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Practical Bioremediation Course – Laboratory Exercises on Biodegradation of Cationic Surfactant †

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INTRODUCTION

Bioremediation is an optional course for final year Biology students at the Faculty of Science, University of Zagreb. Because it was difficult to design and conduct a set of exercises to fit the course curriculum and satisfactorily demonstrate bioremediation basics through practical laboratory work, the bioremediation course students, with the help of a teaching assistant, designed the experiment to explain how natural bioremediation differs from engineered bioremediation. This paper provides a simulated bioremediation of Jarun lake in Zagreb, Croatia, if it were contaminated with benzalkonium chloride (BAC). The BAC was chosen because it was possible to estimate its concentration using simple analytical measurements.

Safety issues

Working with environmental samples may result in contact with pathogenic bacteria. Students and the instructor in charge are therefore required to follow the BSL-2 procedures, explained in detail in Appendix 1.

PROCEDURE

The course is designed for a class of 8–12 students, divided into four groups or workspots (Appendix 1). The course was conducted over a period of five weeks, with a 2-hour exercise each week. Exercises 1 (Experimental part of EC_{50} determination) and 2 (Calculating the EC_{50} using software) were intended to demonstrate the toxicity of BAC towards laboratory strain and wild type bacteria, and are described in detail in Appendix 2.

Exercise 3: Isolation of BAC-degrading bacteria from the environment

Practical (Fig. 1): Each group received a Schott bottle containing 100 mL of mineral salt medium (MSM) (see Appendix 1). In every bottle, 0.5 mL of BAC stock solution (10 g/L) was added to obtain a final BAC concentration of 50 mg/L. The BAC serves as a source of carbon in the media. Then, 10 mL of activated sludge (MLSS) and 10 mL of wastewater (both from a municipal wastewater treatment plant), 1 g of soil, and 1 mL of previously prepared suspension of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were added to the bottles. The bottles were incubated for one week at room temperature (22–25°C) with aeration (Fig. 2). If one wishes to condense the setup or omit the experiments with wastewater and activated sludge due to safety issues or impracticality, the whole course could be reduced to Exercises 4 and 5 and/or pure bacterial cultures of *Pseudomonas*. The final result should be the same, although less challenging for the students.

Exercise 4: Isolation of BAC-degrading bacteria on solid media

Practical: MSM agar plates were prepared beforehand by adding bacteriological agar (20 g/L) to liquid MSM media. A volume of 0.2 mL of BAC solution (50 mg/L) was spread onto the plates as the sole source of carbon in the media. From each bottle, serial dilutions were made (-1 to -4) and 0.1 mL was inoculated onto MSM agar plates. The plates were set for incubation at room temperature for one week.

Results: After incubation in liquid mineral media and subsequent isolation on solid media, the colonies that grew on the MSM plates were counted and designated as BAC degraders.

Exercise 5: In-situ bioremediation simulation

Practical: The lake water was distributed into four Schott bottles, 100 mL each. The first bottle was the control, and 0.5 mL of BAC solution (10 g/L) was added to the other bottles to obtain a final BAC concentration of

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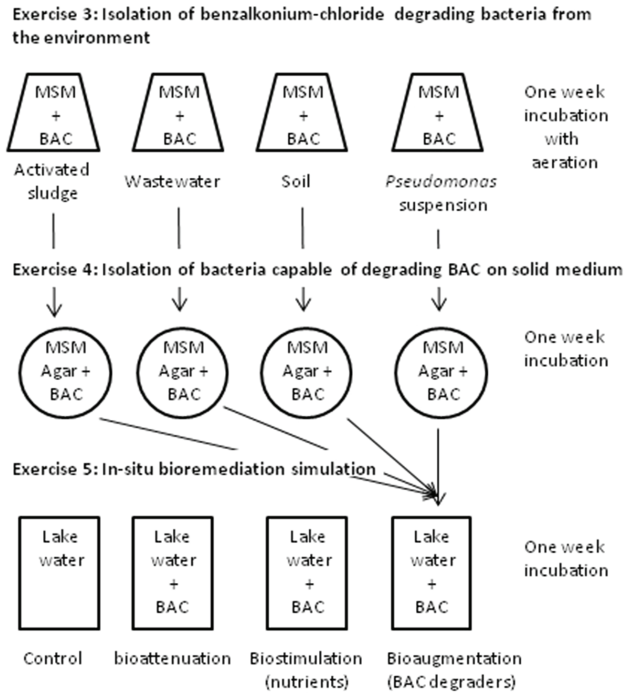

FIGURE 1. Schematic diagram of Exercises 3–5. MSM = mineral salt medium; BAC = benzalkonium chloride.

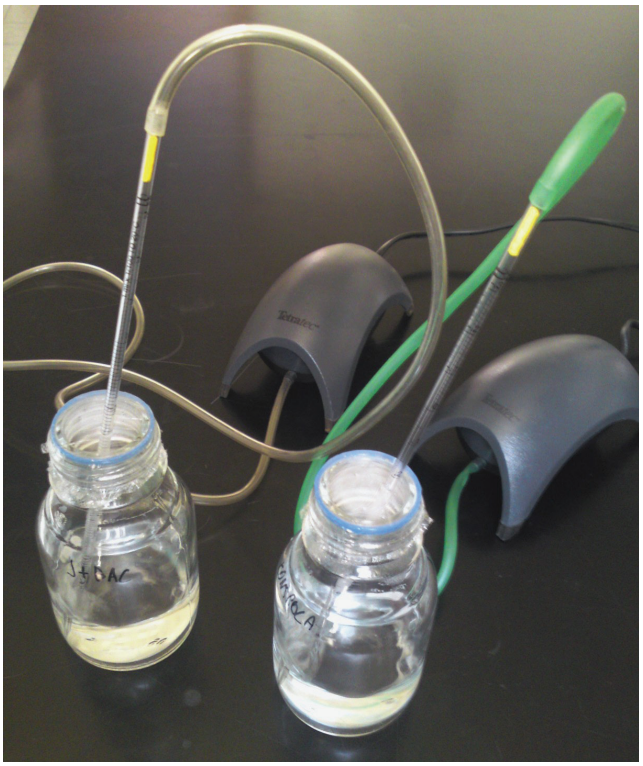

FIGURE 2. Aeration apparatus. The bottles were covered with food wrapping plastic foil and sealed with adhesive tape. The air is powered with aquarium pumps through serological pipettes.

50 mg/L. The following items were then added to one BAC bottle each (Fig. 1):

- 10 mL of nutrient solution (see Appendix 1) (source of N and P) – this system simulates **biostimulation**.
- 1 mL of suspension of BAC degraders – this system simulates **bioaugmentation**. The bacterial suspension was obtained by scraping a few isolated colonies from each MSM agar plate from Exercise 4 and resuspending the colonies in 10 mL of sterile saline solution.
- nothing – this simulates **bioattenuation** by indigenous heterotrophic bacteria.

The bottles were sealed with cotton caps and set for incubation at room temperature on a mechanical shaker (150 rpm). The colony forming unit (CFU) of heterotrophic bacteria was determined at the start of the experiment and after one week of incubation (Table 1). The concentration of BAC was determined after 1 hour and 1 week of incubation using Hach DR2500 spectrophotometer (method 8337) (Figs. 3 and 4).

TABLE 1.
Number of heterotrophic bacteria and BAC (benzalkonium chloride) concentrations in lake water bioremediation experiment after designated incubation time.

	Heterotrophic bacteria (CFU/mL)	BAC concentration (mg/L)		
		Start	1 hour	1 week
Control	$2.75 \pm 2.33 \times 10^4$	0	0	0
Bioattenuation	55±49	50	35	38
Biostimulation	65±21	50	38	38
Bioaugmentation	$2.49 \pm 2.14 \times 10^8$	50	35	5

Starting number of bacteria in lake water = $1.42 \pm 0.69 \times 10^4$ CFU/mL and in lake water with addition of biodegraders = $1.61 \pm 1.26 \times 10^8$ CFU/mL. CFU = colony-forming unit; BAC = benzalkonium chloride.

RESULTS

The key point was measuring BAC concentration. If only the numbers of bacteria were monitored, as is sometimes the case with bioremediation studies, one could assume that the biodegraders were effective in BAC degradation (Table 1). The more engaged students raised the idea that monitoring of only bacterial numbers could simply show that biodegraders were not killed by BAC and that they multiplied to some extent. The BAC measurements clearly showed that degraders were indeed effective, since only 5 mg/L of BAC remained after the incubation (Table 1). Determining BAC concentration after one hour is necessary since positively charged molecules of cationic surfactants can adhere to any organic matter in the lake water (1). This therefore provides the actual starting concentration. If BAC

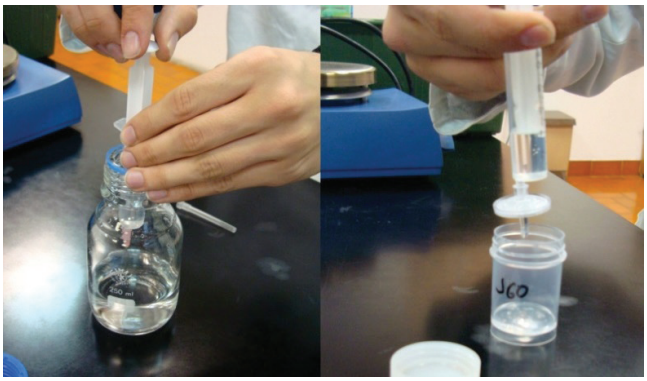

FIGURE 3. Filtration of samples for BAC measurement using syringe nitrocellulose filters (pore size 0.2 µm). BAC = benzalkonium chloride.

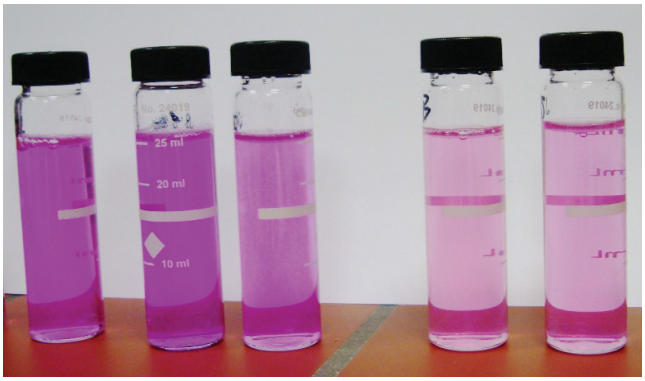

FIGURE 4. Measuring the concentration of BAC in lake water samples. BAC = benzalkonium chloride.

concentration was measured only after one week of incubation, one could assume that 15 mg/L was spontaneously degraded in lake water – which is not true.

Through this simulation experiment, major bioremediation postulates were shown (Table 1), and, more importantly, perceivable to the students:

- Contaminants that are toxic to bacteria cannot be degraded by native microorganisms (bioattenuation).
- Biostimulation has no effect if bacteria incapable of enzymatic degradation of the contaminant are not present in the contaminated area.
- Bioaugmentation is very efficient in the case of toxic contaminants.
- A greater number of bacteria means a greater bioremediation capability.

CONCLUSION

These experiments demonstrate basic bioremediation postulates. Using real natural samples such as wastewater, activated sludge, and lake water made the students much more interested in the course, as they were able to observe that theory from the lectures is applicable to real life. Choosing a quaternary ammonium compound as the contaminant model was a good way to enable easy quantification in the experiments using basic spectrophotometry – a procedure that was easily performed during the class.

SUPPLEMENTAL MATERIALS

Appendix 1: Safety guidelines
Appendix 2: Exercises 1 and 2

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REFERENCES

1. **Nye, J. V., W. F. Guerin, and S. A. Boyd.**1994. Heterotrophic activity of microorganisms in soils treated with quaternary ammonium compounds. Environ. Sci. Technol. **28:**944–951.