



SURVIVAL OF *CANDIDA ALBICANS* AND *PSEUDOMONAS AERUGINOSA* IN OIL POLLUTED TROPICAL COASTAL WATERS*

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Abstract—The effect of environmental abiotic factors on survival and activity of oil degrading isolates, *Pseudomonas aeruginosa* OD-1 and *Candida albicans* OD-2, was determined *in situ* using membrane diffusion chambers. The study sites were located in a tropical bay on the Atlantic Ocean with history of petroleum contamination by oil tankers. Microbial densities were measured by direct counts. ³H-thymidine uptake, microautoradiography, INT-reduction, adenosine triphosphate (ATP), and frequency of dividing cells were used to assess microbial activity. Both organisms showed a significant decrease in cell density over a three day period, although, temporal increases in densities were observed. Significant decreases in total activity were observed for both populations during the study; however, the respiration potential and ATP content per organism remained constant or even increased by the end of the study suggesting that a significant fraction of these populations were capable of withstanding *in situ* environmental conditions. Results suggest that increases in ambient phosphorus concentrations played a role in the prolonged *in situ* survival of these petroleum degrading isolates at some sites. The traffic of oil tankers might have played a significant impact on microbial survival in this bay, as violent agitation of sediments increased phosphorus concentrations available to microorganisms. Published by Elsevier Science Ltd

Key words—survival, microbial activity, marine, topic, oil contamination, diffusion chambers

INTRODUCTION

Public awareness of petroleum contamination and its concomitant problems has increased in recent years to a great extent as the result of the increasing numbers of massive oil spills. Accidents like the blowout of the well off Santa Barbara (Tissier, 1973), the wrecks of the Torrey Canyon off the Brittany coasts and of the Amoco Cadiz affecting the coast of northern France (Atlas, 1981; Gundlach *et al.*, 1983) and more recently the T/V Exxon Valdez oil spill in Alaska (Lindstrom *et al.*, 1991) have fomented public attention on petroleum spill as great potential ecological disasters. Indeed, contamination of marine habitats with petroleum

hydrocarbons has increased significantly as our demand for fuel oils has increased and the world has strived to become more industrialized. Thus our ability to remediate these important habitats has become a problem of global concern.

Oil spills have the greatest impact when they occur near shorelines since these areas normally have a higher density and diversity of benthic and pelagic organisms. Tropical shorelines support the highest levels of primary productivity on Earth, largely maintained by a fine equilibrium between consumers and producers. Thus it is reasonable to expect that they are more sensitive to petroleum contamination than shorelines located in temperate regions. Despite documented spills along tropical and subtropical shorelines, eg. the east coast of Puerto Rico (N.O.A.A., 1979) and the Gulf of Mexico (Payne and Phillips, 1985), nearly all remediation strategies have been based on guidelines developed for temperate regions.

One approach for remediation of polluted shorelines focuses on addition of nutrients (e.g., phosphorus and nitrogen) to stimulate the degradative microbiota present (Lindstrom *et al.*, 1991). While areas that have a history of petroleum contami-

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nation might have developed microbial communities capable of degrading a significant fraction of crude oil, petroleum degradation is normally a slow process that could require decades to restore to pre-release conditions. Newly exposed areas might not intrinsically possess the microbial remediation potential needed or might need long periods of acclimation before any substantial degradation can be detected. Therefore, the introduction of degradative microbes to these areas (i.e., bioaugmentation) is, in theory, an option that might accelerate remediation efforts. Microbial candidates for such *in situ* clean up projects must not only be capable of degrading the pollutants of interest, but must also survive long enough to achieve acceptable levels of decontamination. Thus, a thorough understanding of potential inocula in these marine environments is essential for bioremediation of these contaminants using bioaugmentation.

Our understanding of how petroleum degrading microorganisms survive in marine environments remains unsatisfactory. In contrast, the survival of degradative bacteria in soil has been studied in great detail (Acea *et al.*, 1988; Briglia *et al.*, 1994). Most survival studies of marine microbes have been performed in the lab with microcosms (Dupray and Derrien, 1995; Mezrioui *et al.*, 1995). While this approach allows the investigator to control several physicochemical parameters, microcosms are closed systems that can not simulate normal *in situ* conditions for extended periods of time. Membrane diffusion chambers represent an adequate short-term alternative to microcosms (McFeters and Stuart, 1972; Biamon and Hazen, 1983). These chambers can be submerged in the habitat of interest and thus the organisms under study are subject to the dynamic environmental fluctuations that they would encounter in such a habitat (Hazen and Esch, 1983). Diffusion chambers have been extensively used to evaluate the survival of pathogens and indicators of fecal contamination in tropical waters (Hazen *et al.*, 1986). For example, several studies have shown that *Escherichia coli* and *Streptococcus faecalis* can survive in tropical freshwater and marine waters, suggesting that these bacteria might not be suitable indicators of recent human fecal contamination in the tropics (Santo Domingo *et al.*, 1989; Muñiz *et al.*, 1989). These studies also underscored the importance of evaluating microbial activity in survival studies since microbial densities might not always reflect the survival potential of microorganisms.

The present study focuses on the survival of a petroleum degrading marine bacterium and yeast using membrane diffusion chambers. The objective was to monitor the effect of the prevailing *in situ* conditions of a petroleum-contaminated marine bay in Puerto Rico on the metabolic activity of microbial strains isolated from the same contaminated site. To our knowledge, this represents the first

study showing the survival of petroleum degrading microorganisms in tropical marine waters contaminated with petroleum hydrocarbons.

MATERIALS AND METHODS

Survival studies

Survival studies were performed using membrane diffusion chambers as described by Hazen and Esch (1983). Two marine strains were used in these studies *Pseudomonas aeruginosa* OD-1 and *Candida albicans* OD-2. These strains were isolated from waters near the oil refinery depot in the Yabucoa Bay and have been shown to degrade petroleum hydrocarbons as determined by gas chromatography, O₂ uptake and CO₂ evolution studies (Fuentes, 1987). Cultures of *P. aeruginosa* OD-1 and *C. albicans* OD-2 were grown on Marine broth and Saubouraud broth, respectively, at 34°C for 24 h. Cells were harvested by centrifugation and resuspended in sterilized phosphate buffered saline (pH 7.0) to a final cell concentration of 10⁸ and 10⁷ cells/ml for *P. aeruginosa* and *C. albicans*, respectively. Diffusion chambers were filled with 100 ml of the corresponding cell suspension just prior to the study. Three chambers were submerged (0.6 m depth) for each test organism at two different sites. Aliquots were drawn from each chamber at decreasing intervals over 70 h and analyzed for densities and physiological activity.

Study site

The Yabucoa Bay, located on the east coast of Puerto Rico, lat. 18°3'18"N and long. 65°50'W was selected as the site for survival studies. An artificial basin was excavated in this area to be used for docking for oil tankers. The continuous traffic of oil tankers for more than 20 y enriches the hydrocarbon load in the bay. In addition, this bay receives the treated effluents from an adjacent oil refinery plant. Experiments were conducted at two different study sites in the Yabucoa Bay (Fig. 1). Site A was located at the docks of the oil refinery, whereas Site B was located on the opposite southern shoreline.

Water quality analyses

Water quality was monitored at various times during the survival study. *In situ* measurements of temperature, pH, dissolved oxygen and conductivity were performed with a Hydrolab surveyor model 4041 (Hydrolab, Austin, TX). Salinity was also measured *in situ* using a model 33 salinity meter (Yellow Springs Instruments, Yellow Springs, OH) or alternately with a hand-held refractometer (model 10419, American Optical Scientific Instruments, Buffalo, NY). The following chemical analyses were performed according to APHA (1980): total phosphorus, orthophosphates, ammonia, nitrates, nitrites, turbidity, alkalinity, silica, chlorophyll *a*, dissolved and total organic carbon. Samples for analyses were collected and stored following standard methods recommended by APHA (1980). For details of analytical techniques see Fuentes (1987).

Microbial direct counts and cell activity measurements

Direct counts were determined by the acridine orange direct count (AODC) method of Zimmermann *et al.* (1978). Samples were fixed with 3.7% formaldehyde (final concentration) and stored at 4°C until processed. The total number of bacteria was estimated by using the average of 10–15 randomly selected fields containing 50 to 75 cells per field. A Zeiss epifluorescent microscope equipped with a 100 W mercury lamp, KP-490 blue exciter filter and phase contrast capabilities was used for the observation and enumeration of microbial cells.

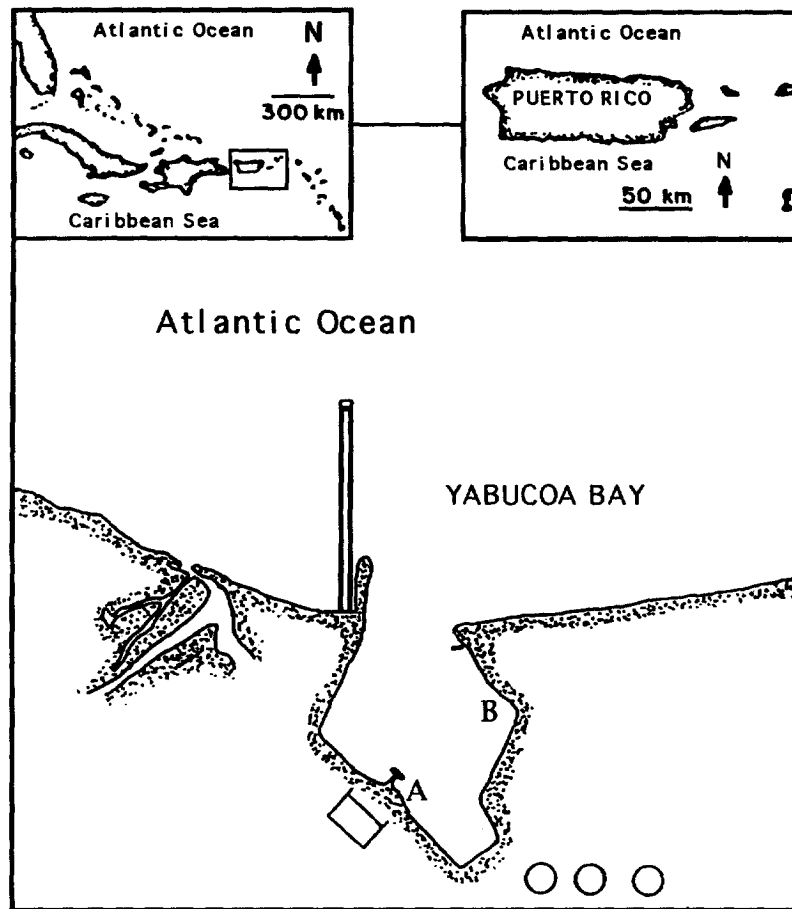


Fig. 1. Map of the Yabucoa Bay survival stations.

The ability of microbial cells to reduce 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan (INT-F) was used to measure the electron transport activity (ETS). Two different methods were used to detect and quantify the formation of intracellular INT-F deposits. The method of Zimmermann *et al.* (1978) was used to determine the percent of individual respiring cells. In addition, ETS activity of the whole population was estimated spectrophotometrically after methanol extraction. Subsamples amended with INT were filtered through 0.2 μm pore size polycarbonate membrane filters. Membranes were transferred to glass vials containing one ml of 95% ethanol and vortexed for 5 min to extract INT-F granules. The concentration of reduced INT in each extract was determined by measuring the absorbance at 485 nm and calculated from a standard curve ($r^2 = 1.0$; Nineham, 1955).

The frequency of dividing cells (FDC) was calculated as the percentage of dividing cells from the total direct cell counts following the method of Newell and Christian (1981). Cellular ^3H -thymidine incorporation was also used as an indicator of the physiological status. One ml samples were amended with ^3H -methyl-thymidine (0.36 mCi/ml; New England Nuclear Corp., Boston, MA) within 30 min after collection and incubated in the dark at *in situ* temperatures for 2 h. Samples were then poisoned with 0.4% formaldehyde (final concentration) and processed as described in Santo Domingo *et al.* (1989). The ability of individual cells to incorporate ^3H -thymidine was determined by microautoradiography following the method of Tabor and Neihof (1982), with modifications described

elsewhere (Santo Domingo *et al.*, 1989). Microautoradiograms of formaldehyde killed cells were used as negative controls. Total population uptake was measured using a Beckman LS 7500 liquid scintillation counter (Beckman, Fullerton, CA) as previously described (Santo Domingo *et al.*, 1989).

The intracellular concentration of ATP was determined using the luciferin-luciferase method (Stevenson *et al.*, 1979) with the following modifications. Samples were kept on dry ice during transport to the laboratory. Subsamples from diffusion chambers were filtered through 0.22 μm pore size, 25 mm diameter, polycarbonate membrane filters. A quantitative assay of the ATP content in each sample was performed using a firefly (*Photinus pyralis*) lantern extract (Sigma, St. Louis, MO) by liquid scintillation counting (Santo Domingo *et al.*, 1989). Light emission unit values were transformed to ATP concentrations by preparing calibration curves using known amounts of ATP added to sterile buffer blanks.

Biodegradation studies

Sea water microcosms were used to determine the effect of nitrogen and phosphorus on petroleum biodegradation. Two hundred microliters (200 μl) of AMNA crude oil, a Venezuelan light crude, were added to 200 ml of sea water and incubated at 22°C in a rotary shaker (250 rpm). Duplicate samples were supplemented with 2 ml of filter sterilized nitrogen-phosphorus (NP) solution (59 g $\text{PO}_4^{3-}/\text{l}$; 100 g $\text{NH}_4\text{NO}_3/\text{l}$ pH 7.4). Microcosms containing filtered sea water, preserved with 1% HgCl_2 (final concentration) and crude oil were used to detect abiotic losses of pet-

Table 1. Water quality in the Yabucoa Bay during the survival study

Time (h)	Water quality parameters ^a												
	Atemp	Wtemp	Cond	DO	Alk	Chl <i>a</i>	NO ₂ + NO ₃	NH ₄	TP	Sal	SiO ₂	Turb	
5	Site A	24.5	29.1	507	5.9	100	1.59	0.07	1.95	0.011	35.0	1.88	94.5
	Site B	19.7	28.0	511	6.5	100	1.78	0.06	2.20	0.002	34.5	1.92	97.0
18.5	Site A	29.4	29.8	501	5.4	100	2.78	0.07	2.5	0.024	35.0	2.04	95.0
	Site B	28.3	28.7	508	6.2	100	4.04	0.05	2.05	0.002	34.0	2.48	97.0
23.5	Site A	28.9	29.2	504	5.8	100	ND	0.06	2.27	0.022	NI(1)	2.40	70.0
	Site B	22.9	28.2	512	6.4	ND	ND	ND	ND	ND	ND	ND	ND
36	Site A	18.5	29.2	506	5.7	100	3.16	0.07	2.45	0.009	34.5	2.40	94.5
	Site B	21.6	28.3	507	6.0	100	1.94	0.04	3.45	0.002	34.5	1.88	95.5
47	Site A	25.4	28.7	498	6.2	100	1.90	0.05	2.27	0.002	33.0	2.88	93.0
	Site B	24.6	26.8	517	6.2	80	4.42	0.08	3.40	0.004	34.0	2.04	87.5
65	Site A	28.8	28.5	503	6.2	100	3.61	0.08	2.59	0.005	34.0	2.88	54.5
	Site B	28.1	28.3	509	6.2	100	3.15	0.07	3.40	0.002	34.0	2.04	95.5

^aAtemp = air temperature (°C); Wtemp = water temperature (°C); Cond = conductivity (×1000 μΩ cm⁻¹); DO = dissolved oxygen (mg l⁻¹); Alk = alkalinity (mg l⁻¹ CaCO₃); Chl *a* = chlorophyll *a* (mg l⁻¹); NO₂ + NO₃ = nitrites + nitrates (mg l⁻¹); NH₄ = ammonium (mg l⁻¹); TP = total phosphates (mg l⁻¹); Sal = salinity (ppt); SiO₂ = silica (mg l⁻¹); Turb = percent turbidity (%T).

roleum hydrocarbons. All the serum bottles were tightly closed with butyl serum stoppers and sealed with teflon tape.

An analysis of the residual oil in each microcosm was performed using an adsorption chromatography procedure described by Fedorak and Westlake (1981). The chemical composition of hydrocarbon extracts was analyzed by gas chromatography (GC) and using a mass spectrometer in series with the GC. Combined gas chromatography/mass spectrometry was performed on a Hewlett Packard model HP 5995 quadropole mass spectrometer system. Hydrocarbon mixtures were resolved using a SE-54 quartz capillary column (dimensions: 15 m length × 0.2 mm i.d., Hewlett Packard). Oven temperature was programmed to start at 90°C increasing up to 260°C at 10°C/min. Retention times were converted to equivalent chain lengths by analysis of the corresponding mass spectra data.

The effect of nutrient addition (NP) on the hydrocarbon degrading potential of natural mixed populations was also tested by measuring the evolution of ¹⁴C₂O₂ from microcosms supplemented with a radiolabeled hydrocarbon. Microcosms consisted of 10 ml sea water collected from Yabucoa Bay amended with 20 ml of AMNA crude oil and 10 ml of ¹⁴C-hexadecane (specific activity 10 μCi/ml of oil). Aliquots (0.1 ml) of NP solution were added to sea water samples and microcosms incubated at 30°C for 10 d in the dark. Triplicates were run for each variable tested. Serum bottles were tightly sealed using butyl rubber stoppers. The ¹⁴C₂O₂ evolved in each microcosm was collected using internal KOH traps (Shuttleworth and Cerniglia, 1996). The amount of ¹⁴C₂O₂ trapped as K₂¹⁴CO₃ in each microcosm was determined by pipetting 0.5 ml of the absorber (KOH) into 20 ml scintillation vials containing 10 ml of Aquasol scintillation cocktail (New England Nuclear, Chicago, IL). Sample radioactivity was measured by liquid scintillation counting. Microcosms poisoned with 0.2 ml of concentrated HCl were used as controls.

Data analysis

Data on water quality were correlated with changes in total cell densities and cell activity using multiple correlation and regression analyses. Two factor analysis of variance (FANOVA) were used to test differences between sites and time in survival studies. *t*-test and one way ANOVA were used to test differences between means of experimental groups. Data heteroscedascity was reduced by using the appropriate transformations (e.g. cell densities, log(*x* + 1); percents, arcsine square root (*x*); counts per minute, square root (*x* + 0.5)). Statistical tests for significance were performed at *p* < 0.05, (Zar, 1984).

Table 2. Water quality of study sites

Parameter	Site A	Site B
AODC	8.85 (8.73)	8.44 (7.26)
TCC	4.83 (4.55)	5.60 (4.95)
ODB	2.29 (0.49)	0.16 (0.08)
CHL-A	2.07 (0.6)	3.66 (0.05)
TOC-water	2.10 (0.12)	1.08 (0.58)
TOC-sediment	16.86 (2.14)	3.58 (0.53)
DOC-water	0.12 (0.03)	BDL
NH ₄ -sediment	4.00 (0.092)	15.23 (2.87)
TP-sediment	0.15 (0.01)	0.08 (0.02)

AODC = acridine orange direct counts, log cells/100 ml; TCC = total culturable counts, log colony forming units (CFU)/100 ml on Marine Agar plates; ODB = oil degrading bacteria (% relative to total heterotrophic plate counts); CHL-A = chlorophyll *a*, mg/ml; TOC = total organic carbon (for water in mg/l; for sediments in mg/g); DOC = dissolved organic carbon, mg/l; NH₄ = ammonia, mg/g; TP = total phosphorus, mg/g.

RESULTS AND DISCUSSION

Water quality

Table 1 describes the water quality status of each site, over the time course of this study. Site A had higher temperature readings and total phosphorus levels, while Site B had higher chlorophyll *a* and dissolved oxygen concentrations. The latter was probably due to the abundant growth of several macro-algae observed along the shoreline at this site. The sediment composition was quite different

by site. Sediments at Site A were muddy and dark colored, whereas Site B presented a light colored sandy bottom. Site A had a significant increase in water turbidity during the departure of two oil tankers, at 23.5 and 47 h, respectively. Indeed, turbidity and total phosphorus were the only two parameters that showed significant differences over time at Site A. Site B did not show significant differences in water quality over time, with the exception of a significant increase in water turbidity at 47 h. Analyses prior to this study determined

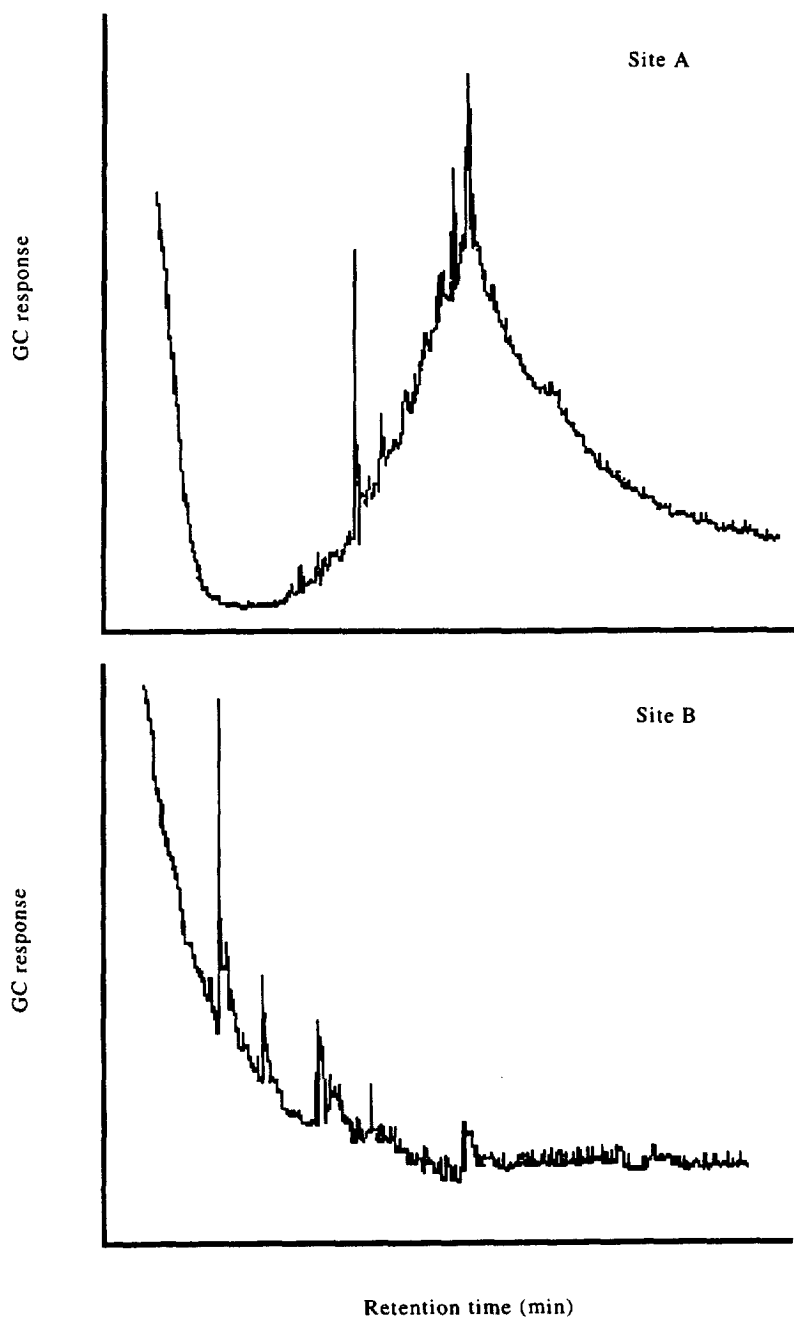


Fig. 2. Gas chromatography profiles of sediment extracts from Site A and Site B at the Yabucoa Bay.

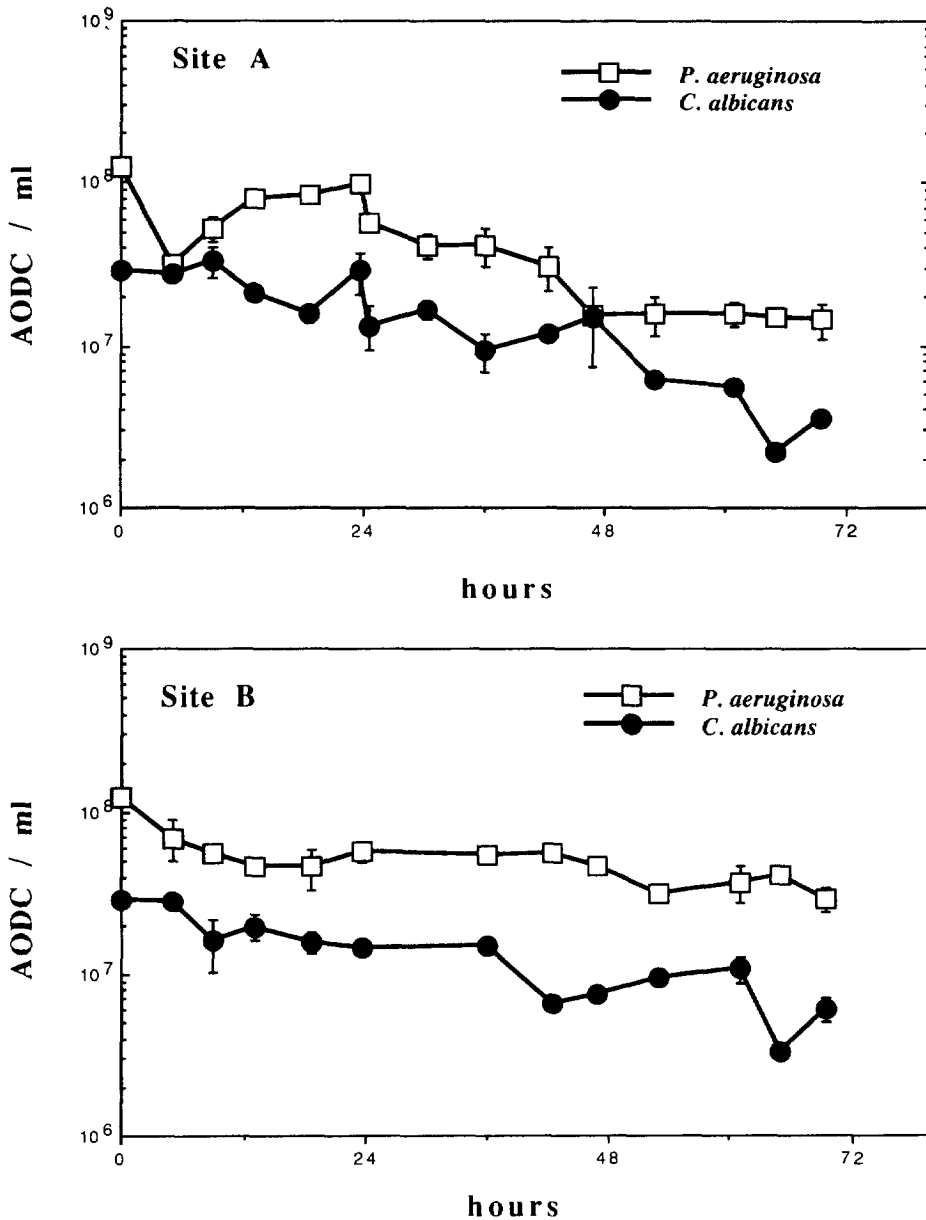


Fig. 3. Direct counts of *P. aeruginosa* and *C. albicans* at Site A and Site B as determined by AODC method.

that Site A normally has higher levels of total organic carbon in sediments, and dissolved organic carbon in the surface waters (Table 2). In addition, gas chromatography analyses of sediment extracts showed that Site A sediment has a richer and more complex carbon load as compared to Site B (Fig. 2).

Survival

The total cell densities of *P. aeruginosa* and *C. albicans* in diffusion chambers at Sites A and B decreased significantly over time ($p < 0.001$) with a reduction of more than 75% for both organisms at both study sites. However, there were no significant

differences in densities for either organism by site (Fig. 3). Densities of *P. aeruginosa* at Site A had an initial steep decline followed by a period of cell regrowth which lasted 18.5 h. After the first 12 h, the highest densities of *P. aeruginosa* and *C. albicans* at Site A coincided with a significant increase in water turbidity and with high total phosphorus concentrations (23.5 h after chamber submersion). Cell densities at Site A declined as total phosphorous concentration also declined over time.

A simultaneous decrease was observed in cell reproductive activity as determined by the FDC for both cells (Fig. 4). This activity measurement showed a positive correlation with AODC densities

of *P. aeruginosa* and *C. albicans* ($r = 0.78$, $p < 0.01$ and $r = 0.71$, $p < 0.01$, respectively) only at Site A. Similarly, the percentage of *P. aeruginosa* respiring cells decreased significantly over time ($p < 0.0001$), although this measurement was not significantly different by site for either organism (Fig. 5). The proportion of respiring yeast cells that were active in respiration after 69.5 h declined by more than 88%, when compared to the initial proportion of respiring yeast cells, despite the fact that the overall differences were not significant by time. In contrast, the population respiratory activity of *P. aeruginosa*, as measured by INT-F extraction, was significantly

different ($p < 0.03$) by site (Fig. 6). A significant decline ($p < 0.03$) was observed in the respiratory activity of bacterial suspensions in chambers at Site A over time, but not for the bacterial cells at Site B. This pattern of population activity at Site A correlated positively with AODC ($r = 0.89$, $p < 0.0001$). For *C. albicans* suspensions, population activity at Site B, had a significant increase during the first two sampling periods. Despite this temporary increase, yeast cells at Site B did not show significant changes in total population activity over time (Fig. 6). On the other hand, the yeast population at Site A had a significantly higher res-

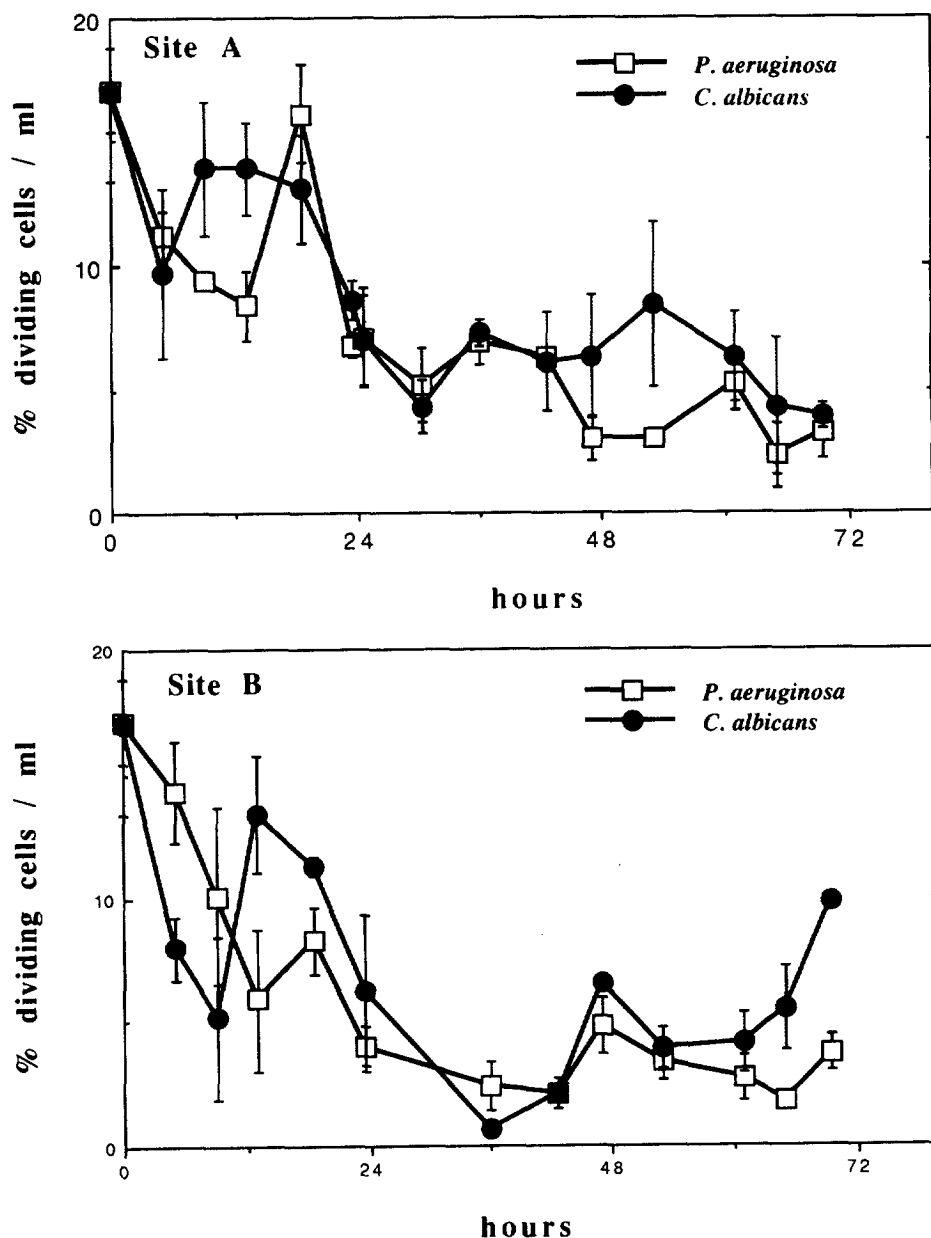


Fig. 4. Percent of *P. aeruginosa* and *C. albicans* dividing cells at Site A and Site B as determined by FDC method.

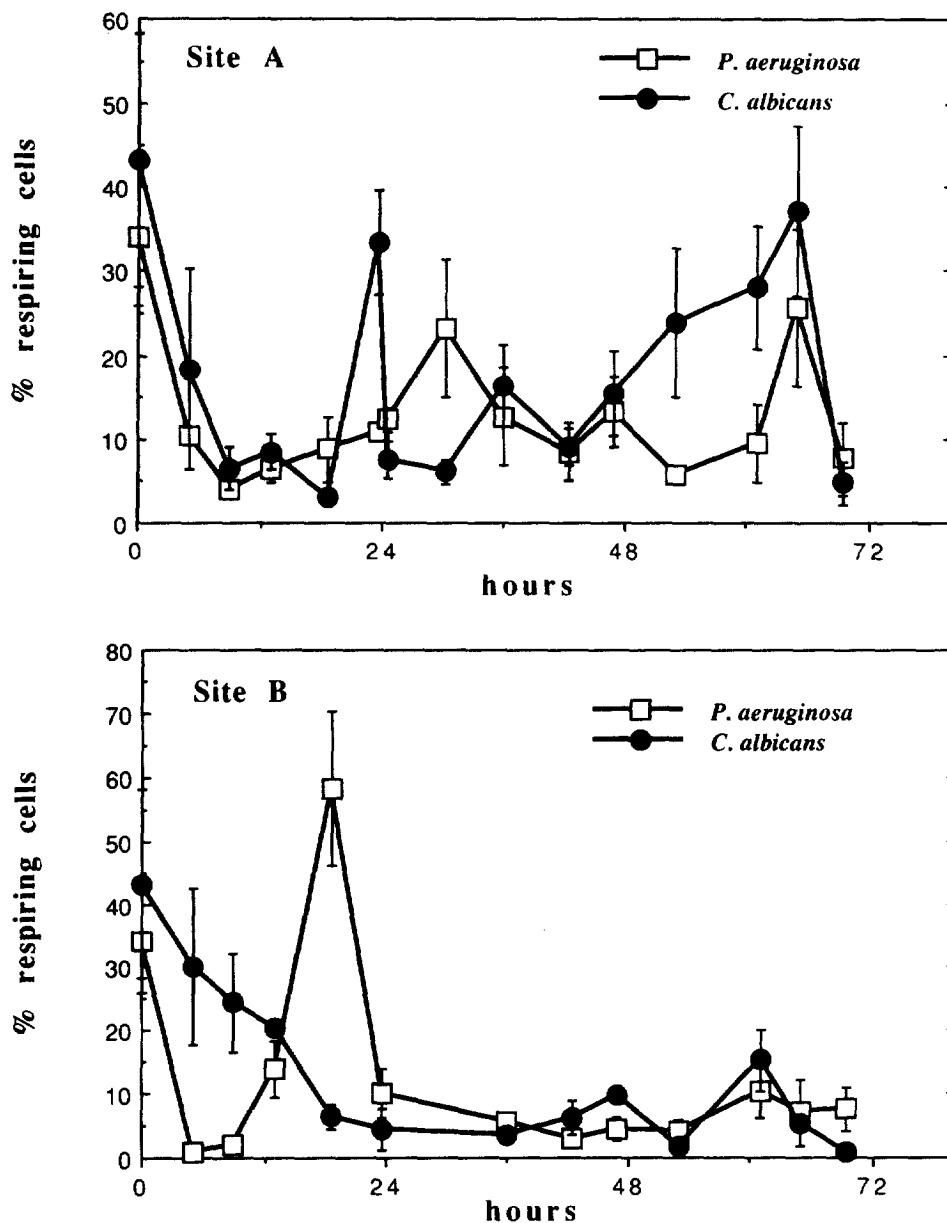


Fig. 5. Percent of *P. aeruginosa* and *C. albicans* respiring cells at Site A and Site B as determined by INT reduction method.

piratory activity over time ($p < 0.05$), in spite of the high variability observed between samples.

Respiratory activity of *P. aeruginosa* individual cells, as estimated from INT-F extraction assays, significantly increased over time after 47 h ($p < 0.01$), but showed no significant differences by site (Fig. 7). A high bacterial respiratory activity was observed at Site B, during the 13–18.5 h time interval. For *C. albicans*, the respiratory activity of individual cells showed significant differences by site ($p < 0.05$), but not over time (Fig. 7). Yeast cells at Site A showed significantly higher concentrations of INT-F per cell, after 53 h. Cell suspensions at Site B did not show significant changes in INT-F con-

centration over time. Thus, although cell densities indicate that yeast cells were declining with time, the respiratory activity is maintained at high levels suggesting that they are capable of adapting to the *in situ* conditions.

The results of ^3H -thymidine uptake did not show significant differences in the survival response of *P. aeruginosa* and *C. albicans* by site nor over time (Fig. 8). The percent of cells active in the incorporation of ^3H -thymidine, as determined by microautoradiography, was not significantly different over time, nor between sites for either organism (Fig. 9). However, the incorporation of ^3H -thymidine by *P. aeruginosa* after 42.5 h, as calculated from popu-

lation uptake determinations was significantly different by site ($p < 0.01$, Fig. 10). Since there was a significant positive correlation between thymidine uptake and the FDC ($r = 0.79$, $p < 0.02$) it is very likely that the radiolabeled nucleoside was incorporated into *P. aeruginosa* DNA molecules. This argument is supported by Pollard and Moriarty (1984) in which several *Pseudomonas* spp. were shown to incorporate ^3H -thymidine into DNA. In contrast, no significant correlation was observed between FDC and the ^3H -thymidine uptake in *C. albicans* cell suspensions. However, a significant increase in the number of individual cells incorporating ^3H -thymidine was observed for *C. albicans* ($p < 0.003$, Fig. 10). We suspect that this was due to the non-

specific labeling of cell macromolecules other than DNA since thymidine kinase, the enzyme required to incorporate thymidine directly into DNA, seems to be rare in fungi (Pollard and Moriarty, 1984).

The ATP content in *P. aeruginosa* and *C. albicans* cell suspensions was also significantly different by site ($p < 0.05$). At Site A, a significant decline was observed for both populations, while cells in chambers at Site B had no significant change over time (Fig. 11). In contrast, the ATP content per *P. aeruginosa* cell at Site B had a significant increase over time (data not shown), although cells at Site A had a significant reduction over time. The intracellular ATP content of *C. albicans* cells at Site A did not change significantly over time, although a

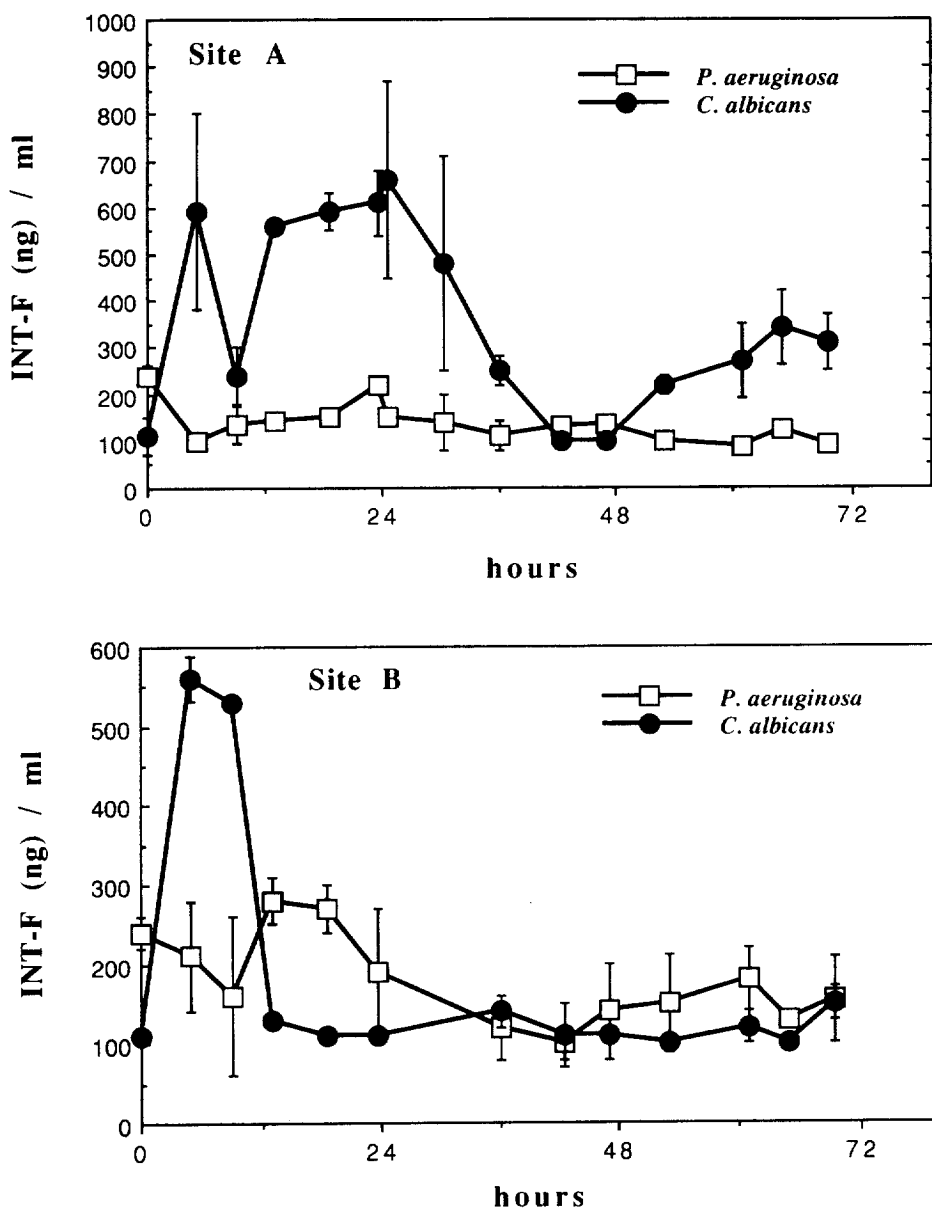


Fig. 6. INT reduced by populations of *P. aeruginosa* and *C. albicans* at Site A and Site B.

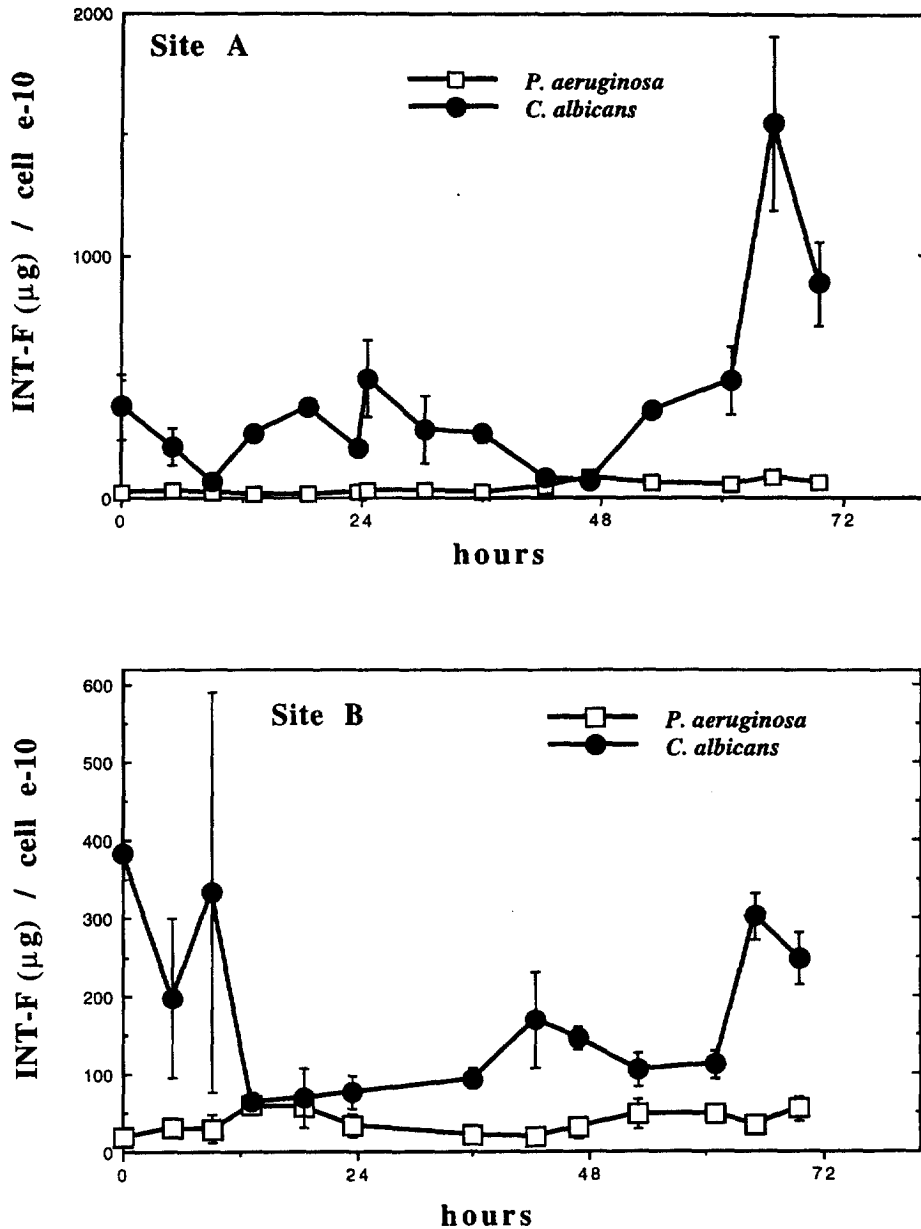


Fig. 7. INT reduced by individual cells of *P. aeruginosa* and *C. albicans* at Site A and Site B.

significant increase was observed at 18.5 h. Yeast cells at Site B showed a significant increase in the ATP content per cell at the end of 18.5 h. The intracellular ATP levels at Site B became consistently higher than at Site A after 47 h.

Although a comparison of the survival rates of *P. aeruginosa* and *C. albicans* at the Yabucoa Bay showed significant declines in cell densities (AODC) and FDC at both sites over time, both organisms showed significant differences in their survival response by site. The biomass of living cells as estimated from ATP content, showed no significant differences over time in diffusion chambers of both organisms at Site B, whereas a significant reduction was observed at Site A. Moreover, an analysis of

the ATP content per cell showed a significant decline in chambers at Site A, whereas cells at Site B had a significant increase over time. In theory, ATP content determinations cannot be used to assess cell metabolic activity in the absence of cell size determinations (Karl, 1980). No significant differences were observed in the size of *P. aeruginosa* and *C. albicans* cells by site at the end of 69.5 h, as determined by the AODC method. Thus, estimates on ATP content per cell suggested that the physiological status of survivors in chambers at Site B improved over time, whereas cells in chambers at Site A showed an increase in metabolic stress over time. Additionally, *C. albicans* and *P. aeruginosa* cells at Site A also showed a significant

increase in respiratory activity (i.e., INT-F/cell) over time, as total cell densities and cell biomass decreased. These results suggest that both organisms increased their respiratory activity to cope with the relatively high hydrocarbon levels at Site A. It is also implicit that although both organisms have shown the ability to degrade crude oil under poor nutrient conditions in the laboratory (Fuentes, 1987), they were subjected to great metabolic stress upon exposure to petroleum hydrocarbons in natural oligotrophic environments.

P. aeruginosa has been isolated from very different environments, ranging from soils and water to human respiratory and urinary tracts (Römling *et al.*, 1994; Palleroni, 1994). However, several investigators have reported poor survival of *P. aeruginosa*

in sea water, both *in situ* and in laboratory microcosms (Vasconcelos and Swartz, 1976; Yoshpe-Purer and Golderman, 1987). Interestingly, our study showed that even when densities of *P. aeruginosa* decline significantly over time in sea water, a small population can still survive in good physiological state in the presence of major stressors (e.g. low nutrients). Thus, this bacterial species seems capable of adapting to quite different habitats. In addition, *P. aeruginosa* has been identified by some investigators as a non-marine bacteria, which is frequently associated with fecal indicator bacteria in estuarine and coastal waters (Cabelli *et al.*, 1976; Robertson and Tobin, 1983; Yoshpe-Purer and Golderman, 1987). Our data suggests that *P. aeruginosa* would not be a good indicator of recent fecal

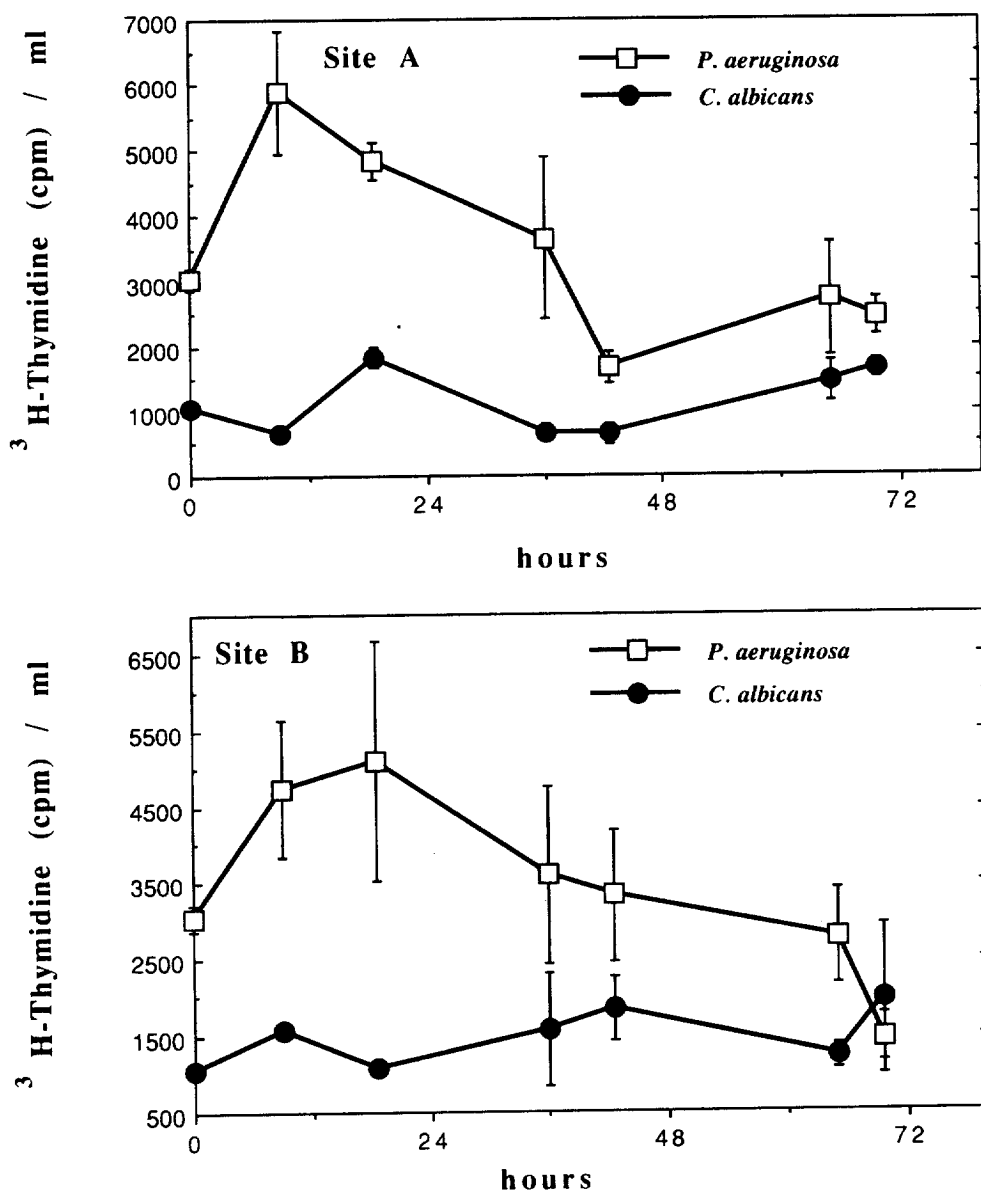


Fig. 8. Thymidine uptake by populations of *P. aeruginosa* and *C. albicans* at Site A and Site B.

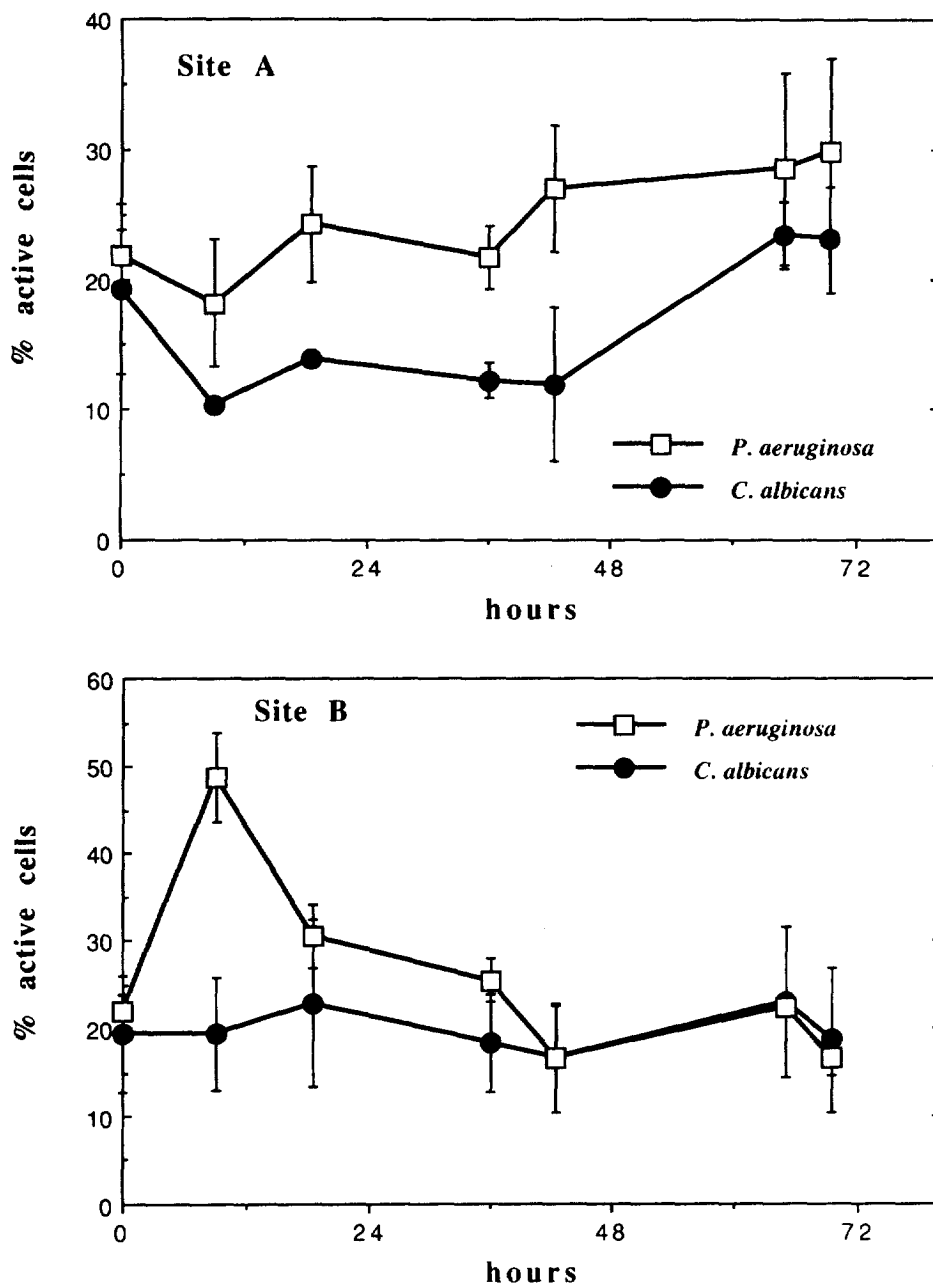


Fig. 9. Percent of *P. aeruginosa* and *C. albicans* active in thymidine uptake at Site A and Site B as determined by microautoradiography.

contamination, due to its capacity to survive for prolonged periods in sea water. Moreover, this bacteria can regrow if essential nutrients are provided (e.g. phosphorus).

Candida albicans OD-2 showed a survival response similar to that described for *P. aeruginosa* OD-1 at both study sites. This organism has been shown to survive for extended periods of time in pristine aquatic environments, including marine and freshwater habitats and also in strongly polluted marine habitats (Valdés-Collazo *et al.*, 1987). In contrast to our study, these investigators reported

no significant changes in *C. albicans* total densities over time. Although the present study indicated a significant reduction in total cell densities over time, the results from both studies are not necessarily antagonistic. There were significant differences in the initial inoculum used in both survival studies. The initial cell densities in the present study were approximately 10 times greater than the inoculum used by Valdés-Collazo *et al.* (1987) in their survival study of a non-polluted marine habitat. No significant differences were observed between the densities reported in the present study for Site B

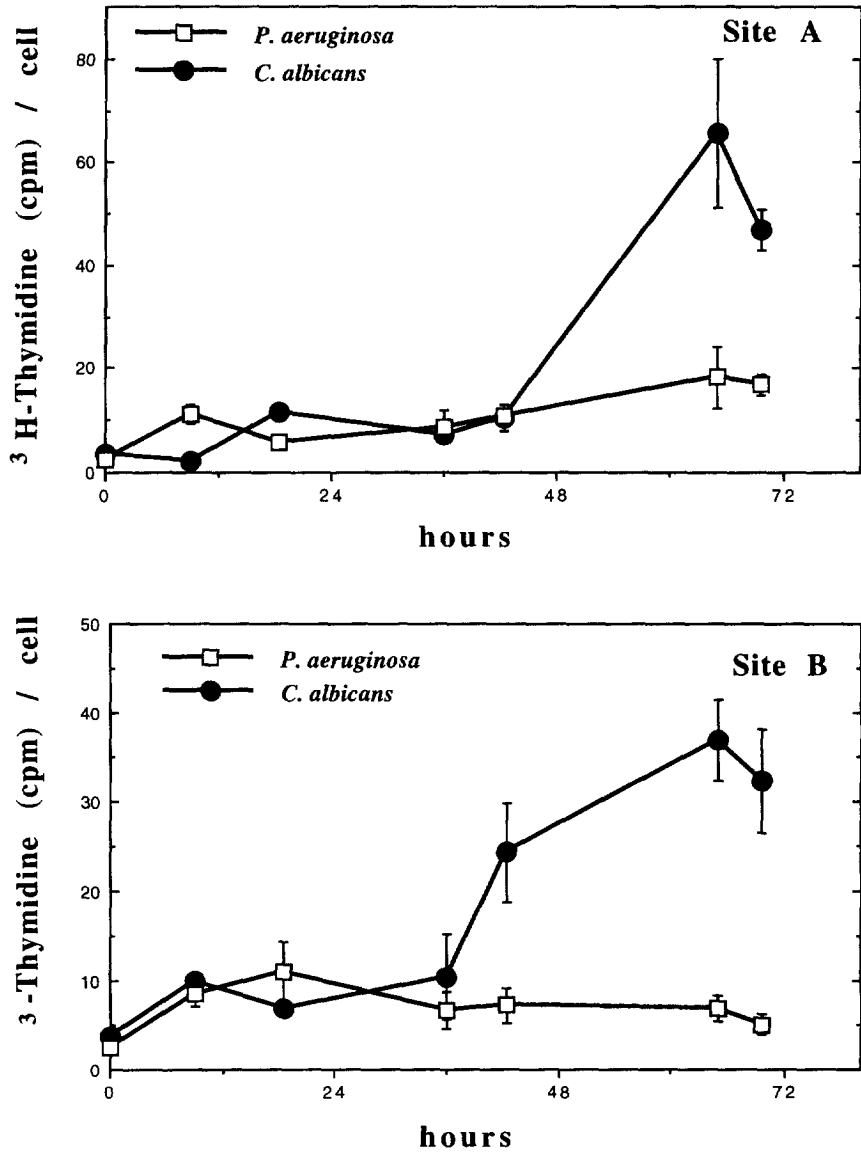


Fig. 10. Thymidine uptake by individual cells of *P. aeruginosa* and *C. albicans* at Site A and Site B.

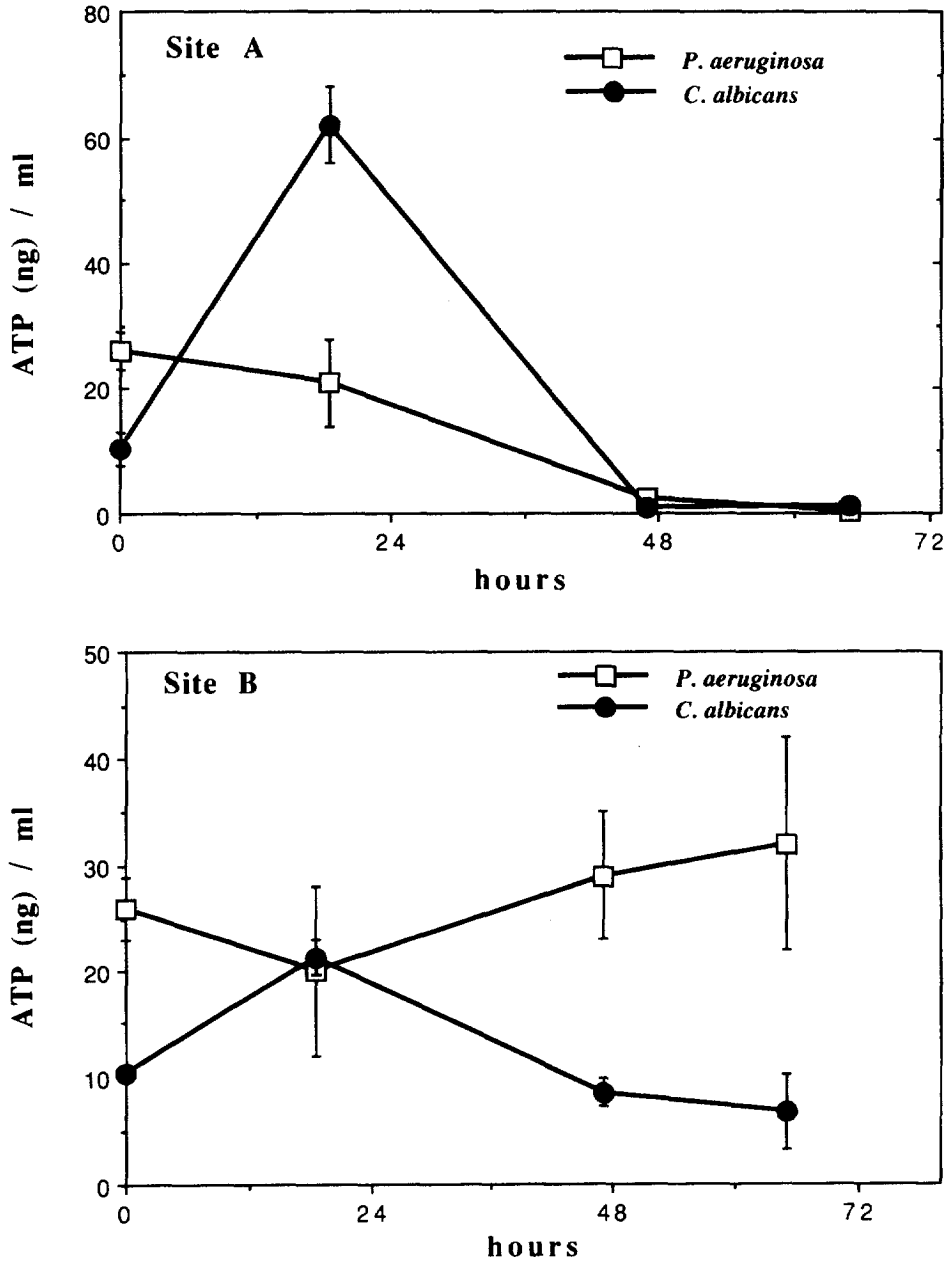


Fig. 11. Total ATP in populations of *P. aeruginosa* and *C. albicans* at Site A and Site B.

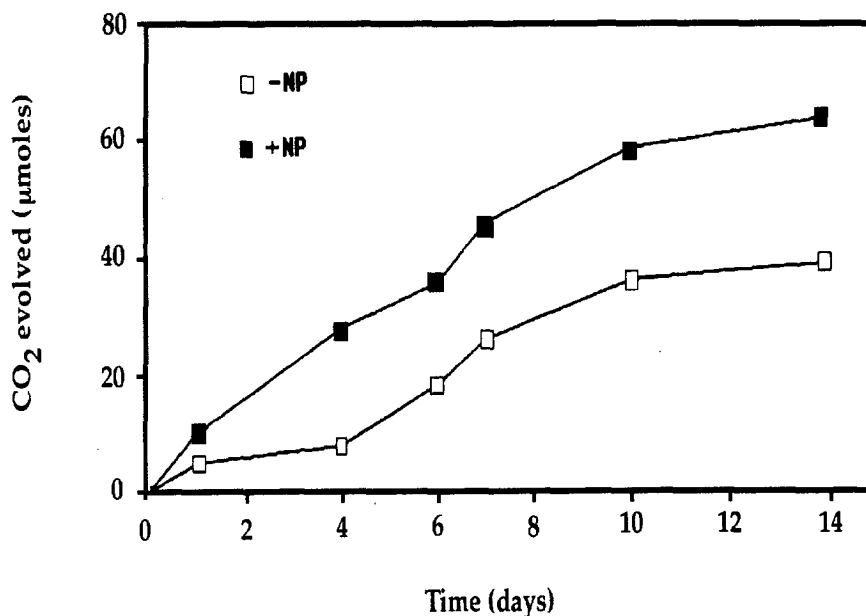


Fig. 12. Effect of nutrient addition in the degradation of hydrocarbons. Experiments were performed in microcosms containing sea water from the Yabucoa Bay amended with ¹⁴C-hexane. Solid squares represent samples amended with nitrogen and phosphate while open squares represent unamended samples. Values represent means of triplicates.

and those reported by Valdés-Collazo *et al.* (1987) for their non-polluted marine habitat after 70 h. Moreover, this study shows that the ATP content for *C. albicans* at Site B did not change over time, whereas the ATP content per cell actually increased, thus confirming that *C. albicans* can survive in non-polluted sea water for extended periods of time in a good physiological condition. These results also confirm the conclusion of Valdés-Collazo *et al.* (1987), that *C. albicans* can not be used as an indicator of recent fecal contamination in tropical marine environments.

The results from this study also provide some information on the nutritional requirements of natural microbial populations in the Yabucoa Bay. There is a marked difference in the levels of oil pollution between Sites A and B in the Yabucoa Bay (Fig. 2). It has been suggested that nutrient supplies, in particular nitrogen and phosphorus, are key limiting factors for hydrocarbon biodegradation in marine environments (Atlas and Bartha, 1972). The continuous input of low concentrations of crude oil hydrocarbons into Site A alters the carbon/nitrogen (C/N) and carbon/phosphorus (C/P) ratios, consequently affecting microbial growth and metabolism. Hydrocarbon mineralization studies have shown that nitrogen-phosphorus (N + P) salt supplements stimulated the degradation of the radiolabeled hydrocarbon in microcosms incubated with sea water samples from Yabucoa Bay (Fig. 12). However, a higher response towards N + P amendments was obtained with Site A microcosms (Fuentes, 1987). Moreover, gas chromatography

analysis of N + P supplemented sediments from this study site indicated a substantial reduction in petroleum hydrocarbons, especially on the n-alkane fraction (Fig. 13). Interestingly, a significant increase in total cell densities and FDC was observed in chambers at Site A, during the 9–23.5 h interval. An increase in total phosphorus was observed during the same period of time. This increase coincided with the departure of an oil tanker from the oil refinery docks. Both events might be closely related, since total phosphorus concentration in water decreased after the tanker departure, followed by a simultaneous decrease in total cell densities and frequencies of dividing cells. Thus, violent agitation of sediments at Site A provided *P. aeruginosa* and *C. albicans* with a transient source of phosphorus, allowing them to grow for a limited period of time. These results suggest the role of phosphorus as key limiting nutrient for oil degrader populations in the Yabucoa Bay oligotrophic environment and suggests that perturbations like the traffic of oil tankers have a significant impact on the ecology of petroleum contaminated environments and the survival of oil degrading bacteria in marine habitats.

CONCLUSIONS

Although a substantial fraction of the microbial populations used in this study maintained *in situ*, a relatively high degree of activity for several days, it is important to note that *P. aeruginosa* or *C. albicans* are not recognized as marine microbes, and

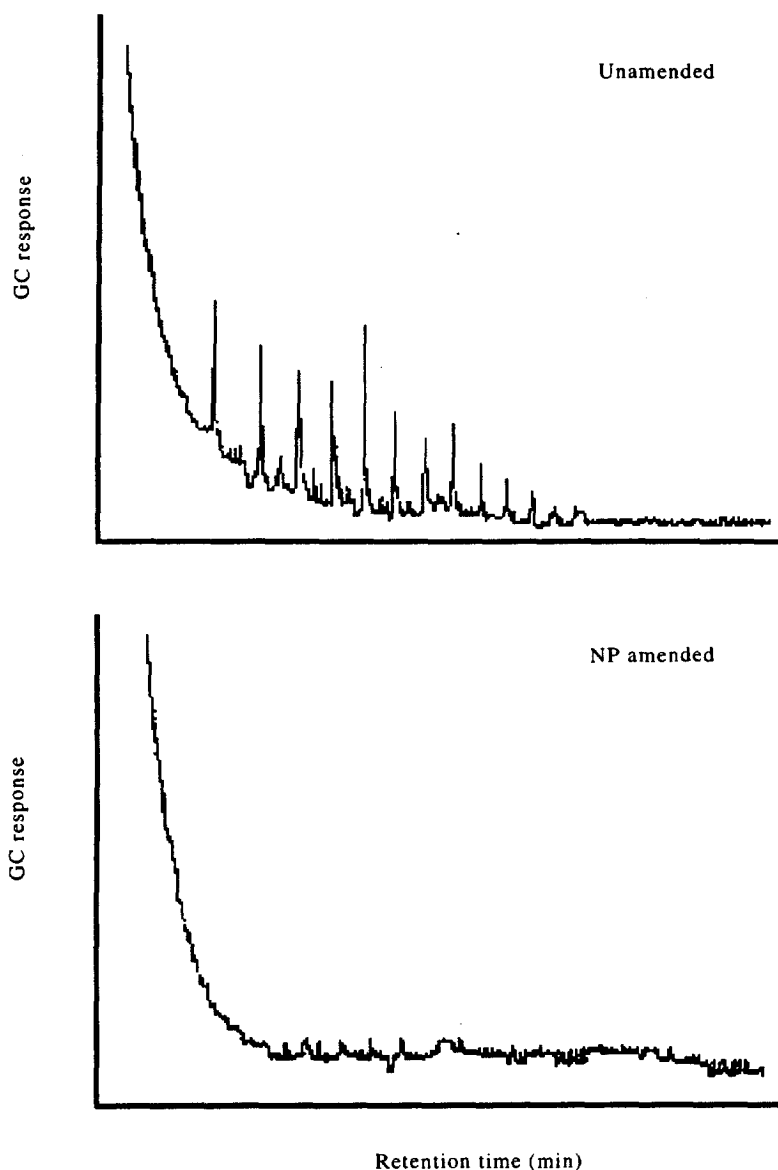


Fig. 13. Effect of nutrient addition in the degradation of the petroleum n-alkane fraction by microbial communities from Yabucoa Bay. Gas chromatography profiles shown in the upper panel are typical of unamended samples extracts while bottom panel shows a typical profile of a sample amended with nitrogen and phosphate.

thus, they might not be capable of maintaining this level of activity for long periods of time. This is indirectly supported by the fact that these genera represent a relatively minor component of the natural community of the Yabucoa Bay (Fuentes, 1987). However, their ability to maintain an unexpected high activity throughout this study suggests that allochthonous populations have developed means for adapting to the natural conditions prevailing at this contaminated site and thus might be adequate for *in situ* bioaugmentation. Since these experiments were carried out using concentrated pure culture suspensions, it is difficult to estimate the possible role of factors like competition, dilution, and preda-

tion on their survival in petroleum contaminated marine waters. Future studies should address these factors in order to better assess the potential use of degrading microbes in these environments.

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