NEPTUNE

New sustainable concepts and processes for optimization and upgrading municipal wastewater and sludge treatment

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under the Thematic Priority 'Global Change and Ecosystems'

Work Package 3 · Contaminant and toxicity identification and assessment

Deliverable D3.1 · Ecotoxicity and microbiological testing methods (OECD and DIN/ISO guidelines) optimized for whole effluent testing

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REFERENCES

1 Introduction

Within Workpackage 3 the following tests will be performed:

• Whole effluent testing (WET) and Toxicity Identification Evaluation (TIE) in fractionated samples for up to 200 samples with **tier 1 screening assays**: YES (Yeast Estrogenic Screen) and YAS (Yeast Androgenic Screen) for the identification of receptor-mediated (anti-) estrogenic and (anti-) androgenic activities, Yeast Mutagenicity Screen and Cytotoxicity Screen.

• WET for up to 10 samples with **tier 2 tests (chronic and life-cycle exposure),** representing key species for the structure and function of aquatic ecosystems in Europe. The following tests will be performed in flow-through design at partner institutes developing the new waste water treatment technologies: (1) Primary producer test (*Lemna minor*) according to OECD guideline draft, (2) worm (*Lumbriculus variegatus*) 28-d growth and reproduction test according to OECD guideline draft (3) mollusc (*Potamopyrgus antipodarum*) 28-d reproduction test according to a new OECD guideline proposal, (4) arthropod (*Chironomus riparius*) life-cycle test to OECD guideline 218 and (5) Fish Early Life Stage Toxicity (FELST) test with *Danio rerio* according to an OECD guideline draft.

• Identification and enumeration of bacterial indicators such as total coliforms, *E. coli* and enterococci as well as viral indicators such as somatic coliphages according to DIN/ISO guidelines adapted to effluent. The test set will be performed in up to 50 whole effluent samples. Bacterial tests will be performed using membrane filtration (MF) techniques, somatic coliphages using the double-layer agar technique.

2 Ecotoxicological testing methods

2.1 Whole effluent testing (WET) and Toxicity Identification Evaluation (TIE)

2.1.1 Tier 1 screening assays

Tier 1 screening assays will be processed for up to 200 samples. The samples will be sent by our project partners in brown glass vessels preferably within 24 hours cooled at 4°C. The tier 1 are selected and developed/adapted for waste water assessment. The tier 1 screening assays can be performed as from July 2007. No deviation from the schedule is anticipated.

2.1.1.1 YES/YAS test

For the identification of receptor-mediated (anti-) estrogenic and (anti-) androgenic activities the yeast screens will be performed according to a standard operation procedure of the Department of Aquatic Ecotoxicology at the University in Frankfurt am Main.
The procedure is the same for YES and YAS. The only thing to consider is that the YAS strain grows slower.

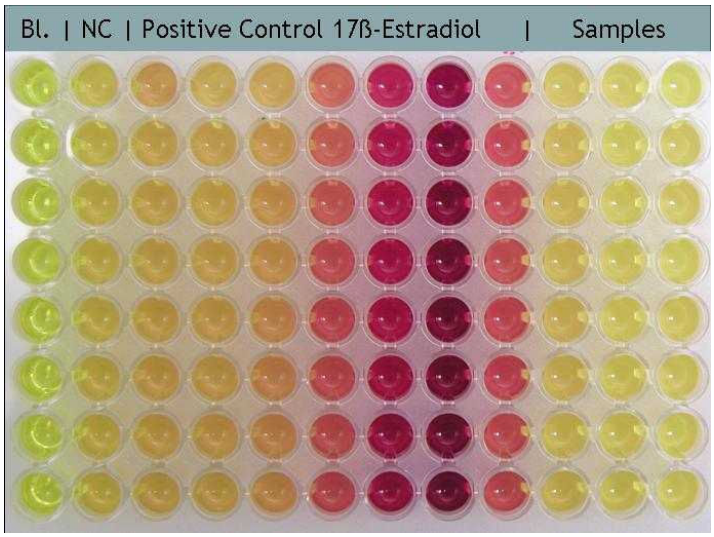


Figure 2.1 YES test set up

2.1.1.1.1 Material

Table 2.1 Solutions

Solutions	Ingredients and handling
growth medium (minimal)	1 mL 10xSD + 1 mL 10xDO + 8 mL ultra pure water/A. demin. (sterile)
	use immediately
10xSD	13,4 g Yeast Nitrogen Base w/o amino acids (10x = 67 g/L) 40 g D-Glucose (20% w/v) 200 mL ultra pure water/A. demin. filtrate sterile in 50 mL aliquots
	store at 4°C
10xDO	100 mg adenine (200 mg/l) 100 mg arginine (200 mg/l) 500 mg asparagic acid (1000 mg/l) 500 mg glutamic acid (1000 mg/l) 100 mg histidine (200 mg/l) 150 mg isoleucine (300 mg/l) 500 mg leucine (1000 mg/l) 150 mg lysine (300 mg/l) 100 mg methionine (200 mg/l) 250 mg phenylalanine (500 mg/l) 2000 mg serine (4000 mg/l) 1000 mg threonine (2000 mg/l) 150 mg tyrosine (300 mg/l) 750 mg valine (1500 mg/l) 500 mL ultra pure water/A. demin. filtrate sterile in 50 mL aliquots
	store at 4°C
lac Z-Puffer	8,52g Na_2HPO_4 (0,06M) 0,75g KCl (0,01M) 0,25g $MgSO_4$ x $7H_2O$ (0,01M) 950mL ultra pure water/A. demin. (takes some time) adjust pH to 7,0 with 25% H_2SO_4 autoclave add 1g SDS under the bench, fill with sterile water to 1L
	store at room temperature (maximum 1 year)
10 mM copper (II) sulphate	solve 2,497 g $CuSO_4$ x 5 H_2O in 1L A. demin., autoclave
	store at room temperature (maximum 1 year)

Table 2.2 Positive control (YES): 17β-estradiolestradiol (CAS: 50-28-2; >99%; Merck, Darmstadt; MW = 272,4 g)

	Ethanol (abs., undenat.)	Substance	Concentration	Concentration in the well (/480)
stock	1000 µL	5,45 mg estradiol	20 mM	/
A	500 µL	+ 500 µL stock	10 mM	/
B	990 µL	+ 10 µL A	100 µM	/
C	520 µL	+ 480 µL B	48 µM	/
D	990 µL	+ 10 µL C	480 nM	1 nM
E	700 µL	+ 300 µL D	144 nM	300 pM
F	900 µL	+ 100 µL D	48 nM	100 pM
G	900 µL	+ 100 µL E	14,4 nM	30 pM
H	900 µL	+ 100 µL F	4,8 nM	10 pM
I	900 µL	+ 100 µL G	1,44 nM	3 pM
K	900 µL	+ 100 µL H	480 pM	1 pM
L	900 µL	+ 100 µL I	144 pM	0,3 pM
M	900 µL	+ 100 µL K	48 pM	0,1 pM

Table 2.3 Positive control (YAS): Testosterone (CAS: 58-22-0; >98%; Merck, Darmstadt; MW = 288,4 g)

	Ethanol (abs., undenat.)	Substance	Concentration	Concentration in the well (/480)
stock	1000 µL	5,768 mg testost.	20 mM	/
A	500 µL	+ 500 µL stock	10 mM	/
B	990 µL	+10 µL A	100 µM	/
C	520 µL	+ 480 µL B	48 µM	100 nM
D	500 µL	+ 500 µL C	24 µM	50 nM
E	700 µL	+ 300 µL C	14,4 µM	30 nM
F	500 µL	+ 500 µL E	7,2 µM	15 nM
G	500 µL	+ 500 µL F	3,6 µM	7,5 nM
H	900 µL	+ 100 µL D	2,4 µM	5 nM
I	500 µL	+ 500 µL H	1,2 µM	2,5 nM
K	800 µL	+ 200 µL H	480 nM	1 nM
L	900 µL	+ 100 µL H	240 nM	0, 5 nM

2.1.1.1.2 Culturing informations

Liquid culture

- Prepare growth medium in a sterile 50 mL vial
- Pick a single colony from an agar plate stored in the fridge and transfer it to the vial
- Mix until the colony has been completely resuspended
- Place in the incubator (30°C, 750 rpm)
- Grow over night

Culture on agar plate

- Autoclave 200 mL ultra pure water/A. demin. + 5 g Agar (2% w/v) sufficient for approx. 8 plates
- Cool down to approx. 50°C add 25 mL 10xSD + 25 mL 10xDO
- Pour the plates and let it cool down
- Plates can be stored in the fridge for 4 weeks maximum (check for contaminations)
- Plate 20 µL of a fresh liquid culture (no lag-phase!) and let it grow at 30°C until colonies at approx. 1 mm in diameter
- Seal the yeast culture on agar plate with Parafilm and store it in the fridge

Cryo culture

- place 500 µL 80% Glycerol (sterile) in a 2 mL-cryo vial
- add 500 µL of a grown liquid culture
- store at -80°C
- for recovery plate 125 µL of the cryo culture on an agar plate (see above)

2.1.1.1.3 Test procedure

Things to be ready

- Liquid culture of YES/YAS-strain (maximum 3 weeks old) for growth medium see table 2.1.
- Positive controls (E2 and/or Testosterone, shouldn't be older than 4 weeks) see table 2.2 or 2.3
- Water samples/dilutions of the substances to be tested (remember the dilution factor of 480 in the assay itself)
- Arrangement of the samples/concentrations etc. on the plates Excel sheet
- Appropriate uncontaminated reference water

Test procedure of day 1

- Rinse all reservoirs with A. demin. once and absolute EtOH twice, place under the bench for drying
- Label the sterile 96-well-plates according to the sample arrangement
- Prepare 5 x medium as follows in table 2.1

Table 2.4 Test procedure day 1

Testing water samples				
no. of plates	1	2	3	4
Ampicillin	/	10 mg	15 mg	20 mg
Streptomycin	/	10 mg	15 mg	20 mg
10xSD	/	7,5 mL	11,25 mL	15 mL
10xDO	/	7,5 mL	11,25 mL	15 mL
1mM $CuSO_4$	/	148 µL	222 µL	296 µL

Testing water samples

Get the water samples ready, add 1 mL in 8fold-reservoir each. Add 75 µL reference water to the wells of blank, negative control and positive control. Add 75 µL of water sample to the wells according to sample arrangement. For preparing positive control: add 990 µL 5 x medium to appropriate 8fold-reservoir. Add 10 µL of the E2/testosterone concentration to the appropriate well. Add 25 µL of the solved E2/testosterone in each well (Multipette). Add solvent to 5 x medium (undivided reservoir):

4 plates: 9,9 mL 5 x medium + 100 µL EtOH

3 plates: 7,43 mL 5 x medium + 75 µL EtOH

2 plates: 4,95 mL 5 x medium + 50 µL EtOH

Add 25 µL of this solution to all well except the positive control. Put 0.5 mL yeast liquid culture (per plate) in an Eppendorf vial centrifuge for 7 min at 14,5 rpm. Add 2.5 mL 5 x medium (per plate) in an undivided reservoir. Carefully decant the supernatant and resuspend the culture in the same volume (0.5 mL per plate) of 5 x medium, add to the reservoir. Add 20 µL of yeast culture in each well, except the blank (20 µL of pure 5 x medium). Note the time in the Excel sheet. Seal plates with Breathe-Easy membrane. Incubate for 24 h at 30°C and 750 rpm.

Day 2

Table 2.5 Preparation of lacZ-solution

No. of plates	1	2	3	4
CPRG	4 mg	8 mg	12 mg	16 mg
Lyticase	1,75 mg	3,5 mg	5,25 mg	7 mg
lacZ-buffer	10 mL	20 mL	30 mL	40 mL
ß-mercaptoethanol	25 µL	50 µL	75 µL	100 µL

Carefully remove the membranes, shake until cells are completely resuspended and bubbles are removed. Determine OD_{595} (optical density at 595 nm wavelength) and copy to Excel-Sheet. Add 100 µL lacZ-solution to each well and place plates again in the incubator (30°C/750 rpm). Determine OD_{540} in intervals of 0.5 or 1 h and copy values to the Excel-Sheet (reaction should be finished after 4 h).

For data analysis, copy all values to the calculation sheet, this should calculate the corrected absorbance. Determine the best fitting dose response relationship for the positive controls in Prism. Use the values corresponding to the best fitting curve for sample interpretation.

2.1.1.2 Yeast mutagenicity screen

For detection of the mutagenic potential of whole effluents, the Yeast mutagenicity screen will be used. The test is based on the work of Srb (1956).
The yeast mutagenicity screen is using a forward mutation in the arginine permease, which also transports canavanine into the cell. This mutation leads to a loss of function of the permease and keeps the yeast resistant to the arginine analogon canavanine.

2.1.1.2.1 Material

The yeast culture used contains the wildtype of *Saccharomyces cerevisiae* (BY4741) and for the positive control, the mutant BY4741 $^{\Delta}$CAN1 which has no functional arginine permease.

Table 2.6 Preparation of media

Media	Ingredients
YPD	2% D-glucose 2% Bacto peptone 1% Yeast Extract in aqua dem.
Synthetic complete medium (SC)	1,6% agar 2% D-glucose 0,17% Difco Yeast Nitrogen Base w/o amino acids 0,51% ammonium sulphate 680 mg /L synthetic complete mixture sterilised in autoclave for 20 min at 121°C
Synthetic complete mixture	40 mg adenine 30 mg arginine 20 mg histidine 20 mg isoleucine 30 mg leucine 30 mg lysine 20 mg methionine 50 mg phenylalanine 100 mg serine 150 mg threonine 30 mg tyrosine 100 mg valine 30 mg tryptophane 20 mg uracil 1000 mL A. demin.
Drop out media (canavanine plates)	Synthetic complete mixture without arginine 40 mg/L canavanine

2.1.1.2.2 Test procedure

It is necessary to work under sterile conditions to prevent microbial contamination.

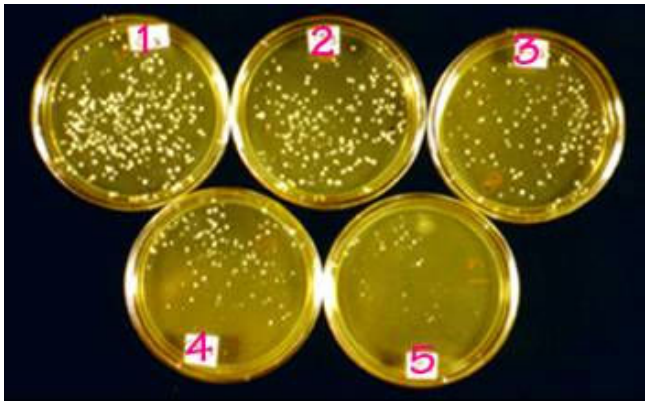


Figure 2.2 Yeast mutagenicity screen

Both yeast strains grow overnight in liquid YPD at 30°C. After measurement of the OD_{595}, dilute the cultures down to 2 x 10^8 cells/mL. Take 2 mL from the diluted yeast culture (BY4741) for each sample in an Eppendorf vial. Same procedure for solvent control, positive control and negative control. Take 1 mL from the BY4741 $^{\Delta}$CAN1 culture for the positive control of the drop out plates. Centrifuge all samples for 1 min at 3000 rpm. Carefully decant the supernatant. To prevent microbial contamination centrifuge the wastewater samples for 15 min at 14500 rpm before treatment. Resuspend with either 2000 µL of the water sample, 1980 µL A. demin. + 20 µL Ethanol (solvent control),

1980 µL A. demin. + 20 µL of 100 µM 4-NQO (positive control) or 2000 µL A. demin. (negative control). Resuspend the BY4741 $^{\Delta}$CAN1 pellet in 1000 µL A. demin.
Incubate all vials for 90 min at 30°C and 450 rpm. Centrifuge 30 s at 3000 rpm. Decant the supernatant. Resuspend with 2 mL A. demin. (cleaning step). Centrifuge 30 s at 3000 rpm. Decant the supernatant. Resuspend in 2 mL YPD (room temperature). Incubate for 3 h at 30°C and 450 rpm. Centrifuge 30 s at 3000 rpm. Decant the supernatant. Resuspend with 2 mL A. demin. (cleaning step). Centrifuge 30 s at 3000 rpm.
Resuspend the 4-NQO and wastewater treated samples, negative control and solvent control in 300 µL A. demin. Plate 50 µL per plate onto 3 canavanine plates.
Make a 1:20000 dilution with the remaining 150 µL of each vial/sample for the SC-plates and the positive control (BY4741 $^{\Delta}$CAN1) for the canavanine plates. Plate 50 µL per plate onto 3 SC/canavanine-plates. Incubate for 3 days at 30°C in dark.

Calculation of the mutation rate. The number of grown colonies on the canavanine plates divided by the number of grown colonies on the SC-plates multiplied by 20000 (dilution step). To get the mutation rate induced by wastewater subtract the spontaneous mutation rate of the negative control.

2.1.1.3 Cytotoxicity screen

The cell density measured within the YES/YAS (see 2.1.1.1.3) as well as the number of colonies grown on SC-plates (see 2.1.1.2.2, mutagenicity screen) act as tool for assessing cytotoxicity. Significant differences between cell densities of negative control and wastewater treated samples are calculated with ANOVA and a sufficient post-test. Significant decreased cell numbers indicate cytotoxicity.

2.1.2 Tier 2 (chronic and life-cycle exposure)

The on-site tests within tier 2 will be performed directly at wastewater treatment plants. For the flow-through design, 24 channel tubing pumps will be used to achieve a water exchange of six times per day (two times per day in the case of the FELST-Test) with a continuous flow through the test vessels. The test waters come from reservoir basins (60 l) with high flow through rates of treated wastewater directly from the effluent. The water body of the reservoir basins are aerated by air tubes. The test chambers are glass aquaria (12 l) divided into 5 compartments with equal size, which can be used as replicates (except for the FELST, see 2.1.2.5). For keeping a constant temperature, the test chambers are located in a basin filled with water. For cooling/heating a flow through cooling-heating system is used. For the

sediment required test organisms - *Lumbiculus* and *Chironomus* - the artificial sediment composition according the OECD guidelines will be adapted to the flow through conditions. Therefore no kaolin clay will be used because its fine graininess could lead to a flush out of sediment and to a clouding of the test waters due to the flow through design. The amount of food necessary to ensure survival and natural growth of the organisms is added to the sediment (see 2.1.2.2).

A pilot test with the flow through test system onsite at a waste water treatment plant is going to start in July. No deviation from the schedule is anticipated.

Table 2.7 Equipment for the flow through system

Equipment	Purpose
Aquarium (60 l)	Reservoir for the test waters
Aquaria (12 l) divided into 5 replicates	Test chamber
Basin (synthetic resin) 220x20x40 cm	For maintaining temperature in the test chambers
RC20 CS - Cooling-/heating pump (Lauda)	For maintaining temperature of the water in the basin
IPC 24 - Tubing Pump (Ismatec)	For a continuous flow through the test vessels

2.1.2.1 Lemna minor

As primary producer test-organism, the duckweed *Lemna minor* is used according the OECD-guideline draft from 2002.

Principle of the test. Plants of the genus *Lemna* are allowed to grow as monocultures in different test waters over a period of seven days. The objective of the test is to quantify different wastewater treatment-related effects on vegetative growth over the investigated period based on assessments of frond number, and also on assessments of biomass (total frond area, dry weight or fresh weight). To quantify wastewater-related effects, growth in the test solutions is compared with growth of the control and the x % inhibition of growth (e.g. 50 %) is determined.

Figure 2.3 *Lemna minor*

Validity of the test. For the test to be valid, the doubling time of frond number in the control must be less than 2.5 days (60 h), corresponding to an approximately seven-fold increase in

seven days. It is anticipated that this criterion will be achievable under flow-through test conditions.

Incubation conditions. Continuous white fluorescent lighting is used to provide a light intensity range of 6500-10000 lux and a photosynthetically-active radiation (400-700 nm) of 85-125 $\mu E^{-2}s^{-1}$ as measured at points with the same distance from the light source as the *Lemna* fronds. The temperature in the test vessels is 24 ± 2 °C. The pH of the control medium does not increase by more than 1.5 units during the test. However, deviation of more than 1.5 units would not invalidate the test when it can be shown that validity criteria are met.

Duration. The test is terminated 7 days after the plants were transferred into the test vessels.

Observations. At the start of the test, frond and colony numbers in the test vessels are counted and recorded, taking care to ensure that overlapping but distinctly visible fronds are accounted for. Frond and colony numbers (normal and abnormal) and their appearance need only be determined at the beginning and end of the test when effects are to be assessed in terms of the average specific growth rate over the full duration of the test. Changes in plant development (e.g. frond size, appearance, necrosis, chlorosis or gibbosity, colony break-up or loss of buoyancy, root length, morphology or breakdown) are noted. Significant features of the test medium (e.g. presence of undissolved material, growth of algae in the test vessel) should also be noted. In addition to determinations of frond number during the test, effects of the wastewater on final biomass are also assessed, based on one the following parameters: total frond area and dry weight. Dry weight is additionally determined at the start of the test from a sample of the inoculum culture, and at the end of the test with the plant material from each test and control vessel. Total frond area can be determined for each test and control vessel at the start, in the course, and at the end of the test.

Total frond area, dry weight and fresh weight may be determined as follows:

- Total frond area: The total frond area of colonies may be determined by image analysis. A silhouette of the test vessel and plants can be captured using a video camera (i.e. by placing the vessel on a light box) and the resulting image digitised. By calibration with flat shapes of known area, the total frond area in a test vessel may then be determined. Care should be taken to exclude interference caused by the rim of the test vessel.
- Dry weight: Colonies are collected from each of the test vessels and rinsed with distilled or deionised water. They are blotted to remove excess water and then dried at 60 °C to a constant weight. Any root fragments should be included. The dry weight should be expressed to an accuracy of at least 0.1 mg.

- Fresh weight: Colonies are transferred, to pre-weighed polystyrene (or other inert material) tubes with small (1 mm) holes in the rounded bottoms. The tubes are then centrifuged at 3000 rpm for 10 min at room temperature. Tubes, containing the now dried colonies, are re-weighed and the fresh weight is calculated by subtracting the weight of the empty tube.

Frequency of analytical determinations and measurements. Light intensity is measured in the growth chamber, incubator or room at points with the same distance from the light source as the *Lemna* fronds. Measurements should be made at least once during the test. The temperature of the medium in a surrogate vessel held under the same conditions in the growth chamber, incubator or room is recorded at least daily. The additional water-parameters as pH-value, currency, ammonium and phosphate will be recorded 2 times per week.

Statistic analysis. To estimate if different wastewater treatment technologies result in different toxic wastewater, ANOVA is used to calculate the mean average specific growth rate or final biomass and pooled residual standard deviation across replicates for each treatment group. The resulting mean for each treatment is then compared with the control and different treated wastewaters mean using an appropriate multiple comparison method e.g. Dunnett's or Williams' tests. Other multiple comparison techniques may be preferred if deemed appropriate. A test for normality of the data is advised e.g. by calculating the Shapiro-Wilk's statistic and, if the replicate data reveal a normally distributed error structure, a test for homogeneity of variances across treatment groups is recommended e.g. using Bartlett's or Levene's test. If the variances are not homogeneous, it may be necessary to carry out a transformation of the data prior to ANOVA calculations. The log transformation is recommended for average specific growth rate, and the square root transformation for final biomass. Non-parametric analysis, e.g. Wilcoxon Rank Sum Test, may be used when the assumptions of normality and homogeneity of variances are not satisfied. If a one-tailed test to compare means is used, rejection of the null hypothesis implies that the mean of the treatment group is less than the mean of the control group. If a two-tailed test is used, rejection of the null hypothesis implies that the mean of the treatment group could be either less (inhibition) or more (stimulation) than the mean of the control group. The type of means comparison test should therefore be described and also whether a one-tailed or two-tailed procedure was employed. In addition, the size of the effect which can be detected using ANOVA (the least significant difference) must be reported.

2.1.2.2 Lumbriculus variegatus

As sediment-ingesting endobenthic test-organism the aquatic oligochaete *Lumbriculus variegatus* will be used according the OECD guideline draft from 2006.

Principle of the test. Adult worms of similar physiological (synchronised) state are exposed to different treated wastewaters in a sediment water system. Artificial sediment is used as media. Test vessels with tap water or - if this is not appropriate – reconstituted water serve as controls. The test organisms are introduced into the test vessels in which the sediment and water concentrations of chemical substances have been equilibrated for seven days. The test animals are exposed to the sediment-water systems for a period of 28 days. Due to the low nutrient content of the artificial sediment, the sediment will be amended with a food source (powder of *Urtica dioica* leaves) to ensure that the worms will grow and reproduce under control conditions. In this way it is ensured that the test animals are exposed through the water and sediment as well as by their food.

Figure 2.4 Lumbriculus variegatus

Validity of the test. For a test to be valid, the following requirements should be fulfilled:
- A ring-test has shown that for *Lumbriculus variegatus*, the average number of living worms per replicate in the controls should have increased by a factor of at least 1.8 at the end of exposure compared to the number of worms per replicate at the start of exposure.
- The pH of the overlying water should be between 6 and 9 at the start and at the end of the test.
- The oxygen concentration in the overlying water should not be below 60% of air saturation value (ASV) at the end of the test.

Sediment. A formulated sediment is used to provide maximum standardisation. The following formulated sediment is based on the artificial sediment according to the official guideline apart from the absence of kaoline clay:
(a) 4-5 % (dry weight) peat: as close to pH 5.5 to 6.0 as possible
(b) 95-96 % (dry weight) quartz sand (fine sand, grain size: ≤ 2 mm, but > 50 % of the particles should be in the range of 50-200 µm).
(c) Deionised water, 30-50 % of sediment dry weight, in addition to the dry sediment components.

(d) Calcium carbonate of chemically pure quality ($CaCO_3$) is added to adjust the pH of the final mixture of the sediment.
(e) The total organic carbon content (TOC) of the final mixture should be 2 % (±0.5 %) of sediment dry weight and is adjusted by the use of appropriate amounts of peat and sand, according to (a) and (c).
(f) Food, a mixture of powdered leaves of *Urtica* sp. with alpha-cellulose (1 : 1), at 0.4 - 0.5 % of sediment d.w., in addition to the dry sediment components.

Exposure conditions. The test is conducted with 10 worms for each replicate. This number of worms corresponds to approximately 50 - 100 mg of wet biomass. Assuming a dry content of 17.1%, this results in approximately 9 - 17 mg of dry biomass per vessel. For the formulated sediment, this corresponds to approximately 43 g sediment (dry weight) per 10 worms at a TOC content of 2.0% of dry sediment. Only worms of similar size, the same source and similar physiological state are selected. The worms to be used in the test are removed from the culture. Adult animals that do not show signs of recent fragmentation are transferred to glass dishes (e.g. Petri dishes) containing clean water. They are subsequently synchronised. After regenerating for a period of 10 to 14 d, intact complete worms of similar size, which are actively swimming or crawling upon a gentle mechanical stimulus, should be used for the test. Since food is added to the sediment, the worms are not fed additionally during the test. The photoperiod in the culture and the test is 16 hours per day. Light intensity should be kept low (e.g. 100-1000 lx) to imitate natural conditions at the sediment surface, and measured at least once during the exposure period. The temperature is 20 °C ± 2°C throughout the test. The dissolved oxygen concentration should not fall below 60% of air saturation value (ASV).

Water quality measurements. The following water quality parameters have to be measured in the overlying water:

- **Temperature** at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period
- **Dissolved oxygen content** at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period; expressed as mg/L and % ASV (air saturation value)
- **Air supply** should be controlled at least once daily on workdays and – if necessary - adjusted
- **pH** at least in one test vessel of each concentration level and one test vessel of the controls once per week and at the start and the end of the exposure period

- **Total water hardness** at least in one replicate of the controls and one test vessel at the highest concentration at the start and the end of the exposure period; expressed as mg/L $CaCO_3$
- **Total ammonia content** at least in one replicate of the controls and in one test vessel of each concentration level at the start of the exposure period, and subsequently 3 x per week; expressed as mg/L NH_4^+ or NH_3 or total ammonia-N.

Biological parameters at the end of the test. The test vessels are observed in order to assess visually any behavioural differences in the worms (e.g. sediment avoidance, faecal pellets visible on the sediment surface) compared with the controls. Observations are recorded. Each replicate is examined. The total number of living and dead individuals per replicate will be recorded and assessed as well as the dry weight. The biomass is determined as total biomass per replicate including adult and young worms.

Treatment of results. The main parameters to be evaluated statistically are the biomass and the total number of worms per replicate. Additionally, reproduction (as increase of worm numbers) and growth (as increase of dry biomass) are evaluated statistically. In this case, an estimate of the dry weight of the worms at start of exposure should be obtained e.g. by measurement of the dry weight of a representative subsample of the batch of synchronised worms to be used for the test. Additionally, mortalities are evaluated as far as possible.

2.1.2.3 Potamopyrgus antipodarum

Introduction. The New Zealand mud-snail *Potamopyrgus antipodarum* is a well established test organism for the in-vivo detection of endocrine disruption and especially estrogenic activity (Schulte-Oehlmann et al. 2001; Duft et al. 2003b; Duft et al. 2003a). Due to the assumedly exclusive parthenogenetic reproduction of this introduced species in Europe, the populations are considered to be solely female and genetically almost equal (Jacobsen et al. 1996; Weetman et al. 2002; Staedler et al. 2005). In these tests a well established population of our laboratory at the University Frankfurt is used.

Figure 2.5 Potamopyrgus antipodarum

Short description of the method. Adult female *P. antipodarum* are exposed to test-waters. Survival of the snails is determined at the end of the test, while reproduction is examined after 14, 28 and 56 days of exposure to the test waters for a sub-group of exposed animals. After removal of the shell, embryos can easily be seen through the epithelia. By opening the brood pouch and subsequently removing the embryos and counting them, the reproductive success of each female is easy to determine.

For a test to be valid the following conditions should be fulfilled:

- mortality in the controls should not exceed 20% and
- the dissolved oxygen concentration must be at least 60% of the air saturation value throughout the test.

Feeding is done at least three times per week with fine grounded TetraPhyll® (2 µg per animal). The photoperiod in the culture and the test is L:D = 16:8 hours and temperature is 16 ± 1 °C throughout the test.

Test animals are taken from the laboratory stock (showing no signs of stress such as high mortality, poor fecundity, etc.). The stock snails must be maintained at culture conditions (light, temperature, medium and feeding) similar to those to be used in the test. The shell height of the test snails should be between 3.7 and 4.3 mm and 80 adult snails have to be allocated randomly to each test vessel containing the exposure water using tweezers. The test vessels are observed at least three times per week to achieve visual assessment of any abnormal behaviour (for example avoidance of water). The reproductive success of 20 snails per replicate are analysed after 14, 28, and 56 days of exposure to the tested substance, respectively. Therefore, the snails are narcotised for 45 to 90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 H_2O) in deionised or distilled water. Shell height and aperture height of snails (in a dry state) have to be measured under a stereomicroscope with an ocular micrometer. The shell of the snails is cracked carefully with a pair of pincers. Subsequently the snails are placed into a dissecting dish, which is filled with a solution of magnesium chloride hexahydrate 2.5%. The soft body can be prepared by removing the shell with dissecting needles or pointed tweezers. The brood pouch of the snails should be opened carefully with a dissecting needle, and the embryos have to be counted, distinguishing shelled and unshelled embryos. The mean and variability parameters such as standard deviation or standard error of the mean, for the shell height, aperture height, number of embryos with shell, number of embryos without shell, and the total number of embryos are calculated. The main parameters to be evaluated statistically are the mortality and the total number of embryos, separated into shelled and unshelled embryos per replicate. An increasing number of embryos compared to the control act as an indicator for estrogenic activity.

2.1.2.4 Chironomus riparius

In this test the sediment-dwelling larvae of the dipteran *Chironomus riparius* is used according the OECD guideline 218 from 2004.

Principle of the test. First instar chironomid larvae are exposed to different treated wastewaters in sediment-water systems. Artificial sediment (2.3.2) and reconstituted water should be used as media. Test vessels with tap water or - if this is not appropriate – reconstituted water serves as control. The test organisms are introduced into the test vessels in which the sediment and water concentrations of chemical substances have been equilibrated for seven days. Chironomid emergence and development rate is measured at the end of the test. These data are analysed by using statistical hypothesis testing to determine significant differences in the toxicity of the investigated wastewaters. This requires comparison of effect values with control values using statistical tests.

Figure 2.6 *Chironimus riparus*, adult

Validity of the test. For the test to be valid the following conditions apply:

- the emergence in the control must be at least 70% at the end of the test;
- emergence to adults from control vessels should occur between 12 and 23 days after their insertion into the vessels
- the water temperature should not differ by more than ± 1 °C

Sediment. see 2.1.2.2

Addition of test organisms. Four to five days before adding the test organisms to the test vessels, egg masses are taken from the cultures and placed in small vessels in culture medium. Aged medium from the stock culture is used. Only freshly laid egg masses are used. Normally, the larvae begin to hatch a couple of days after the eggs are laid (2 to 3 days for *Chironomus riparius* at 20 °C) and larval growth occurs in four instars, each of 4-8 days duration. First instar larvae (2-3 or 1-4 days post hatching) are used in the test. The instar of midges is checked using head capsule width. Twenty first instar larvae are allocated randomly to each test vessel using a blunt pipette. The flow through has to be stopped while adding the larvae to test vessels and remain so for another 24 hours after addition of larvae.

Water quality measurements. see 2.1.2.2

Incubation conditions. The test is conducted at a constant temperature of 20°C (±1°C). A 16 hours photoperiod is used and the light intensity should be 500 to 1000 lux.

Exposure duration. The maximum exposure duration is 28 days. If midges emerge earlier, the test can be terminated after a minimum of five days after emergence of the last adult in the control.

Emergence. The development time and the total number of fully emerged male and female midges are determined. Males are easily identified by their plumose antennae. The test vessels are observed at least three times per week to make visual assessment of any abnormal behaviour (for example leaving sediment, unusual swimming), compared with the control. During the period of expected emergence a daily count of emerged midges is necessary. The sex and number of fully emerged midges are recorded daily. After identification the midges are removed from the vessels. Any egg masses deposited prior to the termination of the test are recorded and then removed to prevent re-introduction of larvae into the sediment. The number of visible pupae that have failed to emerge is also recorded.

Physical-chemical parameters. see 2.1.2.2

Treatment of results. The purpose of this test is to determine and to compare the effects of advanced and conventional treated wastewaters on the development rate and the total number of fully emerged male and female midges. If there are no indications of statistically different sensitivities of sexes, male and female results may be pooled for statistical analyses. For determining different toxicities among the test waters by hypothesis testing, the variability among vessels needs to be taken into account, for example by a nested ANOVA. Alternatively, more robust tests can be appropriate in situations where there are violations of the usual ANOVA assumptions.

2.1.2.5 Danio rerio

Introduction. Tests with the early-life stages of fish are intended to define the lethal and sublethal effects of chemicals - or in this case wastewater - on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sublethal effects of the substance or water sample on other fish species. The following description is

written according to the draft EPA guideline draft for the Fish Early Life Stage Toxicity (FELST-) Test from 1996 (OPPTS 850.1400).

Figure 2.7 *Danio rerio*, adult fish and fertilized eggs

Principle of the test. The early-life stages of fish are exposed to advanced (e.g. ozonation, activated carbon) and conventional treated wastewater as well as to control water (reconstituted water) under flow-through conditions with a water exchange of two times per day. The test starts by placing fertilized eggs in the test chambers (stainless steel, 10 L volume) and is continued at least until all the control fish are free-feeding. Lethal and sublethal effects (malformation, abnormal behaviour) are assessed and compared with control values. The test are performed directly at wastewater treatment plants.

Short description of the method.

Table 2.8 Feeding and handling requirements of brood and test animals

Species	Food			
	Brood Fish	Newly Hatched	Juveniles	Time to first feeding
Danio rerio	BSN48, flake food	protozoa [1] protein [2]	BSN48	6–7 days after spawning

[1] filtered from mixed culture

[2] granules from fermentation process

Table 2.9 Test conditions, duration, and survival criteria

Species	Test Conditions		Recommended Duration of Test	Survival of Controls (minimum percent)
	Temperature (°C)	Photoperiod (hours)		Post-Hatch Success
Danio rerio	25 ± 2	12–16	30 days post-	70

			hatch	

Control. One water control (tap water or if not appropriate reconstituted water made out of demineralised water and reconstituted with Aqua Marin® to a conductivity of 700 µS/cm) will be run in addition to the test series.

Loading. The number of fertilized eggs at the start of the test has to be sufficient to meet statistical requirements. They are randomly distributed among treatments, and 30 fertilised eggs are used per replicate. In cases of low fecundity a higher number of eggs (e.g. 60) have to be introduced into the test vessels, to achieve the recommended number of 30 fertilised eggs.

Frequency of analytical determinations and measurements. During the test, dissolved oxygen, pH, total water hardness and salinity (if relevant), and temperature are measured in all test vessels. As a minimum, dissolved oxygen, salinity (if relevant), and temperature should be measured weekly, and pH and hardness should be measured at the beginning and end of the test.

Observations. The embryonic stage at the beginning of exposure to the test substance is verified as precisely as possible. This is done using a representative sample of eggs suitably preserved and cleared.

Hatching and survival. Observations on hatching and survival are made at least once daily and numbers have to be recorded. Dead embryos, larvae, and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage: For eggs: Particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance. For embryos: Absence of body movement and/or absence of heart-beat. For larvae and juvenile fish: Immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

Abnormal appearance. The number of larvae or fish showing abnormality of body form is recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally

and can be of the order of several percent in the controls in some species. Abnormal animals should only be removed from the test vessels on death.

Abnormal behaviour. Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence, and atypical feeding behaviour are recorded at adequate intervals depending on the duration of the test. These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data and influence a decision to extend the exposure period beyond the recommended duration.

Weight. At the end of the test all surviving fish must be weighed. Individual weights are preferred but, if the fish are especially small, they may be weighed in groups by test vessel. Dry weights (24 h at 60 °C) are preferable to wet weights (blotted dry).

Length. At the end of the test, measurement of individual lengths is recommended. Standard, fork, or total length may be used. If however, caudal fin rot or fin erosion occurs standard lengths should be used.

Data for statistical analysis. These observations result in some or all of the following data being available for statistical analysis: Cumulative mortality. Numbers of healthy fish at end of test. Time to start of hatching and end of hatching. Numbers of larvae hatching each day. Length and weight of surviving animals. Numbers of deformed larvae. Numbers of fish exhibiting abnormal behaviour.

Data and reporting treatment of results. To make a multiple comparison between the results at the different treated wastewater and that for the control, Dunnett's method is used. However, care must be taken when applying such a method to ensure that chamber-to-chamber variability is estimated and is acceptably low. Other useful methods are also available.

Results. Evidence that controls met the overall survival acceptability standard of the test species. Data on mortality/survival at embryo, larval, and juvenile stages and overall mortality/survival. Days to hatch and numbers hatched. Data for length and weight. Incidence and description of morphological abnormalities, if any. Incidence and description of behavioural effects, if any statistical analysis and treatment of data.

2.1.3 Actual Problems

Tier 1 screening assays

In the case of the mutagenicity screen microbial contamination might be a problem because yeast and non-yeast colonies on the agar plates have to be distinguished. Therefore further centrifugation steps of the wastewater samples and treatment with antibiotics should be applied for removing bacterial and fungal contamination. Furthermore a validation of the results of the yeast mutagenicity screen by comparing with a standardized test system (e.g. umu-test) might be useful. Additionally a simplification of the test system is planned to reduce time, place and material needed for the test.

Tier 2 tests

If the use of tap-water as control water is not appropriate for the in-vivo tests (is going to examined in the pilot test in July), reconstituted water has to be applied instead. In this case some basic water parameters (e.g. currency and pH) are able to be adjusted to the waste water conditions to improve the comparability between negative control and water samples.
Additionally the remaining of a constant temperature might be a problem during extreme high or low outside-temperatures, therefore it is recommended to perform these tests during spring and autumn to avoid extreme weather conditions.

3 Microbiological testing methods

It is necessary to work under sterile conditions (clean bench) to prevent microbial contamination of used media and stock cultures. Although all used microbial stock cultures are not human pathogenic, it can not be excluded that other microorganisms that are human pathogens may be present in the wastewater samples. Therefor all samples must be handled with gloves and special care.

3.1 Bacterial indicators

For identification and enumeration of indicators and pathogens, test procedures according to EN ISO standards are adapted to whole effluent of wastewater treatment plants. The detection of *E. coli* and coliforms bacteria are performed according to EN ISO 9308-1:2000, of intestinale enterococci according to EN ISO 7899-2:2000.

Figure 3.1 Membrane filtration unit

In both methods, the water sample is filtered through a membrane which retains the bacteria. After filtration, the membrane is placed on selective and differential media and the cultures are incubated.

The samples have to be examined within 6h after sampling if transported at room temperature- if the samples are transported cooled (5±3°C) and dark, within 24h.

3.1.1 Faecal coliforms and E. coli

3.1.1.1 Material

Table 3.1 Media

Media	Ingredients	Handling
Lactose TTC agar with Tergitol™ (base)	Lactose 20 g Peptone 10g Yeast extract 6g Beef extract 5g Tergitol 0.1g Bromthymol blue 0.05g Agar 12.7g	Add to 1000 mL A. demin. and let completely dissolve in the heat. Adjust pH 7.2 ± 0.1 at 25 °C. Aliquot to 100-250 mL and autoclave at 121 ± 3°C for 15 min
TTC-solution	TCC 0.05%	Sterilize by 0.2µm membrane filtration
Complete Lactose-TCC Agar with Tergitol™ (TCC agar)	basic media 100 mL TCC-solution 5 mL	Let basic media melt completely in the heat, add 5 mL 0.05% TCC-solution sterile and pour 5 mm into petri dishes. Do not store longer than 10 d at 5 ± 3°C in the dark
Tryptophane broth	Caseine 10g L-Tryptophane 1g Sodium chloride 5g	Add to 1000 mL A. demin. and completely dissolve in the heat. Aliquot 3 mL in glass test tubes with lid and autoclave at 121 ± 3°C for 15 min. Final pH 7.5 ± 0.1 at 25 °C
Tryptic-soy-agar (TSA)	Caseine 15g Peptone 5g Sodium chloride 5g Agar 15g	Add to 1000 mL A. demin. and let completely dissolve in the heat. Aliquot to max. 250 mL and autoclave at 121 ± 3°C for 15 min.Final pH 7.2 ± 0.1 at 25 °C. Let cool down to 50 ± 5°C and pour 5 mm into petri dishes
Kovacs Indole reagent	n-Butanol; hydrochloric acid; 4-dimethylamino-benzaldehyde	For the detection of indole production from tryptophan
Bactident oxidase test stripes	N,N-Dimethyl-1,4-phenylenediammonium chloride; naphthol-(1)	For the detection of cytochrome oxidase in microorganisms
1 x Phosphate buffered saline (PBS)	NaCl 8g KCl 0.2g Na_2HPO_4 1.44g KH_2PO_4 0.24g	Add to 1000 mL A. demin and adjust to final pH 7.4 ± 0.2. Autoclave at 121 ± 3°C for 15 min.

If not stated differently, the media can be stored in the refrigerator for up to 1 month.

3.1.1.2 Test procedure

Prepare appropriate dilution steps (1:5 steps), normally up to 10^{-4}, of the wastewater effluent samples with 1x PBS. Mark agar plates with sample ID, volume and dilution. Filter at least 3 subsample volumes of each dilution (25-50mL) through the membrane filter. Place the membrane filter on TCC-agar plate without formation of bubbles between membrane and agar surface. Invert the dish and incubate at 36 ±2 °C for 21± 3h. If no typical colonies get visible, extent incubation time to 44 ±4h.

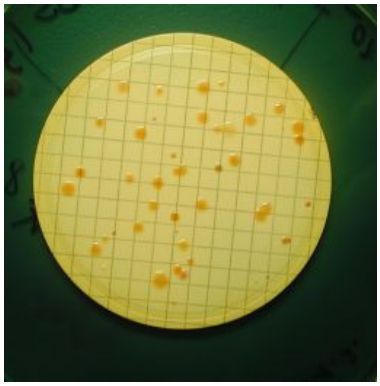


Figure 3.2 Membrane filter with coliform bacteria on TCC-agar

After incubation, check the filters for **colonies under the membrane with a yellowish colour** formation and count them as **lactose-positive**.

Select filters on plates with an acceptable number of positive colonies (20-60). As possible, prepare from all positive colonies (or a representative number) subcultures on non-selective TSA-agar and in Tryptophane broth.

Incubate inoculated TSA plates at 36 ±2 °C for 21± 3h and perform the oxidase test with each isolated colony. Dark blue colour development shows positive oxidase reaction.

Incubate Tryptophane broth at 44 ±0.5 °C for 21± 3h, afterwards add 0.1 mL Kovacs Indole reagent. A red colour formation indicates positive indole production from tryptophane.

Always prepare a negative control (PBS-solution) and a positive control, for example *E. coli* DSM 13127 using the same procedure as for analysing wastewater samples.

Results

Coliform bacteria: all lactose-positive colonies with a negative oxidase reaction on TSA-agar.

E.coli: all lactose-positive colonies with a negative oxidase reaction on TSA-agar and a positive indole test.

For calculation of the results, select countable filters and calculate both coliform and E. coli colonies per 100 mL of wastewater sample as mean values.

3.1.2 Intestinale enterococci

3.1.2.1 Material

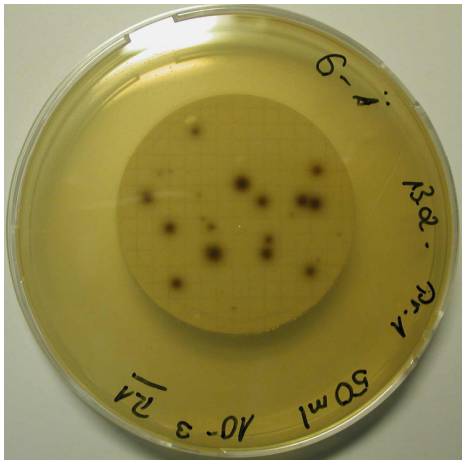
Table 3.2 Media

Media	Ingredients	Handling
Slanetz-Bartley agar	Tryptose 20g Yeast extract 5g Glucose 2g K_2HPO_4 x 2 H_2O 4g Sodium azide 0.4g Tetrazolium chloride 0.1g Agar 10g	Add to 1000 mL A. demin. and bring to boil to dissolve completely. Do not autoclave. Final pH 7.2 ± 0.2 Harmful
Bile-esculin-azide agar	Peptone 25.3g Sodium chloride 5.0g Esculin 1.0g Sodium azide 0.15g Iron(III) citrate 0.5g Bile 10.0g Agar 13g	Add to 1000 mL A. demin. and let completely dissolve in the heat. Autoclave at 121 ± 3°C for 15 min. Final pH 7.1 ± 0.2 at 25 °C. Let cool down to 50 ± 5°C and pour petri dishes. Storable in refrigerator up to 2 weeks
1 x Phosphate buffered saline (PBS)	NaCl 8g KCl 0.2g Na_2HPO_4 1.44g KH_2PO_4 0.24g	Add to 1000 mL A. demin and adjust to final pH 7.4 ± 0.2. Autoclave at 121 ± 3°C for 15 min.

3.1.2.2 Test procedure

Prepare appropriate dilution steps (1:5 steps), normally up to 10^{-4}, of the wastewater effluent samples with 1x PBS. Mark petri dish with sample ID, volume and dilution. Filter at least 3 subsample volumes of each dilution (25-50mL) through the membrane filter. Place the membrane filter on Slanetz-Bartley agar plates without formation of bubbles between membrane and agar surface. Invert plates and incubate at 36 ±2 °C for 44± 4h.

After incubation, all colonies with a pink to red colour are to be regarded as characteristic. Transfer membranes with characteristic colonies to a prewarmed (44°C) Bile-esculin-azide agar plate and incubate at 44 ±0.5 °C for 2h. Pink to red enterococci colonies will develop a black to yellowish-brown precipitate on the underside of the filter.



Always prepare a negative control (PBS-solution) and a positive control, for example *Enterococcus durans* ATTC 11576 using the same procedure as for analysing wastewater samples.

Figure 3.3 Membrane filter with enterococci on Bile-esculin-azide agar

For calculation of the results, select countable filters and calculate enterococci colonies per 100 mL of wastewater sample as mean values.

3.2 Viral indicators

For enumeration of viral indicators, a test procedure according to EN ISO standards has been adapted to whole effluent of wastewater treatment plants. The detection of somatic coliphages will be performed according to EN ISO 10705-2:2001.

Bacteriophages are bacterial viruses that infect only prokaryotic cells. As the determination, detection and cultivation of enteric viruses demands specific laboratorial security facilities (Biosafety level 2), coliphages serve as acceptable indicators of viral removal in wastewater treatment plants (Arraj et al. 2005; Duran et al. 2003).

3.2.1 Somatic coliphages

3.2.1.1 Media

Table 3.3 Media and reagents

Media	Ingredients	Handling
Na_2CO_3- solution	150g Na_2CO_3	Add A. demin. to final 1000 mL and autoclave at 121 ± 3°C for 15 min.
$MgCl_2$- solution	100g $MgCl_2 \cdot 6\ HO_2$	Add A. demin. to final 50 mL and autoclave at 121 ± 3°C for 15 min.
Modified Scholten's broth (MSB)	Peptone 10g Yeast extract 3g Beef extract 12g Sodium chloride 3g Na_2CO_3- solution 5 mL $MgCl_2$- solution 0.3 mL	Add A. demin. to final 1000 mL and let completely dissolve in the heat. Aliquot in 200 mL glass bottles. Autoclave at 121 ± 3°C for 15 min. Final pH 7.2 ± 0.2. Store refrigerated for up to 6 month.
Modified Scholten's agar (MSA base)	Peptone 10g Yeast extract 3g Beef extract 12g Sodium chloride 3g Na_2CO_3- solution 5 mL $MgCl_2$- solution 0.3 mL Agar 15g	Add A. demin. to final 1000 mL and let completely dissolve in the heat. Aliquot 200 mL in glass bottles. Autoclave at 121 ± 3°C for 15 min. Final pH 7.2 ± 0.2. Store refrigerated for up to 6 month.
$CaCl_2$- solution	$CaCl_2 \cdot 6\ HO_2$ 14.6g	Add to 100 mL under carefully heating. Sterilize by membrane filtration (0.2µm). Store in aliquots refrigerated for up to 6 month.
Complete MSA agar	MSA base 200 mL $CaCl_2$- solution 1.2mL	Let MSA base completely melt in a boiling water bath. Cool down to 45-50°C. Add $CaCl_2$- solution aseptically, mix gently and pour petri dishes. Store refrigerated for up to 1 month.
ssMSA	Peptone 10g Yeast extract 3g Beef extract 12g Sodium chloride 3g Na_2CO_3- solution 5 mL $MgCl_2$- solution 0.3 mL Agar 6-10g	Prepare as MSA but with reduced amount of agar (depending on used agar). In our case, 7.5g agar per 1L gave good results. Aliquot 50 mL in glass bottles
Nalidixic acid solution	Nalidixic acid 250mg NaOH (c=1Mol/l) 2mL A. demin. 8mL	Dissolve Nalidixic acid in NaOH, add A. demin and sterilise by membrane filtration (0.2µm). Store at 5±3°C not longer than 8h, at – 20 ± 3°C for up to 6 month
Glycerol solution	Glycerol 87g	Add with A. demin to final 100 mL and autoclave at 121 ± 3°C for 15 min. Store in the dark for up to 1 year.
McConkey agar	Peptone 20g Lactose 10g Bile salts 1.5g Sodium chloride 5g Neutral red 30 mg Crystal violet 1mg Agar 13.5g	Add A. demin. to final 1000 mL and let completely dissolve in the boiling water bath. Final pH 7.1 ± 0.2. Autoclave at 121 ± 3°C for 15 min and pour plates
Peptone salt solution	Peptone 1g Sodium chloride 8.5 g	Add A. demin. to final 1000 mL. Autoclave at 121 ± 3°C for 15 min. Final pH 7.2 ± 0.5. Store refrigerated for up to 6 month.
Nutrient agar	Peptone 5g Beef extract 3g Agar 15g	Add A. demin. to final 1000 mL and autoclave at 121 ± 3°C for 15 min. pH 7.0 ±0.2. Pour plates

Coliphage buffer	Na_2HPO_4 7g KH_2HPO_4 3g Sodium chloride 5g 0.1M $MgSO_4$ 10 mL 0.1M $CaCl_2$ 10mL	Add to 1000 mL A. demin and autoclave at 121 ± 3°C for 15 min.

3.2.1.2 Culturing informations

For samples such as wastewater containing large numbers of bacteria, the nalidixic acid resistant mutant ***E. coli* CN (ATCC 700078)** should be used as **host bacteria** for somatic coliphages to reduce bacterial background.

For preparation of the bacteria cryo host cultures, rehydrate *E. coli* ATCC 700078 stock vial in 3 mL MSB. Add complete volume to 50 mL MSB in a 300 mL Erlenmeyer flask. Incubate for 20 ±4h at 36 ± 2°C and 100 rpm. Add 10 mL sterile Glycerol solution and mix. Dispense into 0.5-1 mL aliquots in 2 mL cryovials and store at – 70 °C.

To prepare frozen host bacteria stock cultures defrost a vial of the cryo host culture at room temperature. Streak culture on McConkey plates to attain isolated colonies. Incubate inoculated plates over night at 36 ± 2°C. Prewarm 50 mL MSB (36 ± 2°C) in 300 mL Erlenmeyer flask and pick 3-5 individual lactose-positive colonies to inoculate the broth. Incubate for 5±1h at 36 ± 2°C and 100 rpm. Add 10 mL sterile Glycerol solution. Dispense into 1 mL aliquots in 2 mL cryovials and store at – 70 °C for up to 2 years.

For calibration of Log-phase host bacteria defrost a vial with frozen host bacteria stock culture at room temperature. To a 200 mL Erlenmeyer flask containing 50 mL MSB add 0.5 mL of host bacteria stock. Incubate at 36 ± 2°C and 100 rpm for approximately 4h. Read absorbance at 600nm every 0.5 h by aseptically dispensing 1 mL of culture in a cuvette. Another 1 mL is used for preparing appropriate dilutions for plate counts on nutrient agar plates (20 ± 4h at 36 ± 2°C).
The calibration procedure is used to determine the OD_{600} and time point at which 10^8 cell forming units (cfu) per mL of the log-phase host bacteria are reached. This time point will be used to inoculate the host bacteria for the somatic coliphages test procedure.
For quality assurance, this procedure needs to be repeated regularly.

For preparation of the bacteriophage cryo stock cultures prepare an over night culture of *E. coli* DSM 13127 in MSB. Next day inoculate 25 mL fresh MSA with 0.25mL of over night culture and incubate at 36 ± 2°C and 100 rpm for 1.5h. Add 25 µL of rehydrated coliphage Phi X174 stock in 2 mL coliphage buffer. Incubate 4.5h. Spin down for 20 min at 3000g.

Decant and add 1.25 mL of glycerol-solution to supernatant that contains the proliferated coliphages. Aliquot in 2mL cryo vials and store at -70°C. Coliphage stock typically should have 10^8 pfu/mL.

3.2.1.3 Test procedure

Pour an appropriate amount of MSA- agar plates a day in advance. Prepare a Log-phase host bacteria culture of *E. coli* ATCC 700078 (10^8 bacteria/ mL) and store it at 4± 1 °C on chilled ice until use.

Let 50 mL ssMSA melt completely in a boiling water bath. Keep warm at 45 ± 1°C. Add aseptically 300 µL of $CaCl_2$- solution and nalidixic acid to a final concentration of 250 µg/mL. Aliquot 2.5 mL in a glass test-tube with caps in a 45 ± 1°C water bath.

The following steps are critical because of different temperature of the media. To ensure viability of bacterial host and coliphages and molten agar, give some time between the working steps to warm up the respective media or samples in the water bath.

Prepare appropriate dilution steps (1:10 steps), normally up to 10^{-4}, of the wastewater effluent samples with peptone-salt-solution or 1XPBS. Mark petri dishes with sample ID, volume and dilution. Add 1 mL of the wastewater sample dilution (room temperature) to the test-tube. Add 1 mL of Log-phase host bacteria (ice-chilled) and mix gently by avoiding air bubbles. The content should remain molten in the water bath. Do not let the inoculated test tubes longer than 10 min in the water bath. Pour the content onto a labelled MSA agar plate. Do at least 3 duplicates per dilution.

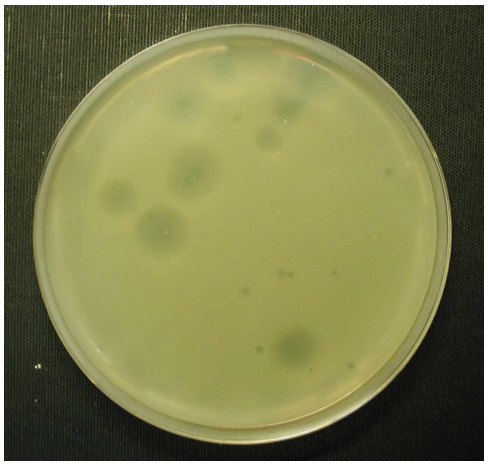


Figure 3.4 Plaques of somatic coliphages on double-layer agar

Let the agar dry by incubation with slightly open lit. Invert plates and incubate at 36 ±2 °C for 18± 2h, possibly after 6h the plaques are countable.

Results. Select plates with more than 30 plaques and count plaques under indirect light and calculate plaque forming units (pfu) in original wastewater sample per mL.

As a quality control, with every test performance, prepare a negative control with dilution media as sample using the same procedure as for analysing wastewater samples. As a positive control, use an appropriate dilution of coliphage Phi X174 cryo stock.

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