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Predation of bacteria by the protozoa *Tetrahymena pyriformis* in toluene-degrading cultures

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Abstract

The influence of the toluene concentration on predation of toluene-degrading bacteria by the protozoa *Tetrahymena pyriformis* was investigated in suspended batch cultures continuously aerated with toluene-contaminated air. At gas phase concentrations of 0.035 to 0.74 g m^{-3}, toluene did not significantly affected protozoan activity and the final bacteria concentration was reduced by growing protozoa by 98 to 99.9% compared to protozoa-free controls. As the toluene concentration was increased to 1.16–1.33 g m^{-3}, the reduction of the bacteria cell concentration was 80%. At 3.35 g toluene m^{-3}, growth of *T. pyriformis* was completely inhibited. Overall, the results presented herein demonstrate that protozoa grazing on bacteria play a major role in controlling bacterial cell concentration, but that the toxicity of the treated pollutants to the protozoa is an important factor that needs to be taken into account in biological treatment processes.

Introduction

Biomass accumulation in biological waste treatment processes such as trickling filters for wastewater treatment, or biotrickling filters for air pollution control is a well-recognized problem. Depending on the particular treatment process and the severity of the problem, several solutions have been proposed ranging from washing trickling filters with various chemicals to physical removal of the accumulated biomass (Weber & Hartmans 1996, Smith *et al.* 1996, Laurenzis *et al.* 1998, Metcalf & Eddy 1991). Recently, we investigated the use of protozoa that prey upon bacteria as a means to slow down the rate of biomass accumulation in a biotrickling filter used for air pollution control (Cox & Deshusses 1997, 1998). The addition of a mixture of protozoa to the liquid of a toluene-degrading biotrickling filter resulted in an increase of pollutant mineralization and a decrease of the biomass accumulation rate. However, the protozoa, *Tetrahymena pyriformis* and *Vorticella microstoma* (two of the protozoa inoculated originally), rapidly disappeared from the biotrickling filter (Cox & Deshusses 1998), which suggested that conditions in the biotrickling filter may not have been optimal for these species.

Our observations raised the question to which extent protozoa could be used to control bacteria accumulation in bioreactors treating high loads of toxic pollutants such as toluene. A literature survey revealed that little information was available on predator-prey relationships in toxic environments. Selivanovskaya *et al.* (1997) observed an increase of the microfauna in the last stages of a six-compartment rotating biological contactor treating a mixture of phenol, acetophenone and styrene. They found that feed toxicity significantly reduced the abundance and the diversity of protozoa and higher organisms. Other studies stressed that the presence of protozoa and other higher organisms in aerobic wastewater treatment systems is an indication of good system health and often correlates with high effluent quality (Curds 1982, Kinner *et al.* 1988, Luna-Pabello *et al.* 1990). Clearly, further research on protozoa-pollutant degrading bacteria interaction was warranted.

In this paper, the results of systematic investigations on the influence of the toluene concentration on

the predation of toluene-degrading bacteria by *T. pyriformis* are presented and discussed. While predation of bacteria by the latter free-swimming ciliate has been well studied in batch and continuous cultures (e.g., Curds & Cockburn 1968, 1971, Habte & Alexander 1978, Sambanis & Fredrickson 1988) no data exist for predation in the presence of toxic pollutants such as toluene.

Materials and methods

Organisms, media and inocula

The bacterium used in this study was isolated from activated sludge using toluene as the sole source of carbon and energy and was tentatively identified as *Pseudomonas corrugata* using the Biolog test (GN Microplate, Biolog, Inc., Hayward, CA). Inocula were prepared by cultivating *P. corrugata* in a mineral medium (Cox & Deshusses 1998) with 30 μL toluene L^{-1}. A stock culture was stored at $-70\,°C$ with 25% (v/v) glycerol. The same mineral medium was used in predation experiments. *T. pyriformis* (ATCC 3005) was cultured and maintained in ATCC medium 357 at room temperature and transferred at least once every month. For predation studies, bacterial cultures were inoculated with 7–30 days old cultures of the protozoa *T. pyriformis*.

Experimental setup

Predation by *T. pyriformis* was investigated in six independent experiments with toluene gas phase concentrations ranging from 0.035 to 3.35 g m^{-3}. In each experiment, duplicate mixed cultures containing *P. corrugata* and *T. pyriformis* were compared over a period of 220 h to a single bacterial control culture containing *P. corrugata* only. Experiments were performed in 1 l Erlenmeyer flasks with 0.5 or 0.75 l mineral medium and inoculated with 0.4 ml l^{-1} bacterial stock culture (controls) or 0.4 ml l^{-1} bacterial stock culture and 2 ml l^{-1} of *T. pyriformis* maintenance culture (mixed cultures for predation studies). The flasks were sealed with a rubber stopper with openings for gas inlet and outlet flow. Toluene-containing air was bubbled through the cultures at an average flow rate of 196 l h^{-1} via a tube extending to the bottom of the flask (Figure 1). Desired toluene gas phase concentrations were obtained by delivering different rates of liquid toluene (Fisher Scientific) into the main air stream using a metering pump (Fluid Metering, Inc., Oyster Bay, NY). The first two experiments at 0.035 and 3.35 g m^{-3} toluene were done aseptically with sterilized medium and sterile inlet and outlet air filters. However, pressure drop over the outlet filters increased rapidly due to condensation, causing difficulties in maintaining a constant air flow rate over time. The air filters were removed in subsequent experiments.

Analyses

Liquid samples were taken once or twice a day depending on the growth phase. Concentrations of bacteria and *T. pyriformis* were determined by direct counting as the average of ten fields in a Petroff-Hausser counting chamber (cell depth 0.02 mm) and the average of nine fields in a hemacytometer (cell depth 0.1 mm), respectively. Cells were fixed by adding 20% (v/v) Lugol solution (Sigma) prior to counting. Specific growth rates were calculated by regression or as the steepest slope between two data points during the exponential growth phase. Bacteria concentration in mixed culture flasks reached a maximum at the end of the exponential growth phase, and then decreased as a result of predation by protozoa to finally reach a steady value, usually between 150 to 220 h after inoculation. At this time, *T. pyriformis* cell concentration was also constant, and final cell concentrations were calculated by taking the average of three to seven samples over a 2–3-day period. Bacteria decay in the control cultures was minimal. Toluene gas phase concentrations were determined with a HP 5890 Series II gas chromatograph (Hewlett-Packard) equipped with a packed column (Supelcowax 10, 30 m $\times$ 0.53 mm $\times$ 1 μm), a gas sampling valve and a FID detector.

Results and discussion

Influence of the toluene concentration on bacterial growth in control cultures

Cultures were aerated at a high flow rate in order to provide an excess of toluene and to minimize the effect of possible depletion of toluene by bacterial growth. GC analysis of the inlet and outlet gases confirmed that toluene was present in excess at all concentrations tested. It was therefore assumed that toluene concentrations in the liquid phase were constant over the entire course of the experiments and in equilibrium with the concentration in the gas phase.

P. corrugata started to grow after about 50 h, except in the cultures with 0.035 and 3.35 g toluene

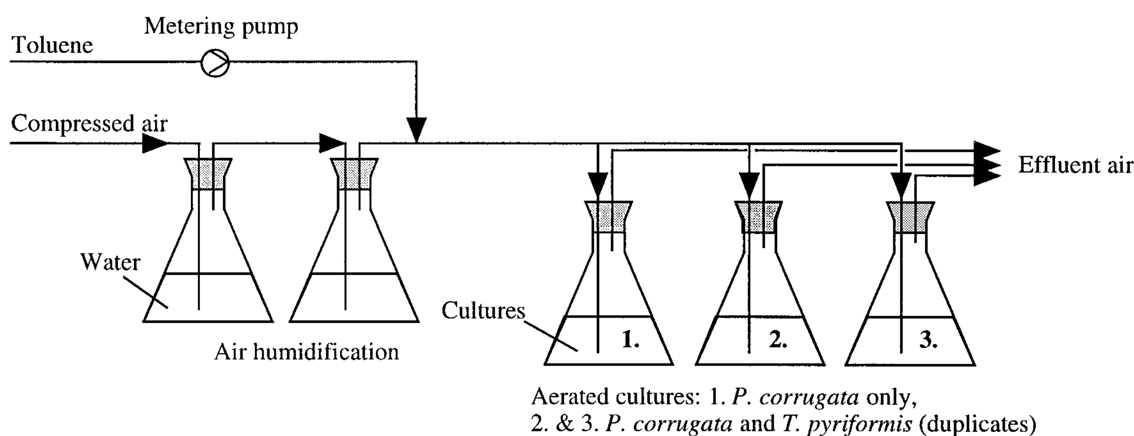

Fig. 1. Schematic of the experimental setup.

m^{-3} for which the lag phases were shorter. These two cultures were run aseptically, which may have caused differences, but the reasons for differences were not clear. All bacterial controls reached a steady state within 100 h after which the bacterial cell concentration remained constant. At this time, nitrogen in the medium became limiting as indicated by lower cell concentrations obtained in other experiments with lower nitrate contents in the culture medium (results not shown). Specific growth rates and stationary phase average cell concentrations for different toluene inlet concentrations are summarized in Table 1. No major effect of toluene concentration was found on the specific growth rate and on the final cell density, although the final cell density tended to be slightly lower in the experiments at low toluene concentrations. The average specific growth rate of 0.24 h^{-1} found herein falls within the range of maximal specific growth rates (0.13–0.86 h^{-1}) reported for other *Pseudomonas* species on toluene (Mirpuri *et al.* 1997, Pedersen *et al.* 1997, and references therein). Toluene concentrations in the liquid phase varied from 0.18 to 17.6 mg l^{-1} assuming a dimensionless Henry coefficient of 0.19 (Peng & Wan 1997). These toluene liquid phase concentrations were well below the Andrews substrate inhibition constants of *Pseudomonas putida* species growing on toluene (Mirpuri *et al.* 1997). Reported toluene half saturation constants of *Pseudomonas* species are between 0.1 and 15 mg l^{-1} (Mirpuri *et al.* 1997, Pedersen *et al.* 1997), which is comparable to the liquid phase toluene concentrations in this study. However, the specific growth rate of *P. corrugata* was the same at all toluene concentrations tested (Table 1), which indicates a low K_s (<0.18 mg l^{-1}) for toluene for this species. It was therefore assumed that toluene at the concentrations tested herein had no effect on growth of *P. corrugata.*

Table 1. Influence of the toluene gas phase concentration on the bacterial specific growth rate (μ), and the final cell density X_{final} (standard deviation in parentheses) in bacterial control cultures.

Toluene (g m^{-3})	μ (h^{-1})	X_{final} (ml^{-1})
0.035	0.21	7.4×10^8 (1.7×10^8)
0.26	0.23	3.0×10^9 (4.7×10^8)
0.74	0.27	3.4×10^9 (5.4×10^8)
1.16	0.27	9.3×10^9 (3.8×10^9)
1.33	0.22	3.1×10^9 (1.1×10^9)
3.35	0.22	2.6×10^9 (6.2×10^8)

Influence of the toluene concentration on predation by T. pyriformis *in mixed cultures*

In batch experiments, *T. pyriformis* failed to grow in mineral medium amended with glucose or toluene, nor was growth observed in the supernatant of a *P. corrugata* culture grown on toluene. Although *T. pyriformis* is capable of growing on dissolved nutrients (e.g., ATCC maintenance medium #357), bacterial cells were most probably the only food source for *T. pyriformis* in mixed cultures with mineral medium. *T. pyriformis* grew equally well in autoclaved and non-autoclaved cultures of *P. corrugata* grown on toluene (results not shown), which suggests that *T. pyriformis* had no preference for living or dead cells.

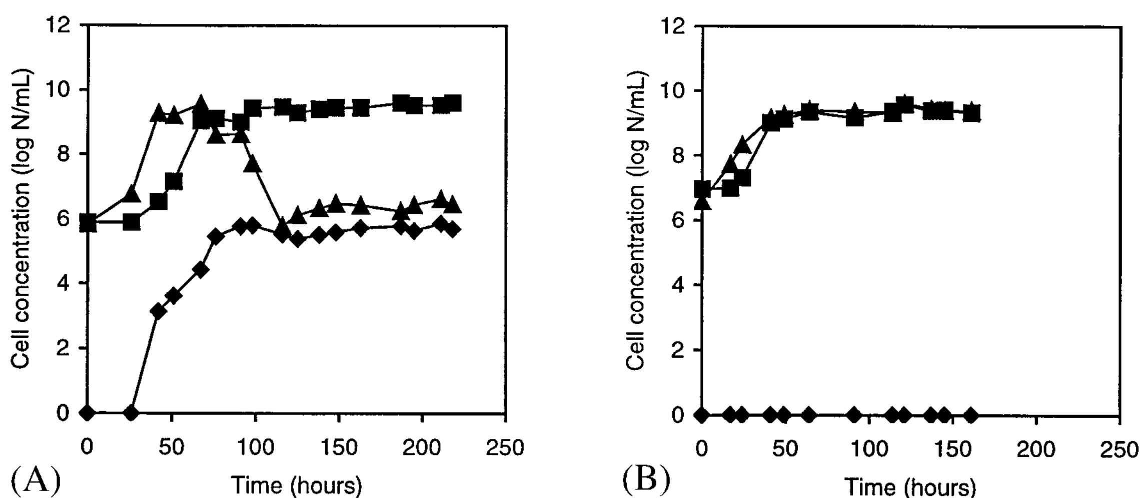

Fig. 2. Development of bacteria in control (■) in mixed (▲) cultures and *Tetrahymena pyriformis* in mixed cultures (♦) continuously aerated with air containing 0.74 g toluene m^{-3} (A) and 3.35 g toluene m^{-3} (B).

Table 2. Influence of the toluene gas phase concentration on the specific growth rate (μ) and stationary phase final cell density (X_{final}) of bacteria and *Tetrahymena pyriformis* in mixed cultures. Standard deviations are shown in parentheses.

Toluene	Bacteria		X_{final} reduction	*T. pyriformis*	
($g\ m^{-3}$)	μ (h^{-1})	X_{final} (ml^{-1})	compared to controls (%)	μ (h^{-1})	X_{final} (ml^{-1})
0.035	0.20	5.2×10^6 (6.8×10^5)	99.3	0.11	9.9×10^4 (5.5×10^4)
0.26	0.12	4.7×10^7 (1.2×10^6)	98.4	0.15	2.1×10^5 (4.6×10^4)
0.74	0.14	2.9×10^6 (7.8×10^5)	99.9	0.17	5.4×10^5 (1.2×10^5)
1.16	0.13	1.9×10^9 (5.1×10^8)	79.6	0.08	4.9×10^5 (4.8×10^4)
1.33	0.15	5.9×10^8 (2.6×10^8)	81.0	0.12	4.6×10^5 (5.0×10^4)
3.35	0.17	2.7×10^9 (7.5×10^7)	−3.8	0	0

Figure 2 shows two examples of cultures at a relatively low and at the highest toluene concentration. At 0.74 g toluene m^{-3}, growth of *T. pyriformis* in the mixed culture started shortly after growth of bacteria (Figure 2A). The protozoa cell concentration reached a maximum after 100 h and remained constant thereafter. Predation of bacteria caused a decline of the bacterial cell concentration starting at the end of the growth phase of *T. pyriformis* until a constant concentration was reached at about three orders of a magnitude lower than in the control culture. At 3.35 g toluene m^{-3}, growth of *T. pyriformis* was not observed, and, consequently, the bacterial cell concentration in the stationary phase of the mixed culture remained constant at a level comparable to those of the control culture (Figure 2B). Specific growth rates, stationary phase cell concentrations of bacteria and *T. pyriformis* in mixed cultures and percentage bacteria reduction due to grazing protozoa are summarized in Table 2. Bacterial specific growth rates in the mixed cultures appeared in general to be lower than in the controls without *T. pyriformis.* This may have been caused by predation since *T. pyriformis* growth started during the bacterial exponential growth phase (Figure 2A). At 0.035 to 1.33 g toluene m^{-3}, the specific growth rate of *T. pyriformis* fluctuated between 0.08 and 0.17 h^{-1} with no apparent relation to the toluene concentration (Table 2). These values are slightly lower than reported by Curds & Cockburn (1968, 1971), who found maximum specific growth rates of 0.22 and 0.38 h^{-1} with *Klebsiella aerogenes* as the substrate in batch and continuous cultures, respectively.

A reduction of the bacterial cell concentration of 98–99.9% was observed in mixed cultures supplied with 0.035 to 0.74 g toluene m^{-3} compared to the control cultures without protozoa. Residual bacterial concentrations in the stationary phase were close to those found to persist in the presence of *T. pyriformis* (Habte & Alexander 1978, Sambanis & Fredrickson 1988). At intermediate toluene concentrations (1.16 and 1.33 g m^{-3}), predation resulted in only 80% reduction of the bacterial cell concentration in the stationary phase (Table 2). At 3.35 g toluene m^{-3}, *T. pyriformis* did not grow and bacterial concentrations in mixed and control cultures were the same. As toluene concentration did not affect bacteria in the range of concentrations examined (Table 1), one can conclude that the reduced predation at toluene concentrations of 1.159 g m^{-3} a nd higher is caused by an inhibitory effect of toluene on *T. pyriformis*.

Conclusions

Toluene inhibited predation of bacteria by the protozoa *T. pyriformis* at gas phase concentrations of 1.16 g m^{-3} (corresponding to 6.1 mg l^{-1} in the liquid) and higher, while complete inhibition of protozoa was observed at 3.35 g m^{-3} (17.6 mg l^{-1} in the liquid). In previous studies with waste air biotrickling filters, *T. pyriformis* unexpectedly disappeared at a toluene gas concentration of only 1 g m^{-3} (Cox & Deshusses 1998). Based on the results presented herein, no toxic effect of toluene on *T. pyriformis* was expected at such a low concentration. Hence, it is likely that other factors such as mechanical stress or simple washout also played a role in the survival of *T. pyriformis* in the biotrickling filters and it suggests that such factors should be considered in the design of the bioreactor.

The toluene concentrations that inhibited protozoa were well below reported Andrews substrate inhibition constants of toluene-degrading *Pseudomonas* species. Consequently, inhibition of protozoa will occur before inhibition of the primary degraders in a biotreatment process. This indicates that for optimum and stable trickling filter operation, the toxicity of the treated pollutant(s) to both the primary degraders and to the secondary degraders such as protozoa should be considered. This is particularly relevant for biotrickling filters used in air pollution control. In these bioreactors, pollutant concentrations and loadings are orders of magnitude higher than in water treatment processes and protozoa have an important function in recycling essential nutrients while slowing down the rate of biomass accumulation (Cox & Deshusses 1998). Failure to consider toxicity of the treated pollutant(s) to both the primary degraders and to the secondary degraders might result in the suppression of predation by higher organisms and might ultimately result process sub optimum operation or in process malfunction.

References

Cox HHJ, Deshusses MA (1997) In: *Biological Waste Gas Cleaning*, Proceedings of an International Symposium, April 28–29, 1997, Maastricht, The Netherlands, pp. 233–240.

Cox HHJ, Deshusses MA (1999) *Biotechnol. Bioeng.* **62**: 216–224.

Curds CR (1982) *Annu. Rev. Microbiol.* **36**: 27–46.

Curds CR, Cockburn A (1968) *J. Gen. Microbiol.* **54**: 343–358.

Curds CR, Cockburn A (1971) *J. Gen. Microbiol.* **66**: 95–108.

Habte M, Alexander M (1978) *Soil Biol. Biochem.* **10**: 1–6.

Kinner NE, Curds CR, Meeker LD (1988) *Wat. Sci. Tech.* **20**: 199–204.

Metcalf, Eddy (1991) *Wastewater Engineering*. New York: McGraw-Hill.

Laurenzis A, Heits H, Wübker S-M, Heinze U, Friedrich C, Werner U (1998) *Biotechnol. Bioeng.* **57**: 497–503.

Luna-Pabello VM, Mayén R, Olvera-Viascan V, Saavedra J, Durán de Bazúa C (1990) *Biol. Wastes* **32**: 81–90.

Mirpuri R, Jones W, Bryers JD (1997) *Biotechnol. Bioeng.* **53**: 535–546.

Pedersen AR, Arvin E (1997) *Biotechnol. Bioeng.* **54**: 131–141.

Peng J, Wan A (1997) *Environ. Sci. Technol.* **31**: 2998–3003.

Sambanis A, Fredrickson AG (1988) *Microb. Ecol.* **16**: 197–211.

Selivanovskaya SY, Petrov AM, Egorova KV, Naumova RP (1997) *World J. Microbiol. Biotechnol.* **13**: 511–517.

Smith FL, Sorial GA, Suidan MT, Breen AW, Biswas P, Brenner RC (1996) *Environ. Sci. Technol.* **30**: 1744–1751.

Weber FJ, Hartmans S (1996) *Biotechnol. Bioeng.* **50**: 91–97.