not exceed 25% within the testing time (cf. para 43).
- test chemical concentrations at which an average OD-increase of more than 5% in the defaunated parallel samples occurs has to be excluded from the effect calculation (cf. para 44).

REFERENCE SUBSTANCES

11. A reference substance is tested as a means of detecting unsatisfactory test conditions. In a 2011 international ring study (1) the EC_{50} of 3,5-dichlorophenol was found to be in the range 1.5 mg/L to 5.1 mg/L for the activated sludge phagocytosis inhibition. As EC_{50} values may vary considerably depending on the sludge used for the test, the aforementioned range of EC_{50} values is only for reference purpose and does not necessarily condition the validity of the test. Results obtained with the reference substance should be given in the test report.

APPLICABILITY OF THE TEST

12. The test method may be applied to water-soluble, poorly soluble and volatile test chemicals. However, it may not always be possible to obtain EC_{50} values with test chemicals of limited solubility and - although the described procedure uses closed test vessels - valid results with volatile test chemicals may only be obtained providing that the bulk (> 80%) of the test chemical remains in the reaction mixture at the end of the exposure period (see (6) for guidance on difficult substances). For poorly soluble test chemicals, a solvent might be used as a last resort (see paragraph 22). Additional analytical support data should be submitted to refine the EC_X concentration when there is any uncertainty regarding the stability of the test chemical or its volatility.

DESCRIPTION OF THE METHOD

Apparatus

13. Normal laboratory equipment and especially the following is necessary:

- glass vials with oxygen permeable caps,

recommended are:

- clear glass screw top vials, 45 x 15 mm (outer dimensions), total volume of 4 mL.
- open top closures (screw caps open tops) with teflon faced silicone liners.
- a rotary shaker (speed adjustable to 250 rpm (4.17 Hz) with racks enabling a slanting position of test vials with an angle of inclination of 20°-40° to horizontal.
- photometer (filter of λ=440 nm) suitable for above mentioned test vials.
- a cabinet or chamber, in which a temperature of 22°C (± 1°C) can be maintained.

Inoculum

14. Activated sludge from the exit of the aeration stage of a well-operated sewage treatment plant treating predominantly domestic sludge is used as the inoculum for the phagocytosis test. Avoid floating scum when collecting activated sludge. Do not collect the sample in dead corners where scum has built up. Take your sample by complete immersion of your sampling tool below the surface of the water. If floating occurs during testing gentle tilting of the glass vials or manual stirring using a glass rod may be successful to let the surface layer drop to the bottom.

15. To perform the test it is necessary to know the dry weight of the sludge. Suspended solids concentrations of 2 g/L to 4 g/L may be considered appropriate. In most cases wastewater facilities continuously perform routine measurements of the sludge concentration. If actual dry weight data are not available, the dry weight has to be determined: Sludge is dried at 60 °C to a constant weight. The dry weight should be expressed to an accuracy of at least 0.1 mg. Alternatively standardized methods for calculation of dry matter fraction after determination of dry residue or water content can be used e.g. DIN EN 15934, 2540 D or equivalents.

From this result, the volume of native activated sludge suspension can be calculated. This must be suspended in the 2 mL assays in order to obtain an activated sludge with a mixed liquor suspended solids value of 0.9 ±0.1g/litre.

16. The activated sludge should be used on day of collection. If this is not possible, the whole batch of native - undiluted - sludge should be stored in the refrigerator at 4-7°C for one week maximum.

Substrate

17. Bacteria showing a low tendency to aggregate, to flocculate and to adhere to activated sludge flocs may be used as substrate. Bacteria which meet these requirements and have been found to be suitable as food source for phagotrophic sludge inhabitants are shown in Annex I. However, it should be noted that any other bacterial strains can be used if they fulfill the validity criteria.

18. On the basis of the results of the ring test a final concentration of the bacteria to be used for feeding the sludge of 0.36 g/L (dry weight) can be recommended. This concentration ensures an adequate food supply and reliable OD-measurements (c.f. chapter 4.2 in the ring test report (1)).

Test medium

19. To dilute the activated sludge and to prepare the chemical dilution series EPA synthetic water is recommended (the detailed composition of the recommended test medium is given in Annex II - Test medium).

20. Modification of the test medium may be necessary for certain purposes, e.g. testing at different pH values. Use of modified media must be described in detail and justified.

Phagocytosis-free controls

21. Some part of the bacterial substrate may form aggregates or attach to the sludge flocs thereby changing the turbidity of supernatant suspension. In addition test chemicals – especially at high concentrations - may have impacts on the floc structure of the activated sludge, leading to an increase in turbidity. Controls without phagocytic activity are therefore necessary, reflecting changes in turbidity due to passive, non-specific reactions in the test system. The eukaryotic inhibitor digitonin (CAS no. 11024-24-1) has proved to be a useful and specific inhibitor of phagocytosis completely defaunating the activated sludge probe (7). To obtain phagocytosis-free data parallels with digitonin at a final of 200 mg/L (preparation see Annex III- Preparation of digitonin solution) should be included in the test for both controls and all chemical concentrations.

Test chemical

22. Pre-dilution series of test solutions are freshly prepared at the start of the study by dilution of a stock solution in test medium. In case of sparingly water soluble test chemicals ultrasonic dispersion or other suitable physical means are the preferred methods to dissolve the test chemical. In some cases the use of solvents may be required in order to produce a suitably concentrated stock solution. Examples of solvents that may be used as vehicles to dissolve test chemicals of low water solubility are acetone, ethyl alcohol and dimethyl sulfoxide. Due to its low toxicity dimethyl sulfoxide may be given preference at lowest concentration possible, i.e. maximum 0.1 mL/L for DMSO, followed by evaporation prior to addition of the sludge. In case a solvent is used, a solvent control should be included in the test. However, every effort should be made to avoid the use of such materials and if organic solvents are used, they must not significantly inhibit the phagocytotic activity.

23. The test should be carried out without adjustment of the pH. If marked change in the pH is expected, the pH value of the stock solution should be adjusted to pH 7 ± 1 (HCl and NaOH may be used for this adjustment if warranted). This pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test chemical is caused.

Conditions of exposure

24.

Duration:	22 hours under continuous shaking.
Vessels:	glass vials with oxygen permeable screw caps.
Light:	the test should be performed in the dark.
Temperature:	22 ± 1°C.
Oxygen supply:	a sufficient oxygen supply has to be maintained throughout the test period by fast shaking the test vials at 250 rpm (4. 4.17 Hz). For the recommended assay the culture volume should not exceed 2 mL in the (4 ml-) test vials which should be closed with oxygen permeable caps and kept at an angle of 40° to the horizontal.

Replicates and controls

25. The test design should include three replicates at each test concentration and six controls. An additional set of six solvent controls will be needed in case a solvent is used.

26. For each assay, a parallel phagocytosis-free test run with the same number of samples (triplicates at each test concentration and 6 controls) containing digitonin as specific inhibitor[1] (200 mg/L final concentration) should be included.

27. A separate set of test solutions (one replicate per concentration) should be prepared for analytical determinations of test chemical concentrations (see paragraphs 32-34), if needed.

28. No less than five concentrations are tested simultaneously, preferably arranged in a geometric series (e.g. spaced by a factor not exceeding 2.0). The lowest concentration should have no observed effect on growth. The highest concentration tested should be 100 mg/L at minimum and inhibit growth by at least 50% relative to the control and, preferably, stop growth completely. For statistical reasons, however, it is desirable to select the concentrations so that they bracket the 50% effect level.

Measurements

29. The tests run for 22 hours. After vigorous shaking the test vials and settling the sludge for 30 min the optical density of the supernatant is measured spectrophotometrically (440 nm) after 2 hrs (t1) and after 22 hrs (t2) of incubation. Since under the experimental conditions optical density is directly proportional to the bacteria content, absorbance is a rapid means of non-invasively estimating the phagocytotic activity which has taken place in the sludge samples.

[1] The detergent digitonin selectively renders the eukaryotic plasma membrane permeability but not bacterial cells (Mooney, 1988). The addition of digitonin provides on the one hand a control with total inhibition of the phagocytic activity and on the other it allowes the measurement of any passive reaction of the added bacterial suspension, i.e. the nutrient substrate.

Range finding

30. If no data is available on the toxicity of the test chemical to bacteria, a range finding test is carried out to determine the 0-100% tolerance range of the phagocytotic activity to the toxicant. It should include at least 5 dilution steps with a dilution factor of 10 from a starting concentration of 1000 mg/L or the maximum solubility of the test chemical. Testing at levels below water solubility is preferred, but also insoluble parts of a test item may contribute to inhibition.

Limit test

31. Under some circumstances, e.g. when a preliminary test indicates that the test chemical is non-toxic at concentrations up to 100 mg/L or up to its limit of solubility in the assay (whichever is the lower), a limit test involving a comparison of responses in a control group and one treatment group (100 mg/L or a concentration equal to the limit of solubility) may be undertaken. It should be supported by analysis of the exposure concentration. All previously described test conditions and validity criteria apply to a limit test, with the exception that the number of treatment replicates should be doubled. Growth in the control and treatment group may be analysed using a statistical test to compare means, e.g. a Student's t-test.

Analytical determinations

32. In some cases, it may be necessary to determine the concentration of the test chemical in the test vials (e.g. for highly volatile or strongly adsorbing test chemicals). Analysis at the start and end of the test of a low and high test concentration and a concentration around the expected EC_{50} may be sufficient where it is likely that exposure concentrations will vary less than 20% from nominal values during the test. Analysis of all test concentrations at the start and end of the test is recommended where concentrations are unlikely to remain within 80-120% of nominal (e.g. for volatile or strongly adsorbing test chemicals). In all cases, determination of test chemical concentrations need only be performed on one replicate vessel at each test concentration (or the contents of the vessels of one concentration pooled by replicate).

33. Test samples prepared specifically for analysis of exposure concentrations should be treated identical to those used for testing, i.e. they should be inoculated with activated sludge, provided with food and incubated under identical conditions. If analysis of the dissolved test chemical concentration is required, it may be necessary to separate the solid constituents from the aqueous phase. Separation should preferably be made by centrifugation, sufficient to settle the activated sludge and the suspended bacterial food substrate.

34. If there is evidence that the concentration of the test chemical has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial concentration. If the deviation from the nominal or measured initial concentration is greater than ± 20%, analysis of the results should be based on average mean measured concentration during exposure.

Other observations

35. Microscopic observation can be performed to verify a normal and healthy appearance of the inoculum activated sludge and to observe any abnormal appearance of the bacterial feeders, especially protozoans.

DATA AND REPORTING

Response variables

36. The purpose of the test is to determine the effects of the test chemical on the phagocytosis activity of activated sludge.

Treatment of results:

37. The content of suspended bacterial food in the test vessels is expressed in units of the surrogate parameter optical density (OD_{440}) used for measurement.

38. The measured optical densities in the test cultures and controls are tabulated together with the concentrations of the test chemical and the time of measurement.

39. The phagocytotic activity is calculated on the basis of the difference between the initial optical density after 2 hrs of incubation and that after 22 hours of incubation;

$$\text{i.e.:} \quad \Delta OD = dOD = OD_{2h} - OD_{22h,}$$

optical density difference between 2 and 22 hours (controls and treatments without digitonin).

40. To correct for unspecific optical density changes (e.g. due to binding. complexing or lysis of the bacterial food or due to deflocculating effects of test chemicals) defaunated, digitonin-treated parallels without phagocytotic activity are included in the test design. Their shift in optical density values between 2 hrs and 22 hrs (ΔOD_{def}) is subtracted from the ΔOD-values;

$$\text{i.e.:} \quad \Delta OD_{def} = OD_{def\ 2h} - OD_{def\ 22h}$$

optical density difference between 2 and 22 hrs of defaunated parallels (treated with 200 mg/mL of the specific eukaryotic inhibitor digitonin).

41. To compensate for unspecific optical density changes the corrected mean OD-difference for all replicates of the control and treatment groups is calculated as:

$$\Delta OD_{corr} = \Delta OD - \text{mean } \Delta OD_{def}$$

where:

ΔOD: ΔOD-value of a single sample without digitonin (6 controls and 3 samples per chemical concentration);

meanΔOD_{def}: mean ΔOD-value of the (defaunated) phagocytosis-free, digitonin-containing control (n=6) and the respective digitonin-containing chemical treatment group (n=3).

Test results

42. The percentage decrease of the OD of controls (important to validity, see para 10) is calculated as:

$$\%\Delta OD(control) = \frac{\text{mean } \Delta ODcorr(controls)}{\text{mean 2h}-\text{OD (controls)}} \text{ x } 100$$

where:

mean ΔODcorr(controls): mean ΔOD(controls) – mean ΔODdef (controls); n=6;
mean 2h-OD(control): mean of the 2h-OD (start-OD at t1=2hrs) for all control replicates (n=6).

43. The percentage 'unspecific' decrease of the OD for phagocytosis-free digitonin-containing controls (important to validity, see para 9) is calculated as:

$$\%\Delta OD_{def}(controls) = \frac{\text{mean } \Delta OD \text{ def(controls)}}{\text{mean 2h}-\text{OD def (controls)}} \text{ x } 100$$

where:

mean ΔODdef(controls): mean of the OD-difference between 2hrs and 22hrs for replicates of digitonin controls (n=6);
mean 2h-ODdef(controls): mean of the 2h-OD (start-OD at t1=2hrs) for replicates of digitonin controls (n=6).

44. The calculation of test chemical effects on the optical density (important to validity. see para 9) is made according to:

$$\%\ \Delta ODdef(treatment)\ = \frac{\text{mean } \Delta OD \text{ def(treatment)}}{\text{mean 2h}-\text{OD def (treatment)}} \text{ x } 100$$

where:

mean ΔODdef(treatment): mean of the OD-difference between 2hrs and 22hrs for replicates of a treatment group (n=3) containing digitonin;
mean 2h-ODdef(treatment): mean of the 2h-OD (start-OD at t1=2hrs) for replicates of a treatment group (3 replicates each) containing digitonin.

Reduction of phagocytosis

45. The inhibition of the phagocytotic activity for each replicate at each test chemical concentration is expressed as a percentage of the mean of the control phagocytotic activities:

$$\%Inhibition_x = 1 - \frac{\Delta ODcorr(\text{replicate x of treatment group})}{\text{mean } \Delta OD \text{ corr(controls)}} \text{ x } 100$$

where:

ΔOD_{corr}(replicate$_x$ of treatment group): corrected OD-difference between measurements after 2hrs and 22hrs for the respective replicate (vial) of the test chemical concentration (treatment group), i.e. ΔOD_{corr}(replicate$_x$ of treatment group) = ΔOD(replicate$_x$ of treatment group) - meanΔOD_{def}(replicates$_{1\text{-}3}$ of the respective treatment containing digitonin);

meanΔOD_{corr}(controls): mean value for corrected OD-differences between controls and controls containing digitonin, i.e. mean of ΔOD(controls) - meanΔOD_{def}(controls).

When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents should be used in calculation of percent inhibition.

Plotting concentration response curves

46. Plot the percentage of inhibition for each individual vial (replicate) against the logarithm of the test chemical concentration (n=3 for each concentration), compare inhibition curve, Annex IV - Concentration-response curves.

47. Include the data of the unspecific OD-change of defaunated parallels for each replicate (n=3 for each concentration) according to:

$$\% \Delta OD_{def}(replicate_x) = \frac{\Delta \text{ OD def(replicate x)}}{\text{mean 2h}-\text{OD def(treatment)}} \text{ x } 100;$$

see also inhibition curve, Figure 1 of Annex IV - .

Evaluation of EC-values

48. EC_X-values are derived by statistical means. All data are normalized by dividing by corresponding data for the controls (average of all control replicates). Concentration-response curves are fitted with nonlinear regression methods on log-transformed concentrations. Calculations of preferably EC_{50}-values should be performed on the basis of three- and four- parameter (symmetrical and asymmetrical) sigmoidal functions. Each replicate is treated as a separate point. The top and bottom plateau are constrained to 0% and 100%, respectively (some test chemicals may stimulate the growth at low concentrations, referred to as hormesis. Only data points indicating effects between 0 and 100% should be considered). EC-values should be reported with 95% confidence limits.

49. Where the data obtained are inadequate for the use of standard curve-fitting methods of calculating the EC_{50}, the highest concentration causing no effect and the lowest concentration producing 100 per cent inhibition should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

Test report

50. The test report must include the following information:

Test chemical

- Mono-constituent substance:
  physical appearance, water solubility, and additional relevant physico-chemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).
- Multi-constituent substance, UVCBs (substances of Unknown or Variable composition, Complex reaction products or Biological materials) and mixtures:
  characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents.

Activated sludge

- origin, conditions of operation of the wastewater treatment plant and influent it receives, dry weight, day of sampling, storage conditions (if relevant), any pretreatment etc.

Test conditions:

- date of the start and the end of the test
- activated sludge concentration (mixed liquor suspended solids)
- temperature
- bacterial strain (culturing. source/origin)
- test vessel and apparatus
- vehicle and method used for solubilizing the test chemical and concentration of the vehicle in the test solutions
- concentrations tested (measured or nominal)
- information of concentrations of test chemicals in the test solutions, analytical method

Results:

- optical density values for each vessel at each measuring point
- mean values of replicates
- graphical presentation of the concentration-response relationships
- EC_{50}-values with 95% confidence limits and method of calculation
- other observed effects, incidents which might have influenced the results

LITERATURE

(1) OECD (2017). Ring-Test to Validate the Protozoa Activated Sludge Inhibition Test. Environmental Health and Safety Publications, Series on Testing and Assessment No.266. OECD, Paris.

(2) OECD (1995). Guideline for the Testing of Chemicals, No. 105: water solubility. Organisation for Economic Cooperation and Development, Paris.

(3) OECD (1995). Guideline for the Testing of Chemicals, No. 107: Partition Coefficient (n-octanol/water). Organisation for Economic Cooperation and Development, Paris.

(4) OECD (2006). Guideline for the Testing of Chemicals, No. 104: vapour pressure. Organisation for Economic Cooperation and Development, Paris.

(5) OECD (1992). Guideline for the Testing of Chemicals, No. 301: Ready Biodegradability. Organisation for Economic Cooperation and Development, Paris.

(6) OECD (2000). Guidance Document on aquatic toxicity testing of difficult substances and mixtures. Environmental Health and Safety Publications, Series on Testing and Assessment Number 23. OECD, Paris.

(7) Mooney, R. A., 1988. Use of digitonin-permeabilized adipocytes for cAMP studies. Methods Enzymol., pp. 193-202.

(8) Wilfried Pauli, Viktoria Poka (2005). Development and standardization of a test system to assess chemical effects on key functions of the protozoan biocenosis in activated sludge of municipal wastewater treatment plants. Environmental Research Plan of the Federal Ministry for the Environment, Nature Conservation and Nuclear Safety - Environmental chemicals / toxic effects. Report 201 67 402.

ANNEX I - SUBSTRATE

Substrate

Commercially available lyophilized cells of the *E.coli*-strains ATCC 9637, ATCC 8739 and K12 (Sigma-Aldrich) have been found to meet the requirements of a low tendency to aggregate, to flocculate and to adhere to activated sludge flocs. They are also suitable as food source for phagotrophic sludge inhabitants.

Additionally it has been shown for both ATCC strains that an appropriate substrate can also be produced by cultivating and subsequent lyophilisation of cells: a spatula tip of *E. coli* powder was added to 2 mL Terrific broth medium (TB) in a 12-mL glass tube with metal cap and shaken overnight in an incubator. 37°C at 200 rpm (3.3 Hz). 100 µL-aliquots were mixed with glycerine (final concentration 15%) and stored at -80°C. 5 µL of this *E. coli* stock culture were used as inoculum of 200 or 500 mL-Erlenmeyer flasks containing 50 or 100 mL TB, respectively. The cultures were incubated at 37°C and continuously shaken for aeration (200 rpm/ 3.3 Hz). After 16-18 h. the early stationary-phase cells were spun down ($10^4 \times g$), washed in 0.9% (w/v) NaCl and lyophilized, i.e the cell mass was frozen at -80°C and the remaining fluid vaporized under vacuum in a lyophilisator (Alpha 1-4 LD, Martin Christ). It should, however, be noted, that not all preparations showed satisfactory results[2] and that each preparation has to be examined with regard to a low binding of the bacterial substrate to sludge flocs under testing conditions.

Other bacterial strains and other preparation methods are of course not excluded if they fulfill the validity criteria.

[2] It is known that environmental conditions and the bioavailability of nutrients may considerably shift the adhesive properties of bacterial cells (e.g. Faille, et al., 2002; Bonaventura, et al., 2008) and that bacterial strains have quite considerable possibilities of hydrophobic property variations in the course of growth (e.g. Jorand, et al., 1994).

ANNEX II - TEST MEDIUM

Test medium: moderately hard EPA synthetic water (EPA, 2002), pH-buffered

I. preparation of chemical stock solutions

1) prepare 100 mL of a 40-fold concentrated stock solution for each reagent 1-5:

reagent	final conc. in test	conc. factor	reagent per 100 mL
(1) NaHCO3	96 mg/L	40x	384 mg ad 100 mL H2O
(2) CaSO4▪2H2O	60 mg/L	40x	240 mg ad 100 mL H2O
(3) MgSO4	60 mg/L	40x	240 mg ad 100 mL H2O
(4) KCl	4 mg/L	40x	16 mg ad 100 mL H2O
(5) Hepes, pH 7.5	10 mM	40x	9.532 g adjust pH → ad 100 mL H_2O

2) adjust the pH of the HEPES buffer-solution to pH 7.5 (NaOH)

a) dissolve 9.532 g HEPES reagent in ca. 90 mL of H_2O

b) adjust the pH to 7.5 with a concentrated NaOH solution (ca. 2.8 mL of a 5 M (200g/L) NaOH solution)

c) fill up to 100 mL with deion. (or distilled) water

3) store the test medium stocks at -20°C (for practical reasons it is recommended to aliquote the stocks)

II. preparation of the standard moderately hard EPA synthetic water, pH-buffered

1) Fill a 200 milliliter volumetric flask with approximately 160 ml deionized (or distilled) water and

2) add 5 mL of each concentrated stock solution, in the sequence 1 to 5.

3) Add deionized (or distilled) water up to the 200 ml mark and shake to homogenize the medium.

→ store the (diluted) test medium at 4-6°C for no longer than 2 weeks.

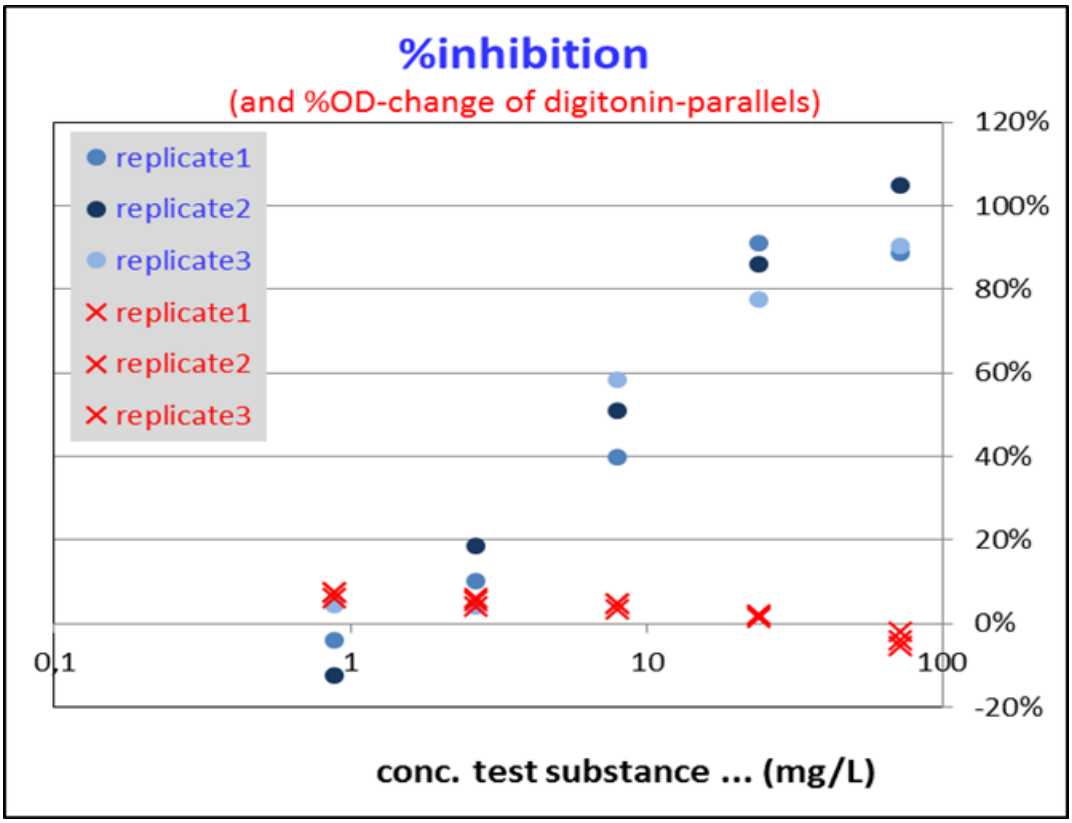
ANNEX III- PREPARATION OF DIGITONIN SOLUTION

*Digitonin (*CAS Number 11024-24-1) tends to *form precipitates* in solution, but it dissolves readily in boiling water. After cooling the solution remains clear for several hours.
Add 100 mg digitonin to 10 mL H_2O (= 10 g/L = 50-fold concentrated solution), heat the solution to about 95°C-100°C for 5 minutes and vortex slowly to dissolve the precipitate. Cool to room temperature prior to use (*Note*: Commercial digitonin powder is a mixture consisting of about five glycosides (Fukunaga, et al., 1988). One of the main components is digitonin, which amounts to about 50% of the preparation (TLC) from Sigma-Aldrich used in the ring study).

ANNEX IV - CONCENTRATION-RESPONSE CURVES

Concentration-response curves

Figure 1: Exemplary concentration-response plot with %inhibition data (triplicates) for the phagocytosis



activity (filled circle) and the %change of the defaunated digitonin-triplicates (cross).

LITERATURE

Mooney, R. A., 1988. Use of digitonin-permeabilized adipocytes for cAMP studies. Methods Enzymol., pp. 193-202.