Supplemental Materials

for

Practical Bioremediation Course – Laboratory Exercises on Biodegradation of Cationic Surfactant

Tomislav Ivankovic¹*, Maja Mejdandzic², Sandra Postic², Nikola Malesevic², and Jasna Hrenovic¹

¹Department of Microbiology, Faculty of Science, University of Zagreb, 10000 Zagreb, Croatia, ²Department of Biology, Faculty of Science, University of Zagreb, 10000 Zagreb, Croatia

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*Corresponding author. Mailing address: Department of Microbiology, Division of Biology, PMF, Rooseveltov trg 6, 10000 Zagreb, Croatia. Phone: 003851 4877 700. Fax: 003851 4826 260. E-mail: tomislav.ivankovic@biol.pmf.hr.
Appendix 1: Safety guidelines.

Safety guidelines

In working with environmental samples, especially municipal wastewater and activated sludge, contact with pathogenic bacteria is possible. This is why the students and class leader are required to follow the BSL-2 protocols. The class leader must introduce the students to all the safety protocols and good laboratory practice at the beginning of the class. Detail organization of the laboratory is described below:

The class is organized into 4 workspots. Each workspot is designed as an independent unit. It has a Bunsen burner, vial rack, bacteriological loops, pipette, sterile tip box, autoclavable disposable bag, and waterproof markers (Fig. 1) and is adjacent to a sink. Up to three students can work in one workspot.

All the media, pure cultures, environmental samples etc., used in the exercises are prepared beforehand and are awaiting for the students at their workspots. After the exercise, students deposit all the materials clearly visible on the workspot and leave the laboratory after hand wash and disinfection. Responsibility of the class teacher is to dispose the used materials as required by the safety procedures. The laboratory exercises must be conducted under close supervision of the class teacher.

Used media:

Mineral Salt Medium (g L⁻¹ of tap water): NaCl 2.5, K₂HPO₄ 0.47, KH₂PO₄ 0.56, MgSO₄·7H₂O 0.5, CaCl₂·2H₂O 0.1, NH₄NO₃ 2.5.

Nutrient solution (g L⁻¹ of distilled water): (NH₄)₂SO₄ 10g, K₂HPO₄ 1g.
Figure 1. Workspot.
Appendix 2: Exercises 1 and 2.

Exercise 1: Determining the toxicity of cationic surfactant to bacteria.

Objective: In first exercise the students were introduced with the toxicity of cationic surfactants to bacteria by determining the EC_{50} value of benzalkonium-chloride (BAC) to pure culture of *Micrococcus luteus* and mixed culture of heterotrophic bacteria from real municipal wastewater.

Theoretical: The students were introduced with cationic surfactants, specifically quaternary ammonium compounds (QAC) and learned their disinfectant role in everyday life. Students were encouraged to conclude how exercise is connected to real-life situation; the QACs are used as disinfectants in households and large amounts are thus discharged into sewage system, presenting the real ecological problem which could potentially be solved by bioremediation. Thus, for toxicity testing we used pure bacterial culture as well as bacteria from real wastewater.

Practical: Two sets, 4 Schott bottles each, containing 100 mL of sterile saline solution (0.3 % NaCl) and 100 mL of municipal wastewater were prepared. The wastewater was obtained from inlet canal of the local wastewater treatment plant by the class teacher.

The suspension of *M. luteus* was prepared by re-suspending fresh grown bacterial culture from nutrient agar plate (Tryptic Glucose Yeast Agar, Biolife, Italy) in a plastic vial containing 10 mL of sterile saline, followed by vortex mixing (40 Hz). A 1 mL of suspension was inoculated to each of the bottles containing saline. Starting number of bacteria was determined by decimally diluting 1 mL of sample from a bottle with *M. luteus* and from a bottle with wastewater. From the dilutions a 0.1 mL was inoculated onto nutrient agar plates and set for incubation (25°C/72 h).

After taking the samples for starting number of bacteria, the students added certain volumes of BAC stock solution (10 g L^{-1} of distilled water) to each Schott bottle in order to obtain following BAC concentrations: 1, 10, 50 and 100 mg L^{-1}. The bottles were capped and set for 1 h incubation on a mechanical shaker (150 rpm, Biosan OS-10) at room temperature.
After the incubation, a 1 mL sample from each bottle was diluted, plated on nutrient agar plates and set to incubation (25°C/72 h) to determine the final number of bacteria.

**Exercise 2: Determining the toxicity of cationic surfactant to bacteria.**

The results from Exercise 1 were commented one week later, at the start of Exercise 2. The colonies grown on agar plates were counted and the number of bacteria was determined and reported as CFU mL$^{-1}$. The EC$_{50}$ value was determined collectively; the students were asked to organize a Microsoft Excel sheet and input the collected data. The CFU values were logarithmically transformed to normalize distribution and to equalize variances of the measured parameters. The percentage of bacterial survival was calculated by comparing the final and the starting number of bacteria (log values). Finally, the EC$_{50}$ value was determined using the Microsoft Excel program.

**Obtained results:** The calculated EC$_{50}$ value was 70 mg L$^{-1}$ in experiments with real wastewater and between 1-10 mg L$^{-1}$ in experiments with pure culture of *M. luteus*. Through discussion students were guided to explain the difference between two experiments; conclusions were that wild-type bacteria are more resistant to toxicants than pure bacterial culture from the laboratory. Since cationic surfactants are widely used in households it is logical that significant amounts end up in wastewater and that bacteria inhabiting such water become resistant to them.