University of Zagreb
Faculty of Chemical
Engineering and Technology

FKITMCMXIX

Laboratory exercises manual

Course: **Environmental Engineering**

Prof. Ana Vrsalović Presečki, Ph.D. (avrsalov@fkit.hr)

Department of Reaction Engineering and Catalysis

Assistant: Dino Skendrović (dskendrov@fkit.hr)

Prof. Ana Lončarić Božić, Ph.D. (abozic@fkit.hr)

Department of Polymer Engineering and Organic Chemical Technology

Assistant: Josipa Papac (jpapac@fkit.hr)

Zagreb, December, 2021.

Laboratory exercise: Determination of environmental indicators as a water quality parameters – pH, COD, BOD_5, TOC, TOX

i) determination of Chemical Oxigen Demand (COD)
ii) determination of Biochemical Oxigen Demand (BOD_5)
iii) determination of Total Organic Carbon (TOC)
iv) determination of aquatic toxicity of samples on organism *Daphnia magna*

i) Chemical oxygen demand (COD) is parameter used widely to measure the pollutional strength of domestic and industrial wastewaters. COD is defined as the amount of oxygen required to oxidize organic matter chemically. Potassium dichromate ($K_2Cr_2O_7$) is generally chosen for this purpose due to its strong chemical oxidizing capability. Almost all organic compounds (except for ammonia, aromatic hydrocarbons, pyridine and their related compounds) can be oxidized by dichromate under heated acidic and $AgSO_4$-catalysed conditions, equivalent to 95 – 100% of the theoretical values. One of the main limitations of the COD test is its inability to differentiate between biologically oxidizable and biologically inert organic matter. Nor can it provide any evidence of the biological decomposition rate that proceeds either in natural or man-made conditions. The major advantage of COD test is the short time required for evaluation. The determination can be made in about 3 hr rather than the usual 5 days required for the measurement of BOD.

ii) Biochemical oxygen demand (BOD) is defined as the amount of oxygen required by bacteria while stabilizing decomposable organic matter under aerobic conditions. The BOD test is widely used to determine the pollutional strength of domestic and industrial wastewaters in terms of the oxygen that they will require if discharged into natural watercourses in which aerobic conditions exist. The test is one of the most important both in regulatory work and in studies designed to evaluate the purification capacity of receiving water bodies. Its disadvantage is the long time required by the test, generally taking 5 days.

The BOD test is essentially a bioassay procedure involving the measurement of oxygen consumed by living organisms (mainly bacteria) while utilizing the organic matter present in a waste, under conditions as similar as possible to those that occur in nature. The requirements of the environmental conditions for the test can be summarized as follows:

- Sufficient nutrients, e. g. N, P, S, K, Na, and certain trace elements.
- Free from toxins.
- Presence of a mixed culture of microorganisms (seed).
- Dissolved oxygen must be available in the sample throughout the period of the test.
- No interference due to re-aeration.
- 20 ^oC incubation.

It is possible to interpret BOD data in terms of organic matter, as well as the amount of oxygen used during its oxidation. This concept is fundamental to an understanding of the rate at which BOD is exerted.

iii) TOC measures the organic carbon concentration in the water and wastewater. The test can be performed very rapidly (only several minutes) and conveniently (by TOC instrument) and is becoming more popular. For a given wastewater, if a repeatable empirical relationship is established between its TOC and BOD or COD, then measurements in TOC can be used to estimate the accompanying BOD or COD. However, this relationship must be established independently for each set of conditions, such as at various points in a treatment process.

iv) Determination of acute aqueous toxicity includes determination of EC50 and TU (Toxicity Units).

INDICATORS	SHOWN AS:	UNIT OF MEASUREMENT	ALLOWED AMOUNT
pH	-	-	6.5 - 9.5
COD	O_2	mg/L	700
BOD_5	O_2	mg/L	250
Daphnia magna toxicity	LID_D*	Dilution factor	2

*smallest dilution of wastewater that doesnt have any effect on tested organism

Table 1. Croatian wastewater ordinance for emission of wastewater into public sewer system.

Determination of the pH value of samples

For the determination of the pH value of the samples, portable pH meterHandylab pH/LF, Schott Instruments GmbH is used.

Fig.1. Portable pH meterHandylab pH/LF, Schott Instruments GmbH

Chemical Oxygen Demand (COD)

COD KIT LCK 1414 (5-60 mg/L O_2)
COD KIT LCK 514 (100-2000 mg/L O_2)
COD KIT LCK 314 (15-150 mg/L O_2)

Sample preparation

Initially dilute the sample 1/100.
Then, add 2 mL of that sample to the cuvette, shake it vigorously and put it into preheated thermoblock (170° C) for 120 min.
After the period of cooling down (?)

Fig. 2. Spectrophotometer DR 2800, Hach Lange (left) and mobile thermoblock (right).

Biochemical Oxygen Demand (BOD)

Inoculation BOD5 BioKIT LZC 55

Sample preparation
Initially dilute the sample 1/100.
Adjust the pH value of the sample to around 7.

1.step
Put 1 dose of blue spoon inoculation material in 10 mL buffer solution.
Vigorously stir for 1 minute, then leave it for 1 h on ambient temperature (18-24° C).

2. step
After the period of 1 h, take 0.2 mL of prepared suspension (caution: free from any visible particles) and put it into the clean glass bottle (with RED cap), dilute with 10 mL distilled water. This suspension is used for the inoculation of samples.
For each sample we need two test cuvettes: 1.one is analysed immediately
2.one is analysed after 5 days of the incubation

1.cuvette
Cuvette is filled with sample fully.
With the belonging plastic funnel put „tablets" from the cap and vigorously stir for 3 minutes. Leave it for 1 minute and then proceed with measurements.

2.cuvette
Add 0.2 mL of the inoculation suspension (not older than 2 weeks) to the cuvette.
Fill cuvette to maximum with the sample; on the top there should be „liquid bubble" in accordance with the law of surface tension to make sure that there is no air bubbles.
Clog the cuvette and put it on incubation for 5 days (temperature was previously set on 20° C).

After 5 days of incubation, carefully with the help of plastic funnel put „tablets" (reagents) and vigorously shake for 3 min straight. Leave it for 1 minute and then proceed with the measurements.

Results of the measuring is expressed in mg O_2/L.
BOD is expressed as a difference of initial value and after 5 days of incubation.

Toc Analysis

Using a TOC analyzer, TOC-VCPN, Shimadzu (Figure 3.2), total organic and inorganic carbon is measured, respectively, and accordingly mineralization of organic compound is monitored. The instrument calculates the concentration of total organic carbon (TOC) from the difference of measured concentrations of total carbon and inorganic carbon. There are two basic methods of TOC analysis: direct and indirect. In our work, the non-purgeable organic carbon (NPOC) direct method was used. The degree of CBZ mineralization was determined using the NPOC method of sample measurement.

Principle

The measurements were performed using a Total Organic Carbon Analyzer TOC – TOC-VCPN Total Organic Carbon Analyzer, Shimadzu.

Fig. 3. Total Organic Carbon Analyzer TOC – TOC-VCPN Total Organic Carbon Analyzer, Shimadzu

Two types of carbon are present in water: total organic carbon (TOC) and inorganic carbon (IC). Organic carbon binds with hydrogen or oxygen to form organic compounds. Collectively, the two forms of carbon are referred to as total carbon (TC) and the relationship between them is expressed as: TOC = TC – IC.

After acidifying the sample to pH 2-3, pure gas is bubbled through the sample to eliminate the IC component. The remaining carbon is measured to determine total organic carbon, and the result is generally referred to as TOC. TOC represent non-purgeable organic carbon and refers to organic carbon that is present in a sample in a non-volatile form [1]. Sample is introduced in the combustion tube, which is filled with an oxidation catalyst and heated to 680 °C. In the samples, carbon is first converted to CO_2 by the combustion furnace for TOC and TC analysis or by the IC sparger for IC analysis. Carrier gas flows to the combustion tube and carries the sample combustion products from the combustion tube to an electronic dehumidifier, where the gas is cooled and dehydrated. The gas then carries the sample combustion products through a halogen scrubber to remove chlorine and other halogens.

A carrier gas then sweeps the derived CO_2 thought a non-dispersive infrared (NDIR) detector. Sensitive to the absorption frequency of CO_2, the NDIR generates a non-linear signal that is proportional to the instantaneous concentration of CO_2 in carrier gas. That signal is then plotted versus the samples analysis time. The peak area is proportional to the TC concentration of the sample. Calibration curve equation that mathematically expresses the relationship between the peak area and the TC concentration can be generated by analyzing various concentrations of a TC standard solution. The TC concentration in a sample can be determined by analyzing the sample to obtain the peak area and then using the peak area in the calibration curve equation. The resulting area is then compared to the stored calibration data of a sample with concentration in parts per million.

Sample preparation and analysis

Check the pH value of the sample (20 mL), adjust the value around 3 and put it on the analysis.

Experimental

The principle is to oxidize the organic carbon (C) in wastewater to CO_2 by combustion at 720 °C. There are two methods for TOC determination: a differential and a direct method. In addition to organic carbon, the wastewater sample may contain CO_2 and ions of carbonic acid. Prior to the TOC determination, it is essential to remove total inorganic carbon (TIC) by purging the acidified sample with a gas that is free of CO_2 and organic compounds. In the presence of these substances the TOC concentration is determined separately or differential method may be applied:

$$TOC=TC - TIC$$

The remaining TC is then measured to determine TOC, and the result is generally referred to as TOC. This value is referred to as a non-purgeable organic carbon that is present in a sample in a non-volatile form (NPOC) to distinguish it from the TOC value obtained by calculating the difference between TC and TIC. We can assume that purgeable organic compounds are not present in our samples, thus:

$$TOC=NPOC=TOC$$

and the direct method can be applied. The determination of CO2 can be carried out by a number of different methods. In our case, a non-dispersive infrared detection (NDIR) was applied [2], where the carrier gas delivers the sample combustion products to the cell of a nondispersive infrared gas analyzer, where the CO_2 is detected. The detector is filled with sample gas component (CO_2) to a specified concentration (8% CO_2 in N_2) and divided into two connected chambers. A microflow sensor is fitted in the connection tube of the chambers. The incident radiation is absorbed selectively only in the specific absorption bands of the CO_2 gas in the detector. The absorbed energy is instantaneously transformed to thermal energy through molecular collision. Due to the selectivity of the detector, variations in the absorbed energy directly associated with the CO_2 concentration in the sample cell alone causes temperature and pressure differences between the two detector chambers. This results in a gas flow between the two chambers that is detected by the microflow sensor situated between the chambers. Since the beam is interrupted at a specified frequency by a rotating sector measurement signal, the variation is also periodic and generates an AC voltage in the milivotage range, which is then processed on the NDIR board (peak area). The amount of rays absorbed is proportional to the density of the gas (Lambert–Beer's Law).

D A P H T O X K I T F *MAGNA*

CRUSTACEAN TOXICITY TEST FOR FRESHWATER
BENCH PROTOCOL

Principle:

The **Daphtoxkit F *magna*** contains all the materials, including the test species ***Daphnia magna*** in the form of "dormant eggs (ephippia)", to perform 6 complete acute toxicity tests according to internationally accepted Standard Methods (e.g. OECD and ISO). The tests make use of the "neonates" which are hatched in about 3 days from the eggs.

1. Preparation of Standard Freshwater (ISO formula according to ISO 6341) as hatching and dilution medium:

Fill a 2 liter volumetric flask with approximately one liter deionized (or distilled) water and add the contents of one of the two sets of 4 vials of concentrated salt solutions, in the sequence 1 to 4 (as indicated on the labels). Add deionized (or distilled) water up to the 2000 ml mark and shake to homogenize the medium. Two liter Standard Freshwater largely suffices to perform 3 complete bioassays.
N.B. The Standard Freshwater medium can also be prepared in "double strength" in a one liter flask and subsequently diluted by half at the time of use.

Fig. 4. Standard freshwater preparation

2. Storage of the medium:

If the 3 tests are not carried out within a few days after preparation of the medium, store the Standard Freshwater in the refrigerator. Take care to bring the cooled medium (gradually) back to room temperature prior to use.

3. Hatching of the ephippia:

Hatching of the ephippia should be initiated 3 days prior to the start of the toxicity test. Pour the contents of one vial of ephippia into the microsieve and rinse thoroughly with tap water to

eliminate all traces of the storage medium. Transfer the ephippia into the hatching petri dish in 15 ml Standard Freshwater (one can also use a 10 cm diameter dish with 50 ml Standard Freshwater), preaerated by air bubbling. Cover the petri dish and incubate for 3 days, at 20-22°C, under continuous illumination of min. 6000 lux.

The largest percentage of hatching will occur between 72h and 80h of incubation. Since standard testing procedures indicate that the neonates should not be older than 24h at the start of the toxicity test, the young Daphnids must be collected at the latest 90h after the start of the incubation.

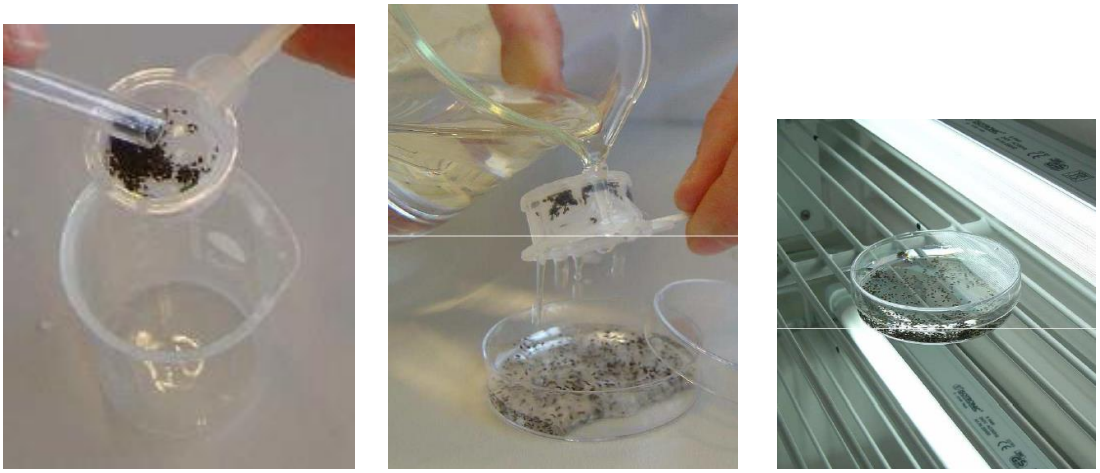

Fig. 5. a) and b) hatching ephippias and c) incubation in Petri dish on 20 °C

4. Preparation of the toxicant dilution series:

Dilution series of the test compound or effluent should be prepared according to standard procedures. A minimum volume of 50 ml is needed for each toxicant dilution.

5. Pre-feeding of the neonates prior to the test

"Starvation to death" of the weakest individuals of the test population may occur when the exposure period is prolonged from 24h to 48h. In order to avoid this problem (which can make the 48h assay invalid due to too high control mortality) a 2h pre-feeding with dry algae can be applied.

Fill one of the tubes containing Spirulina powder with Standard Freshwater and shake thoroughly to homogenize the contents. Pour the contents of the tube into the hatching petri dish 2 hours prior to collecting the neonates for the toxicity test. Swirl the petri dish gently to distribute the algal food evenly.

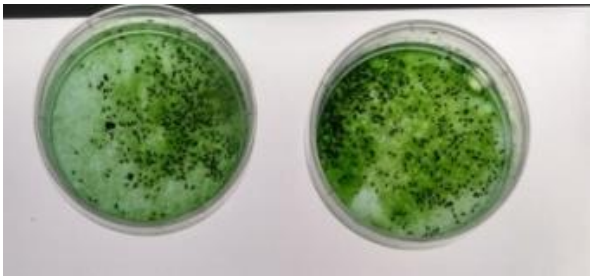

Fig. 6. Pre-feeding of the neonates with the spirulina

6. Filling of the test plate:

The bioassays are conducted in disposable multiwell test plates with 30 test wells (see Figure). Each plate is provided with 4 wells for the controls and 4 wells (A,B,C,D) for each toxicant concentration. Additionnally, the plates are provided on the left side with a column of "rinsing wells" to prevent dilution of the toxicant during the transfer of the neonates from the hatching petri dish to the test wells. The wells are labelled vertically as rows X (for the controls) and 1 to 5 for the toxicant dilutions. Each well of the test plates has to be filled with **10 ml** toxicant solution (or Standard Freshwater in the control column).

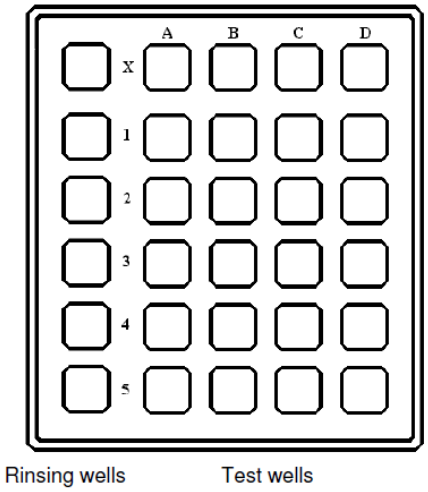

Fig. 7. Test plate scheme

7. Transfer of the neonates to the test wells:

Put the hatching petri dish on the stage of a dissection microscope or on the transparent stage of a light table provided with a black strip to enhance the contrast (see figure). Transfer 20 (actively swimming) neonates with a micropipette into each rinsing cup in the sequence : row X (control), row 1 to row 5 (increasing concentrations of toxicant).

Put the multiwell plate on the stage of the dissection microscope or on the transparent stage of the light table and transfer exactly 5 neonates from the rinsing wells into each of the 4 wells of each column. This transfer shall also be performed in the order of increasing test concentrations.

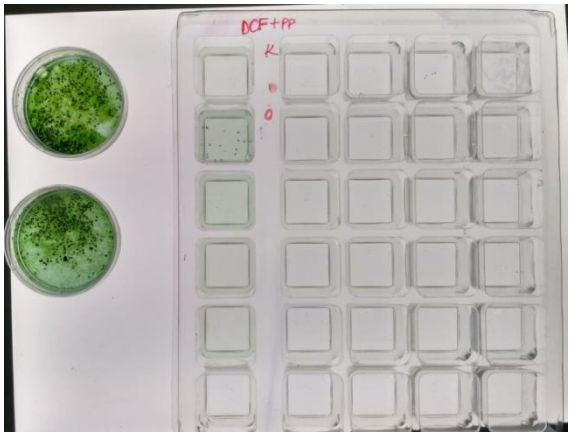

Fig. 8. Daphtoxkit F magna, MicroBioTest Inc., test plate with wells filled with samples (from left to right: 1. row – control; 2.- 4. rows - sample).

Important remark:
SURFACE FLOATING
Daphnids are quite susceptible to being trapped at the surface of the liquid medium in the test wells, by "surface tension" phenomena. "Floating" test organisms may die and hence jeopardize the outcome of the bioassay. In order to avoid "surface floating", it is important, during the transfer of the neonates, to put the tip of the micropipette into the medium and not to drop them onto the surface of medium in wells.

8. Incubation of the test plate and scoring of the results:

Put the Parafilm strip and cover on the multiwell plate and incubate in darkness at 20°C. After 24h and 48h incubation, put the test plate under the dissection microscope or on transparent stage of light table with dark light strip, and determine the number of dead and immobilized* test organisms.
** The neonates which are not able to swim after gentle agitation of the liquid for 15 seconds shall be considered to be immobilized, even if they can still move their antennae.*
Score the data on the Results Sheet and calculate the % effect and the 50% effect threshold, using any standard data processing method.

9. Validity of the test:

Besides all other specific validity conditions prescribed in standard Daphnia bioassay protocols, the number of dead + immobile organisms in the controls should not exceed 10%.

10. Reference test:
In order to check the correct execution of the test procedure and the sensitivity of the test animals, it is advised to perform a reference test from time to time. Quality control tests can e.g. be performed with the reference toxicant potassium dichromate ($K_2Cr_2O_7$), using the following dilution series: 3.2 - 1.8 - 1 - 0.56 - 0.32 mg/L. The 24h-48h EC50 in the quality control should be situated within the limits stipulated in the specification sheet of each Daphtoxkit.