AN INVESTIGATION OF THE AEROBIC MICROBIAL DEGRADATION OF SULFUR, NITROGEN, AND OXYGEN HETEROCYCLES BY THREE LOCAL MARINE BACTERIAL CONSORTIA

JAMES DEREK MEADE

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BY

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Department of Biology
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Now the sea's days of giving have all gone
we've been taking for so long
and the poisons that we pour
in her veins can't be ignored
we can't fish there anymore.

-Lennie Gallant
Days of Giving, 1993

ABSTRACT

Bacterial samples were collected from three marine beaches in coastal Newfoundland and enriched by growth on 1-methylnaphthalene. The most prominent bacterial cell type for each consortium was isolated in a serial dilutions test, and a substrate utilization profile was obtained for each using the Biolog MicroStation System. Each bacterial community was tested for its ability to degrade sulfur heterocycles (benzothiophene: BT, 3-methyl-benzothiophene: 3-MBT, and dibenzothiophene: DBT), a nitrogen heterocycle (carbazole: CARB), and an oxygen heterocycle (dibenzofuran: DBF). Incubations were carried out at an optimum temperature for culture (25 °C) and at a temperature more typical of a northern environment (4 °C). Degradation of the compounds was determined using gas chromatography-mass spectroscopy (GC-MS) and degradation products were identified using GC-MS and Fourier transform infrared spectroscopy (FTIR). Bacterial growth was monitored using optical density measurements to determine the dry weight (μg) of cells/ mL and the number of colony forming units/ mL (CFU/mL). The 2-ringed heterocycles were degraded faster and to a greater extent than the 3-ringed compounds. Degradation of BT was not statistically different from that for 3-MBT and, likewise, a comparison of the 3-ringed heterocycles showed no significant differences in degradability at either incubation temperature. There was a significant difference in degradation of the compounds at the two incubation temperatures as biodegradation was 3 to

5 times greater at 25 °C than at 4 °C. Statistical examination revealed that no one culture demonstrated a significantly greater ability to degrade the 5 heterocycles studied which means that the bacterial consortium isolated from a beach in Bonne Bay, NF., where the sediments exhibited no visible signs of hydrocarbon contamination, demonstrated the ability to degrade the heterocyles as efficiently as bacterial communities from visibly contaminated soils at Come by Chance and Port aux Basques, NF. This study represents the first comprehensive investigation of the ability of local bacteria to biodegrade a range of aromatic compounds. It provides a preliminary understanding of the fate of aromatic compounds in sediments, in terms of their half-life versus environmental temperature.

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1.1 Heterocyclic Compounds in Petroleum

Heterocyclic compounds are those which have a cyclic structure containing at least one heteroatom (an atom other than carbon-generally sulfur, nitrogen, or oxygen) (Davies, 1992). Heterocycles are widely distributed in nature, for example in nucleic acids and indole alkaloids. They play a vital role in the metabolism of all living cells and are therefore essential to life (Katritzky, 1985). Synthetic heterocycles have widespread uses as herbicides, fungicides, insecticides, dyes, organic conductors and pharmaceutical products (Davies, 1992).

Petroleum is a naturally occurring gaseous, liquid or solid mixture, predominantly composed of hydrocarbons. However, appreciable amounts of sulfur-, nitrogen-, and oxygen-containing compounds are also present. The relative abundances of carbon, hydrogen, sulfur, nitrogen and oxygen in petroleum is 83 to 87%, 10 to 14%, 0.05 to 6%, 0.1 to 2%, and 0.05 to 1.5%, respectively. Although their concentrations may be quite small, the influence of these organic, non-hydrocarbon constituents is important (Speight, 1990).

1.1.1 Sulfur in Petroleum

Sulfur is one of the most plentiful elements in nature, ranking thirteenth in abundance in the earth's crust (Rall *et al.*, 1972). It appears in the elemental

state, combined with other elements as minerals, and as a vital constituent in plant and animal tissue. Polycyclic aromatic sulfur heterocycles (PASH) are formed during the fossilization of biomass (Jacob, 1990) and, accordingly, can be found in coal, crude oil and related products such as petroleum fractions, motor oils, shale oil, coal liquids, coal tars and pitches, and other coal-derived technical products. Even though large numbers of individual PASH have been identified in petroleum, sulfur occurs in the form of only a few functional groups, mainly thiols, cyclic sulfides and thiophenes. Disulfides may arise by oxidation of thiols during processing (Figure 1.1) (Speight, 1990).

The PASH from the above materials may be transferred directly into the environment as a result of oil spills from damaged tankers, combustion processes, or through technical processes such as coking plants. Detectable levels of PASH have been determined in sediments, water, marine and freshwater organisms (fish, mussels, algae) as well as air and sewage (Jacob, 1990). Reports on the harmful effects produced by PASH justify interest in the environmental fate of these compounds. For example, PASH have been implicated in the corrosion of engine parts during combustion of gasoline (Speight, 1980) and the corrosion of metals during the production and refining of high-sulfur crude oil (Foght *et al.*, 1990). A major concern is that the combustion of sulfur-containing fuels yields sulfur dioxide which contributes to acid rain (Manowitz and Lipfert, 1990). More important is the fact that certain PASH are

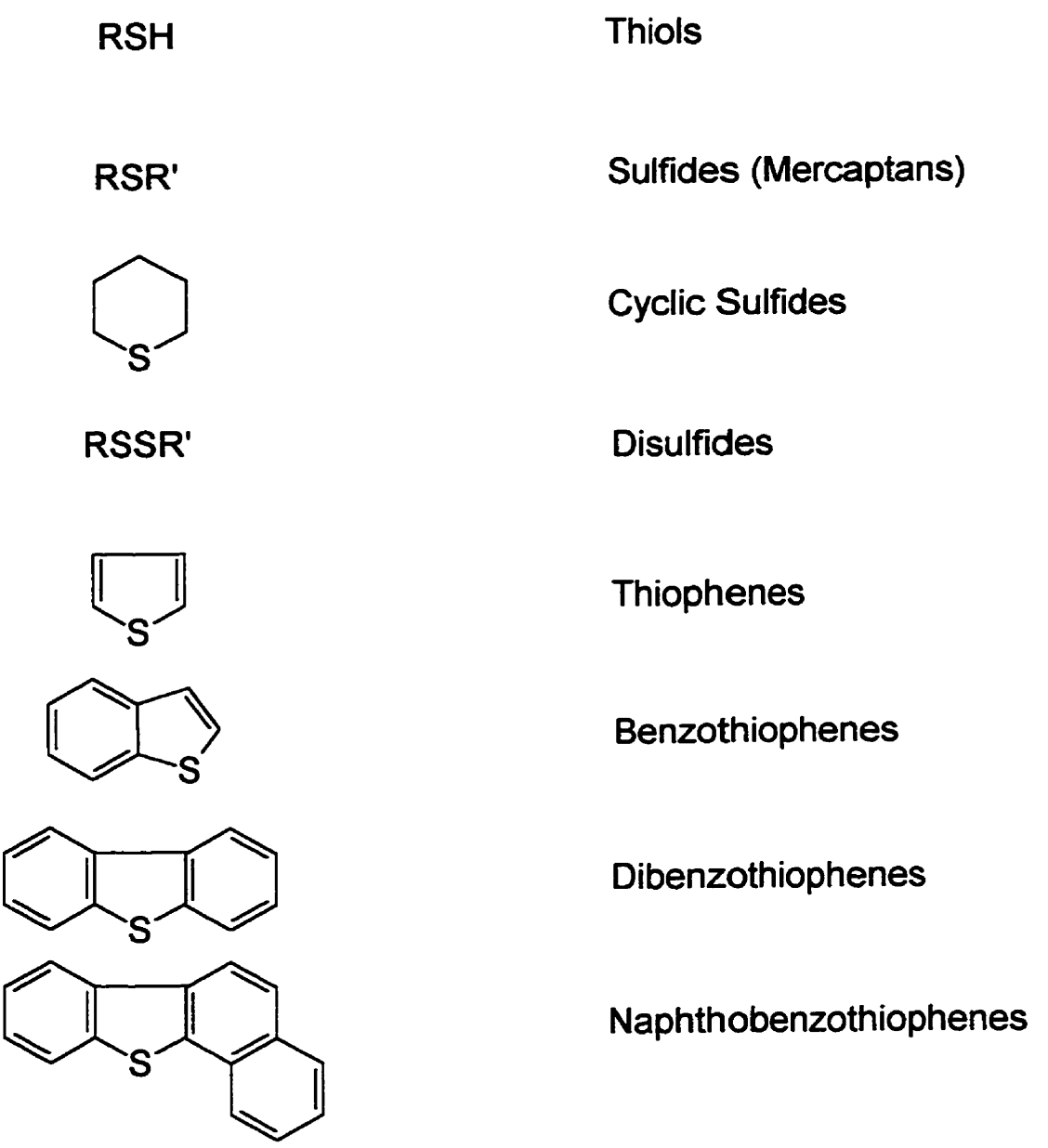


Figure 1.1 Representative sulfur compounds in crude oils.

toxic, some are mutagenic, and others are carcinogenic (Tilak, 1960).

In a study investigating the acute oral toxicity of dibenzothiophene (DBT) to mice, Leighton (1989) reported that DBT is a potent hepatotoxin, it caused necrosis of lymphocytes in the thymus, and also degenerative changes in the walls of small arteries in the lung. Pelroy *et al.* (1983) investigated the mutagenicity of stable tri- and tetracyclic PASH and found that 1 of the 4 three-ring compounds, and 7 of the 13 four-ring compounds tested were mutagenic in the Ames *Salmonella*/microsome test. McFall *et al.* (1984) observed that 5 methyl-substituted 3- and 4-ring PASH demonstrated mutagenic activity in the Ames test. Sinsheimer *et al.* (1992) also used the Ames *Salmonella* test to evaluate the *in vitro* mutagenicity of chryseno[4,5-*bcd*]thiophene and its sulfone derivative. They also investigated, *in vivo*, the extent of chromosomal aberrations in the bone marrow of mice. The results from their genotoxicity studies reinforced the concern that PASH, which are structurally similar to known carcinogenic polycyclic aromatic hydrocarbons (PAH), may also be genotoxic environmental contaminants.

As the world supply of low sulfur petroleum continues to decrease, the use of heavy crude oils and tar sands, which have a higher sulfur content, will be required until alternative energy sources replace fossil fuels. Because sulfur in petroleum has potential harmful effects, the further accumulation of PASH in the environment must be prevented. The proportions of elements in petroleum vary

only slightly over narrow limits but after carbon and hydrogen, sulfur is the third most abundant element in crude oils (Speight, 1990) and, generally, the higher the density of a crude oil, the higher its sulfur content. The average sulfur content based on 9347 samples of conventional crude oil was determined to be 0.65% (Tissot and Welte, 1984). Oils containing less than 1% sulfur are considered low sulfur and those greater than 1% are high sulfur. In an effort to create a more meaningful relationship between the physical properties and processibility of crude oils the American Petroleum Institute (API) devised a measurement of gravity based on the Baumé scale for industrial liquids with the following formula:

$$^{\circ}\text{API} = \frac{141.5}{\text{specific gravity } 60^{\circ} / 60^{\circ}\text{F}} - 131.5$$

The result is that the greater the API gravity the lower the specific gravity and correspondingly, the lower the aromaticity. Consequently, the higher the API gravity the lower the sulfur content. Therefore, crude oils with an API gravity of less than 20° are classified as high sulfur while an API gravity above 20° indicates a low sulfur crude oil (Tissot and Welte, 1984).

The Hibernia oil field, which is expected to be producing oil by 1997, was discovered by Chevron Standard Ltd. and partners in 1979. The discovery well was drilled 325 km east of St. John's, Newfoundland in 80m of water on the Grand Banks (Figure 1.2). Calculations based on initial drilling indicate

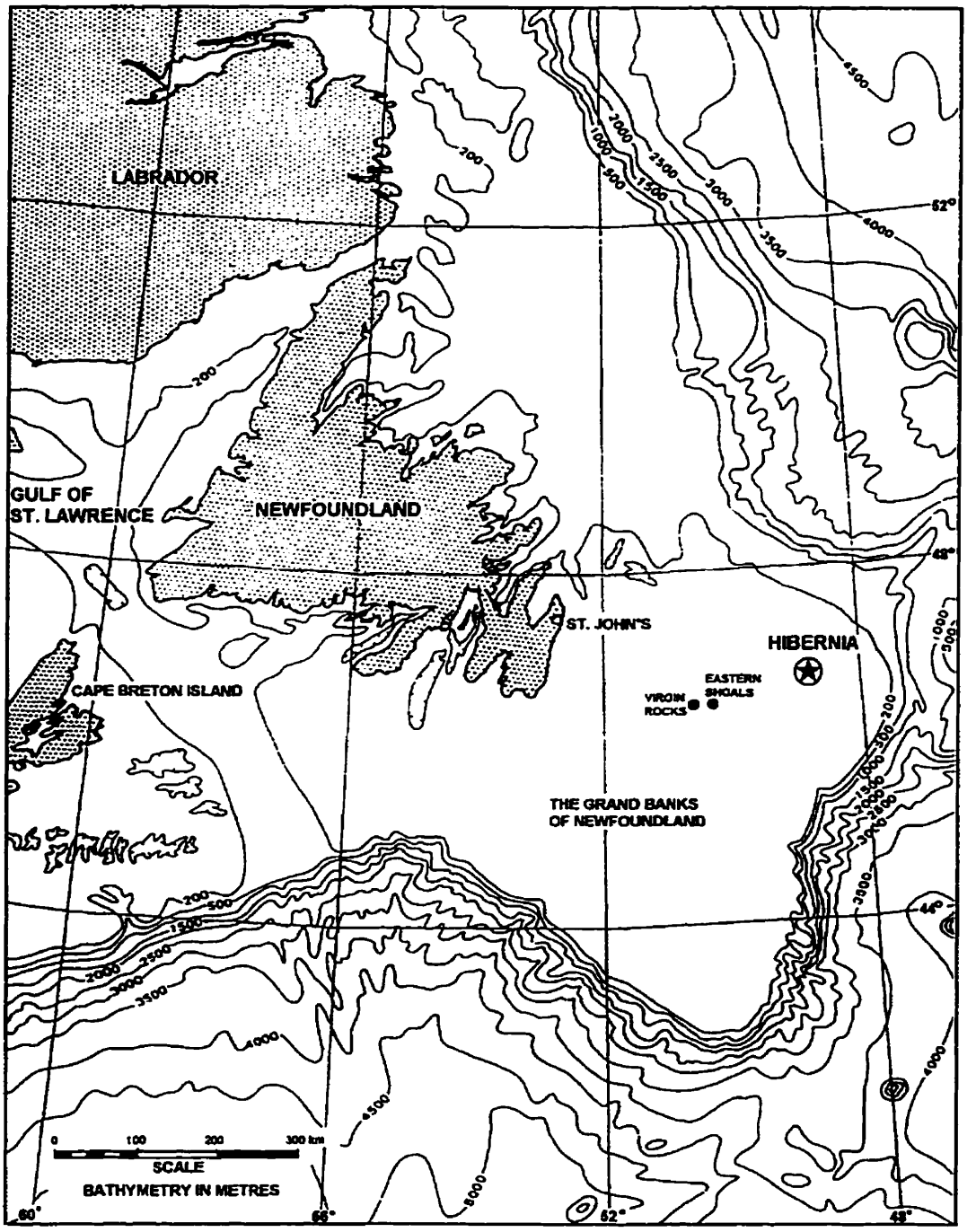


Figure 1.2 Location of the Hibernia oil field on the Grand Banks of Newfoundland

recoverable reserves in excess of one billion barrels of oil (Arthur *et al.*, 1982).

Oil from the Hibernia oil field ranges in gravity from 24 to 60° API (Arthur *et al.*, 1982) and is therefore largely considered a low sulfur crude oil. However, an API gravity of 24 suggests a significant sulfur content to be left contaminating local beaches should oil spillage occur during production, during the filling of tankers, or from tanker damage.

1.1.2 Nitrogen in Petroleum

The nitrogen content of petroleum ranges from 0.1 to 2% by weight and as with sulfur, the heavier the oil the higher the nitrogen content (Speight, 1990). Nitrogen in crude petroleum oil is responsible for corrosion of refining equipment and burning nitrogen-containing compounds releases nitrogen oxidation products into the atmosphere, contributing to acid rain (Kobayashi *et al.*, 1995). Therefore, removal of nitrogen from petroleum, before combustion, is necessary. Figure 1.3 lists the representative nitrogen-containing compounds in crude oil.

Carbazole (CARB) is a polycyclic aromatic nitrogen heterocycle (PANH) that enters the environment in several ways, including combustion and accidental spilling of hydrocarbons and oils, as well as heat and power generation, garbage incineration, fallout from urban pollution, and coal burning processes (Grosser *et al.*, 1991). PANH have been detected in atmospheric samples (Santodonato and Howard, 1981), river sediment (West *et al.*, 1986),

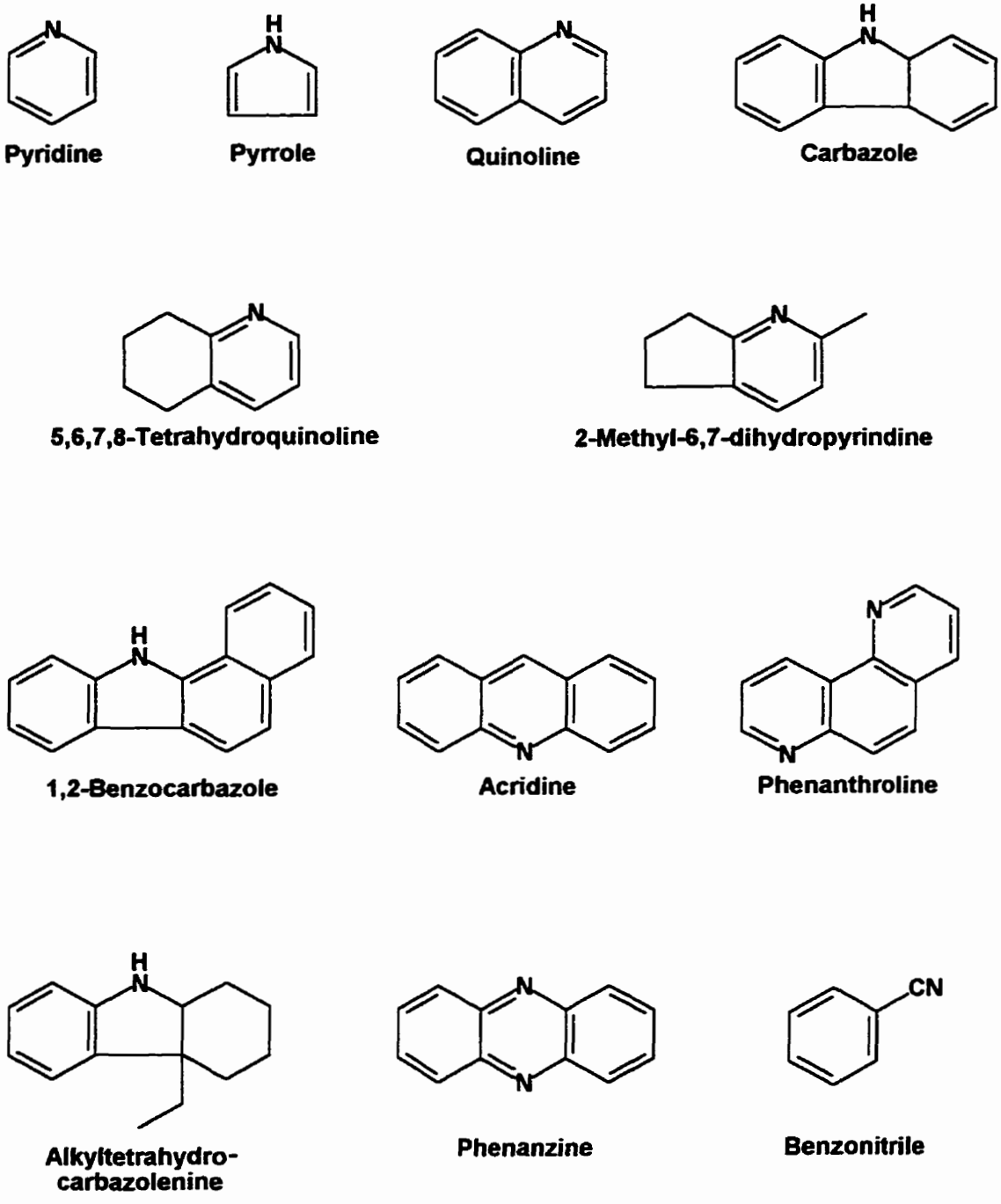
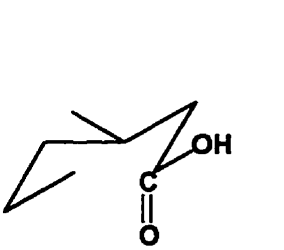


Figure 1.3 Representative nitrogen-containing compounds in crude oil (adapted from Speers and Whitehead, 1969).

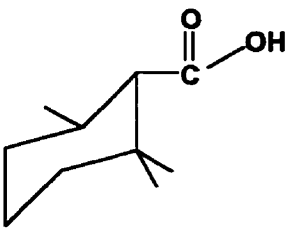
and groundwater (Pereira *et al.*, 1987) and are well known to be mutagenic and toxic (Arcos and Argus, 1968; Santodonato and Howard, 1981). Guerin *et al.* (1980) measured the mutagenicity of petroleum substitutes using the Ames test and reported that the mutagenicity of polycyclic aromatic primary amines was greatest in the 3-4 ring range but significant activity was also observed in the 2-3 and >4 ring ranges. In a subsequent study, Ho *et al.* (1981) reported the significant mutagenicity of the PANH 10-azabenzo[a]-pyrene and dibenz[a,j]-acridine. A study by West *et al.* (1986) to determine the genotoxicity of PANH, using the Ames test and induction of unscheduled DNA synthesis (UDS) in rat hepatocytes, showed that those with 3 to 5 aromatic rings are significantly genotoxic.

1.1.3 Oxygen in Petroleum

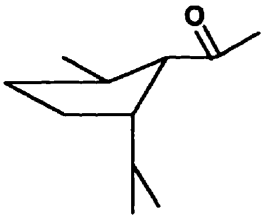
The oxygen content of petroleums generally ranges from 0.05 to 1.5 % by weight but larger amounts have been reported and this may be attributed to prolonged exposure of the oil to the atmosphere either during or after production (Speight, 1990). Oxygen-containing compounds in petroleum occur in the form of carboxylic acids, phenolics, ketones, esters, ethers and anhydrides (Speight, 1990). Polycyclic aromatic oxygen heterocycles (PAOH) have been identified in Ponca City crude oil (Yew and Mair, 1966) and dibenzofuran (DBF) itself has been reported in petroleum (Hartung and Jewell, 1962). Figure 1.4 illustrates some of the oxygen compounds found in crude oil.



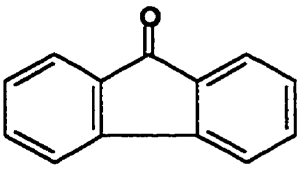
3-methylhexanoic acid



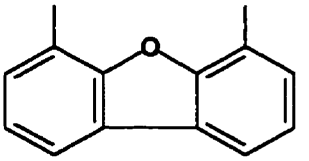
trans-2,2,6-trimethyl cyclohexanecarboxylic acid



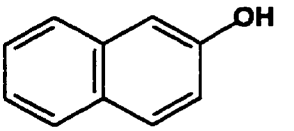
acetylisopropylmethyl-cyclopentane



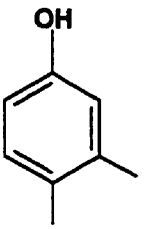
fluorenone



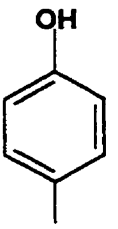
4,6-dimethyldibenzofuran



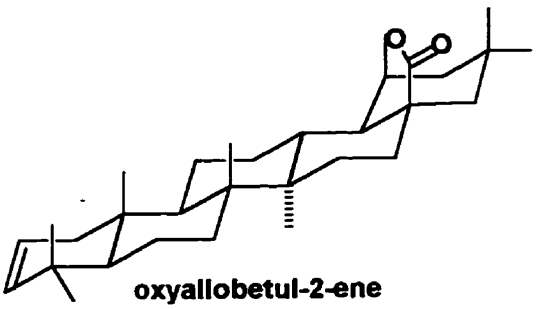
β-naphthol



3,4-xylenol



p-cresol



oxyallobetul-2-ene

Figure 1.4 Oxygen compounds found in crude oil (adapted from Speers and Whitehead, 1969).

DBF is a constituent of coal tar and its derivatives have been found in many natural products including lichen acids, fungal pigments and fruit (Cerniglia *et al.*, 1979). More importantly, toxic polychlorinated derivatives of DBF belong to the most hazardous environmental pollutants. These halogenated heterocycles are generated as undesired byproducts during the synthesis of chlorophenols and some chlorinated herbicides, they constitute a toxic component of wastes derived from the use of the wood preservative pentachlorophenol, and they are formed during the bleaching of pulp at paper mills (Adriaens *et al.*, 1995). They have also been recognized as the major toxic chlorinated organic contaminants of fly ash produced during waste incineration processes (Olie *et al.*, 1977) and once injected into the atmosphere they can be transported long distances before being deposited (Eitzer and Hites, 1989). Consequently, recent studies have reported the detection of these compounds in mussels, crabs, fish, arctic seals, and human breast milk and fatty tissue (Oehme *et al.*, 1988).

1.2 Microbial Degradation of Heterocycles

The microbial degradation of aromatic compounds plays an important role in the earth's carbon cycle. Many aromatic compounds, from natural and anthropogenic sources, are relatively recalcitrant to microbial degradation. If the carbon became locked inside these products it would eventually be exhausted

from the biosphere (Dagley, 1971). The chemical stability of the benzene nucleus is due to the resonance structure of this ring (Gibson and Subramanian, 1984). Several genera of bacteria produce the mono- and dioxygenase enzymes required to insert oxygen within the benzene ring to make it more susceptible to degradation and subsequent utilization as a carbon and energy source, and in the case of heterocycles, as a possible nitrogen and sulfur source. Such enzymes function to degrade a wide spectrum of aromatic compounds including phenolics, PAH, steroids, and alkylbenzene sulfonates. These enzymes are commonly found in bacteria belonging to the genera *Pseudomonas, Bacillus, Acinetobacter, Alcaligenes* and *Nocardia* (Bayley and Barbour, 1984).

When a compound serves as a sole source of carbon for growth the bacteria must contain the enzyme system that produces all of the intermediates and generates the energy yielding reactions essential for biosynthesis and maintenance of cellular activities (Clarke, 1984). Biodegradation is often perceived as the catabolism of a particular compound by a single microbial strain. However, experiments have shown that growth rates and rates of substrate utilization are frequently higher in enriched mixed cultures than in pure cultures isolated from the mixture (Weightman and Slater, 1988). In the natural environment it is more likely that degradation is carried out by mixed microbial communities and it is equally likely that bacteria in the natural environment would be exposed to more than one organic compound (Clarke, 1984).

Consequently, when preparing an experiment to investigate the microbial degradation of aromatic compounds this information must be considered and addressed before samples are collected and bacteria are enriched and isolated for identification purposes.

1.3 Effect of Temperature on Bacterial Growth and Metabolism

Growth of microorganisms is greatly influenced by temperature mainly because the processes for growth are dependent upon chemical reactions that are affected by temperature (Pelczar *et al.*, 1993). Although bacteria grown in the laboratory are usually incubated at a precisely maintained temperature, species tolerate a very broad temperature range - often spanning 40 Celsius degrees (Neidhardt *et al.*, 1990). At favourable temperatures for growth, the growth rate generally doubles for every 10 °C increase in temperature (Pelczar *et al.*, 1993). The optimum growth temperature lies near the upper limit of the temperature range because the rate of enzyme reactions increases with increasing temperature up to the point where enzymes are damaged by heat and cells stop growing (Pelczar et al., 1993). Therefore, higher temperatures that do not kill the bacteria result in higher metabolic activities (Atlas and Bartha, 1987).

Growth temperature profoundly affects the physiological state of bacterial cells. Neidhardt *et al.* (1990) stated that shifting the incubation temperature of an exponentially growing culture within the normal temperature range results in a

growth rate characteristic of the new temperature. Temperature shifts from the normal range, to either the high or low range, results in growth that proceeds at a transitional rate before exponential growth at a rate characteristic of the new temperature begins. However, shifting to or from the low range results in the most marked transient growth periods. This suggests that cellular reactions remain coordinated within the normal temperature range due to changes in enzyme activity, but full growth rate in the high or low range requires that the composition of the cell be altered (Neidhardt *et al.*, 1990). In a study investigating the effects of growth temperature on the cellular content of 133 different proteins of *E. coli*, Herendeen *et al.* (1979) found that the concentrations of very few proteins changed noticeably when growth temperature was varied within the normal range of 23 to 37 °C. However, growth in the high (>40°C) or low (<20°C) range was accompanied by large changes (25 fold or more) in the steady state level of a number of proteins. Proteins that are induced briefly by an increase in temperature are repressed by a decrease in temperature and vice versa (Neidhardt *et al.*, 1990).

More than 200 000 barrels of crude oil from the oil tanker *Exxon Valdez* was spilled in Prince William Sound, Alaska (Leahy and Colwell , 1990). This spill and other high latitude spills, including the *Braer* in the North Sea and the *Bahai Paraiso* in Antarctica, have emphasized the need to better understand the behaviour and effects of naturally weathered and chemically dispersed crude oil

on marine ecosystems in cold environments (Siron *et al.*, 1995). Microbial degradation is the ultimate fate of oil spilled at sea and only a few studies have demonstrated the microbial degradation of hydrocarbons at low temperatures (Atlas and Bartha, 1972; Atlas *et al.*, 1978).

1.4 Research Objectives

Oil spilled in the marine environment is subject to several weathering processes which can alter the chemical composition of the oil, resulting in a new hydrocarbon mixture with different properties (including toxicity) than the original oil (Atlas *et al.*, 1981). The important physical-chemical weathering processes include emulsification, dispersion, evaporation and photooxidation, and the key biological weathering process is microbial degradation (Atlas *et al.*, 1981).

Heterocyclic compounds, in particular dibenzothiophenes, can persist for up to three years after an oil spill, when more susceptible compounds, such as PAH, have been biodegraded (Atlas *et al.*, 1981; Gundlach *et al.*, 1983). This suggests that these compounds are more resistant to physical, chemical and biological degradation processes than are other petroleum constituents. Consequently, the detection of DBT and its derivatives in flat oysters (*Ostrea edulis)* (Friocourt *et al.*, 1982) and mussels (*Mytilus edulis*) (Kira *et al.*, 1983) have been shown to be possible means of monitoring prolonged pollution by oil, such as crude oil or some fuel oils, which contain sulfur heterocycles.

The recalcitrant nature of contaminants may be the result of irreversible binding or desorption hysteresis in which contaminants are readily adsorbed by sediment but the bound contaminants resist desorption. Fu *et al.* (1994) reported that the resistant fraction can be desorbed only by water at a rate one to three orders of magnitude slower than previous predictions with lengthy dichloromethane extractions. They propose that desorption hysteresis could be the rate-limited step in many remediation processes including soil washing, soil venting, pump and treat, and bioremediation where the efficiency of the process relies on the accessibility of the contaminant.

The recent surge in activity to develop the Hibernia and other oil fields has raised public concern over imminent oil spills on the Grand Banks and along the Newfoundland coastline. In the event of oil contamination of coastal beaches, would the local bacterial communities contain the representative species required to degrade these recalcitrant compounds?

This question forms the basis for this research which has five main objectives: (1) to determine if sediments from local marine beaches contain bacteria which can degrade representative sulfur heterocycles; (2) to attempt to characterize the most prominent bacteria in the bacterial consortium; (3) to examine the ability of the bacteria to degrade these compounds not only at optimal temperatures for culture, but also at 4 °C, a temperature more typical of a northern environment; (4) to determine if the bacteria can also degrade a

nitrogen heteroaromatic and an oxygen heteroaromatic, which are also common constituents of crude oil;(5) to identify the products of degradation.

In an effort to expand current knowledge of the fate of minor petroleum constituents, this study investigates the microbial degradation of 3 sulfur-, 1 nitrogen-, and 1 oxygen-containing heterocyclic compound, represented by benzothiophene, 3-methylbenzothiophene, dibenzothiophene, carbazole, and dibenzofuran, respectively. The degradation of these compounds by bacterial communities enriched and isolated from sediments of 3 local marine beaches, at 25 °C and 4 °C, is considered.

Chapter II: Materials and Methods

2.1 Collection, Isolation, and Characterization of the Bacterial Communities

2.1.1 Source of the Cultures

Sediment samples were obtained from three marine beaches in Newfoundland (Figure 2.1). In Come by Chance (CBC) the samples were collected north of the oil refinery filling dock, on the north side of the old dock. The air temperature during sampling was 4 °C and the water temperature was 6°C. Samples consisted of a sand and small gravel mixture and were collected as the waves receded. A close examination of the sediment revealed tiny "tar balls" interspersed in the sediment and larger oil spots on surrounding cobbles.

In Bonne Bay (BB) the samples were collected adjacent to the ferry dock and to the right of the remaining foundation of an old building in Norris Point. The air temperature was 3 °C and the water temperature was 5 °C. A similar sand and small gravel mixture was obtained as above. No trace of hydrocarbon contamination in the form of tar balls or odour was present in the sample.

The samples from Port aux Basques (PAB) were collected on the eastern end of the Marine Atlantic parking lot in an area adjacent to the old railyard which had been contaminated heavily with creosote and petrochemical products. The air temperature at sampling was 7 °C and the water temperature was 6 °C. The sediment composition was similar to the previous two sites but it had a characteristic creosote odour.

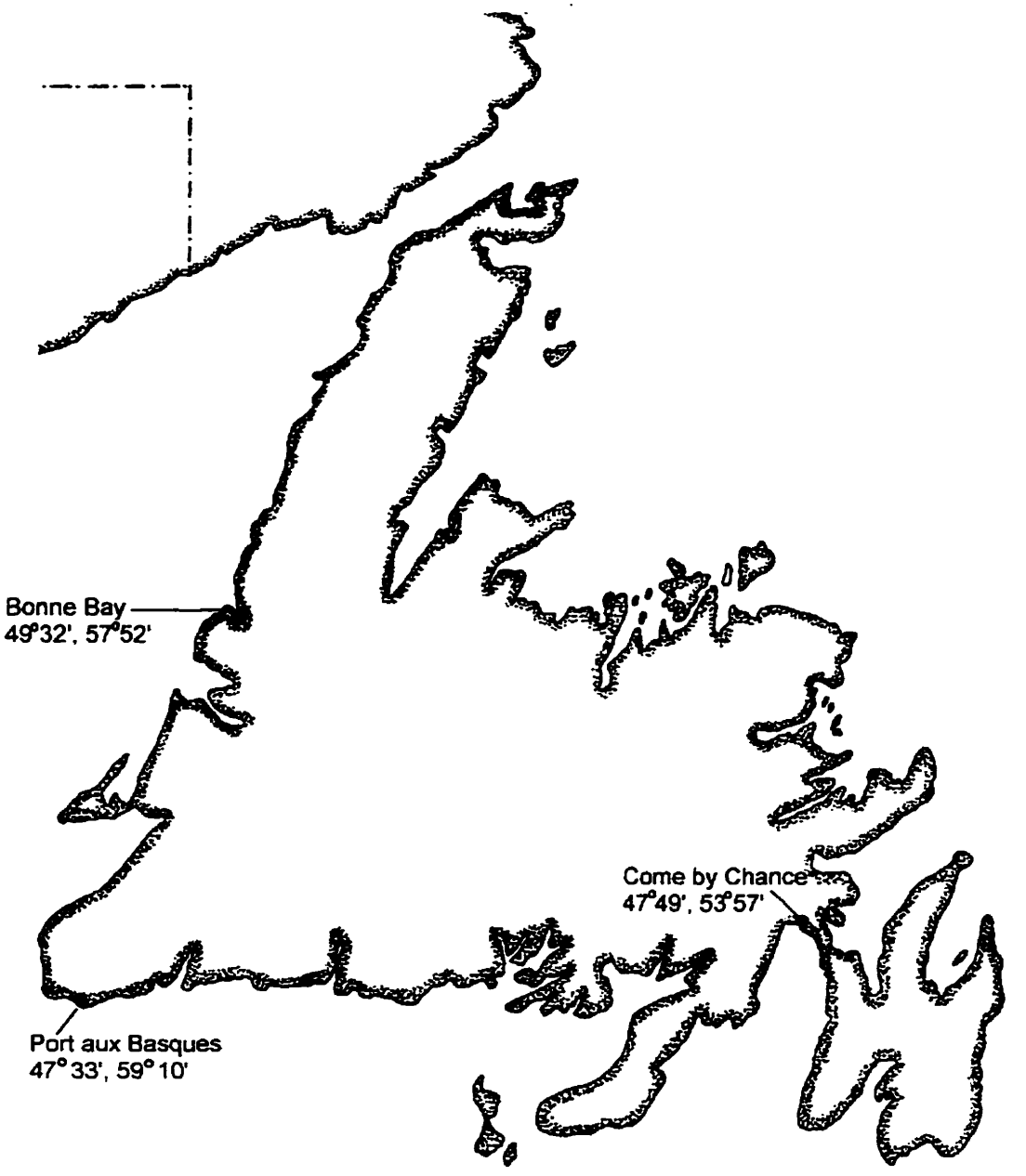


Figure 2.1 Location of the sampling sites

All samples were collected by skimming the top 5 - 10 cm of the sediment with a sterile flip lock sampling bag (Systems Plus, New Hamburg, ON). Three samples were collected at each site and immediately placed in a cooler for transportation to the lab.

2.1.2 Enrichment and maintenance of the 1-methylnaphthalene-utilizing bacteria

In the lab, 5 g of sediment was added to each of two 500 mL screw capped flasks containing 200 mL of a marine mineral salts medium (two flasks per sample location). The mineral salts medium followed the composition of Fedorak and Grbić-Galić (1991) except that a 75:25 natural seawater/ deionized water solution was used. Fifty microlitres of 1-methylnaphthalene (1-MN) was added and represented the sole carbon source and growth substrate.

All flasks were then placed in a rotary shaker bath (200 rpm) at 25 °C, and with a 24 hour light source. The caps of the flasks were closed tightly to prevent volatilization of the 1-MN but, as with Fedorak and Grbić-Galić (1991), the lids were loosened daily for 1 minute to replenish oxygen in the headspace above the culture medium. The cultures were incubated for 14 days but turbidity and a colour change of the media from colourless to beige/ brown were observed by day 2. The cultures were maintained by bi-weekly transfers of 10 mL of turbid culture to 200 mL of fresh mineral salts medium containing 50 µL of 1-MN.

After several transfers, 1 mL of each of the cultures was used for a serial dilutions test. The dilutions were plated on Colwell's agar plates (Colwell and Wiebe, 1970) and incubations showed that one kind of colony morphology remained at the highest dilution. A single colony was streaked onto fresh Colwell's medium to ensure a pure colony for each sample location and these were then used for identification purposes.

2.1.3 Tests employed in the characterization of the bacteria

The isolates from each location were subjected to a plethora of biochemical tests employing various media. Unfortunately, these media supply many nutrients and because the isolated bacteria are acclimated to nutrient poor conditions they failed to grow and yield results. A 24 hour culture grown on Colwell's medium, was used to determine the Gram stain reaction, size, shape and motility of the cells, oxidase and catalase reaction, oxidation or fermentation of carbohydrates and growth at 0 mM and 300 mM NaCl. A 24 hour culture was also used to inoculate a Biolog GN™(Gram-negative) plate (Biolog Inc.).

The Gram reaction and cell morphology were determined using light microscopy, while phase contrast microscopy was used to determine motility at room temperature. The method of Smibert and Krieg (1994) was used for the oxidase and catalase (method 2) tests. The oxidation or fermentation of carbohydrates test was performed using the modified medium of Lemos *et al.*

(1985) for marine bacteria. The test for growth at 0 mM and 300 mM NaCl allows the detection of a sodium requirement for the respective cultures. Growth in 300 mM NaCl but none in 0 mM NaCl suggests sodium is required for growth (Noble and Gow, 1993). Optical density measurements of the sample against a reference standard at 600 nm, using a Spectronic 601 (Milton Roy Co., USA) spectrophotometer, was used to determine growth.

Before suspending in salts solution for inoculation of the Biolog plates, the 24 hour cultures were subcultured twice on Colwell's media to ensure metabolic viability. The salts solution was prepared by dispensing 20 mL of the solution into disposable 20 x 125 mm glass culture tubes which were capped and autoclaved for sterilization.

Before preparing the inoculum, a tube of uninoculated sterile suspending salts medium was placed in the Biolog turbidimeter (590 nm) and adjusted to 100% transmittance. Cell suspensions were obtained by dipping a sterile swab in the sterile salts solution and then rolling it over the colonies, gently to prevent carry over of nutrients from the medium, and transferring the cells to the salts solution by spinning the swab and pressing it against the inside of the tube to break up any clumps of culture. Uniform cell suspensions were achieved by using a vertical stirring motion which was repeated until a transmittance of 53 to 59% was obtained.

The cell suspension was then transferred to a sterile multi-channel pipet

reservoir (Biolog Inc.) while an 8-channel repeating pipettor (Biolog Inc.) fitted with sterile disposable tips (Biolog Inc.) was used to fill the wells of the Biolog GN MicroPlate with 150 µL of inoculum. The slow growing nature of the cultures, necessitated incubation at 25 °C for 24 hours to ensure adequate growth for reading. The GN MicroPlates were read at 24, 48 and 72 hours using a Biolog MicroPlate reader in conjunction with MicroLog Release 3.70 computer software (Biolog Inc.). The lid of the MicroPlate was removed before placing in the drawer of the reader which then, using the "automated variable threshold" option at a set wavelength of 590 nm, was used to obtain a printout of the "breathprint", or record of the carbon sources used by each isolate, and an identification of the culture.

2.2 Bacterial Degradation of the Heterocycles

2.2.1 Degradation Experiments

In all experiments, six 500 mL screw-capped flasks containing 200 mL of sterilized mineral salts medium were spiked with 40 µL of 1-MN (1.4 µM) and 10 mg or 10 µL (3-MBT) of the heterocyclic compound being investigated (approx. 0.3 µM). A 10 mL suspension of the enrichment culture was added to each of two flasks, one to be incubated at 25 °C and the other at 4 °C. Four 125 mL screw-capped flasks containing 50 mL of the same MSM were used as uninoculated controls. To determine any extent of photodegradation of the

substrates, 10 µL of 1-MN and 2.5 mg or 2.5 µL (3-MBT) of the appropriate heterocyclic compound was added to 2 of the flasks. The other 2 flasks were incubated with no further additions and were used to determine if other carbon sources were available in the MSM. One of each of these flasks was incubated at 25 °C and the other at 4 °C. All flasks were then placed in rotary shakers at 200 rpm, provided with a 24-hour light source, and incubated at the appropriate temperature.

Benzothiophene, dibenzothiophene, dibenzofuran, and 1-methyl-naphthalene were obtained from Aldrich Chemical Co. (Milwaukee, WI), the 3-methylbenzothiophene was purchased from Lancaster Synthesis Ltd. (Eastgate, Whitelund, Morecambe, England), and BDH (Toronto, ON) was the source of the carbazole used in this study. All compounds were used without further purification.

2.2.2 Analytical Methods

In order to determine the extent of microbial degradation of 3-MBT and DBT, a 25 mL aliquot was sampled from each flask every second day from day 0 to day 14 of the experiment. These samples were then reduced to pH < 2 with concentrated HCl (Fisher, Nepean, ON) and supplemented with 2.5 µL of 3,3'-dimethylbiphenyl (DMBP)(Aldrich) which served as an internal standard. The samples were extracted 3 times with 15 mL of dichloromethane (DCM)(Aldrich)

to recover the substrates and products and were then dried over anhydrous Na_2SO_4 (Sigma, St. Louis, MO). They were then filtered through Whatman No. 2 filters (Fisher) into 100 mL round bottom flasks and evaporated to dryness using a Büchi rotoevaporator (Büchi, Switzerland). The samples were then redissolved in 2.5 mL acetonitrile (Fisher) and placed in 4 mL amber screw-capped vials (Supelco, Mississauga, ON) before analyzing by GC-MS.

To provide more sampling days for experiments with BT, CARB, and DBF, a 20 mL, rather than 25 mL aliquot was sampled from each flask every second day. Samples were then supplemented with 2.0 µL of DMBP and extracted once with 15 mL and twice with 10 mL of DCM, evaporated, and redissolved in 2.0 mL of acetonitrile. All solvents used were at least HPLC grade.

To determine the amount of microbial degradation, the extracts obtained from every second day were analyzed by GC-MS. This was performed using a 0.25 mm x 25 m CP-Sil 5CB column (Chrompack, Netherlands) housed in a Hewlett-Packard 5890A gas chromatograph coupled to a Hewlett-Packard 5970 mass selective detector. Spectra were processed using a Hewlett-Packard ChemStation software (HPG1034C) package. One microlitre of the extracts was manually injected into the column and the following temperature program was used. An initial oven temperature of 60 °C was maintained for 3 minutes then elevated at a rate of 4 °C/min to 180 °C and held for 1 minute before raising at 24°C/min to 250 °C and holding for 5 minutes, resulting in a total run time of

approximately 40 minutes.

In order to detect any non-volatile products it was necessary to derivatize the samples. Silylation with BSTFA+TMCS 99:1 (Bis(trimethylsilyl)trifluoro-acetamide+trimethylchlorosilane)(Supelco) or alternatively, acetylation with acetic anhydride and pyridine were used.

The relative abundance of the substrate peak was divided by that of the internal standard (DMBP) for each sample on each day. The highest value corresponded to 100% of the compound remaining. As the value decreased throughout the experiment, it was divided by the value at 100% to determine the amount of compound remaining (%) in each flask for the 3 cultures at both temperatures. This method assumes that a similar recovery of the internal standard and the various degradation products was obtained during extraction for all samples. The variability in the response of the mass selective detector was minimized by analyzing the samples from one culture, as one batch. The level of variability expected from the manual injection of the GC-MS was assessed by injecting a sample, in triplicate. The measured coefficient of variation (S.D. / mean x 100%) based on these samples was determined to be 5.33%.

To obtain more information on the mixture, without being limited by volatility, infrared spectroscopy was employed using a Perkin Elmer 1720 Fourier Transform Infrared (FTIR) Spectrometer and a Perkin Elmer IRDM 1700

software package. The samples which had been prepared for GC-MS analysis were evaporated under nitrogen gas and redissolved in DCM for FTIR analysis. The samples were placed in a 40μL NaCl cell (Aldrich) and 16 scans were acquired and fourier transformed.

Errors occur during sampling and analysis of the compounds being investigated and included the binding of the compound to glassware, evaporation / sublimation of the compound, and inaccurate volumes used during sampling, extraction, and injection into the GC-MS. An attempt to measure the level of variability of the sampling process involved taking samples, in triplicate, from the maintenance culture, extracting, and analyzing by GC-MS. The coefficient of variation was determined to be 7.65%.

2.2.3 Measurements of Bacterial Growth

In conjunction with the recording of substrate degradation, the growth of the bacterial cultures was also monitored throughout each experiment. When the flasks were being set up for degradation experiments, duplicate flasks were incubated and received the same experimental conditions as did the experimental flasks except that no samples were removed for analytical purposes. Instead, when the media appeared quite turbid from bacterial growth and/ or when a very noticeable colour change in the medium occurred, the flasks were removed for growth measurement purposes.

The 200 mL of culture was poured into a 250 mL polycarbonate centrifuge bottle and centrifuged at 12 000 x g using a Sorvall RC-5 centrifuge. The supernatant was discarded and the pellet was washed with 200 mL of a sterile 0.15 M NaCl solution. This was repeated 3 times and then the pellet was suspended in 2 mL of sterile 0.015 M NaCl. Using this thick cell suspension, 200 µL was transferred to each of 3 pre-weighed crucibles which were then dried at 105 °C for 24 hours and cooled in a desiccator over P_2O_5 and weighed every 24 hours until constant weight was achieved. Weight measurements were performed on a Sartorius B120S analytical balance (Sartorius-Werke, Germany).

A 100 µL sample of the same suspension was added to a test tube containing 9.9 mL of a sterile 0.15 M NaCl solution. This solution was then mixed and 5 mL was added to another test tube containing 5 mL of sterile 0.15 M NaCl. This was repeated for 5 more tubes which provided a series of doubling dilutions. Using the methods of Lawrence and Maier (1977) the optical density at 600 nm was determined with a Shimadzu UV-Visible recording spectrophotometer (Shimadzu UV-260, Columbia, MD) and this was used to determine the true optical density of the suspension.

Finally, 1 mL of the thick cell suspension was added to a tube containing 9 mL of a sterile 0.15 M NaCl solution. This was mixed and 1 mL was transferred to another 9 mL solution, mixed and 1mL added to another. This was repeated until a dilution of 10^{-8} was obtained. The tubes containing the

dilutions 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were then used to inoculate 5 plates each of Colwell's medium. A bent glass rod was used to spread 200 µL of the culture on each of the plates which were incubated at 25 °C until sufficient colony growth occurred. Plates containing 30 to 300 colonies were counted and recorded.

The information obtained from the above methods was used to create standard curves relating uncorrected OD to true OD, true OD to the number of colony forming units per mL of solution, and true OD to the dry weight (µg) of cells per mL of solution. To determine the growth of the cultures throughout the experiment, a 2 mL sample was collected from each flask each day, centrifuged at 12 000 rpm (Brinkmann Model 5414, Rexdale, ON) for 5 minutes and washed 3 times in a sterile 0.15 M NaCl solution. The pellet was washed finally in 2 mL of sterile 0.015 M NaCl and the OD at 600 nm was measured on a Shimadzu UV-260 spectrophotometer. The OD from the spectrophotometer was matched with the standard curve obtained from the above data to obtain the true OD. This value was then matched with the other standard curves to determine the dry weight (µg) of cells per mL and the number of colony forming units (CFU) per mL of solution each day. Initially, three separate measurements were obtained at 5 minute intervals, and also on three consecutive days to determine the error associated with the measurements of bacterial growth. The measured coefficient of variation was found to be 6.59%.

Chapter III: Results and Discussion

3.1 Characterization of the Bacteria

3.1.1 General Characteristics

Various kinds of bacteria co-exist in the natural environment, yet most of the present knowledge regarding the properties of bacteria comes from studies based on pure cultures (Holt and Krieg, 1994). Consequently, enrichment techniques are required to isolate pure cultures from a mixed-culture source. In this study a chemical method of enrichment was employed as 1-methyl-naphthalene was used to provide a nutrient source to be used preferentially by certain members of the mixed culture. These bacteria were then isolated through a serial dilutions test and through spatially separating the organisms on a solid medium which yielded growth of separate colonies.

According to Smibert and Krieg (1994) certain morphological characteristics should be determined when identifying bacteria to help ascertain the group to which the isolate belongs. All three isolates were determined to be Gram negative, motile rods which is consistent with the morphology of several genera of known PAH (Bayley and Barbour, 1984) and PASH (Fedorak, 1990) degrading bacteria. The bacteria from the Bonne Bay sample were Gram negative, rod-shaped cells with the dimensions 1.76 μm x 0.44 μm (Figure 3.1a). Gram negative, rod-shaped cells (1.79 μm x 0.45 μm) were characteristic of the bacteria from Come by Chance (Figure 3.1b). Gram negative, rod-shaped cells

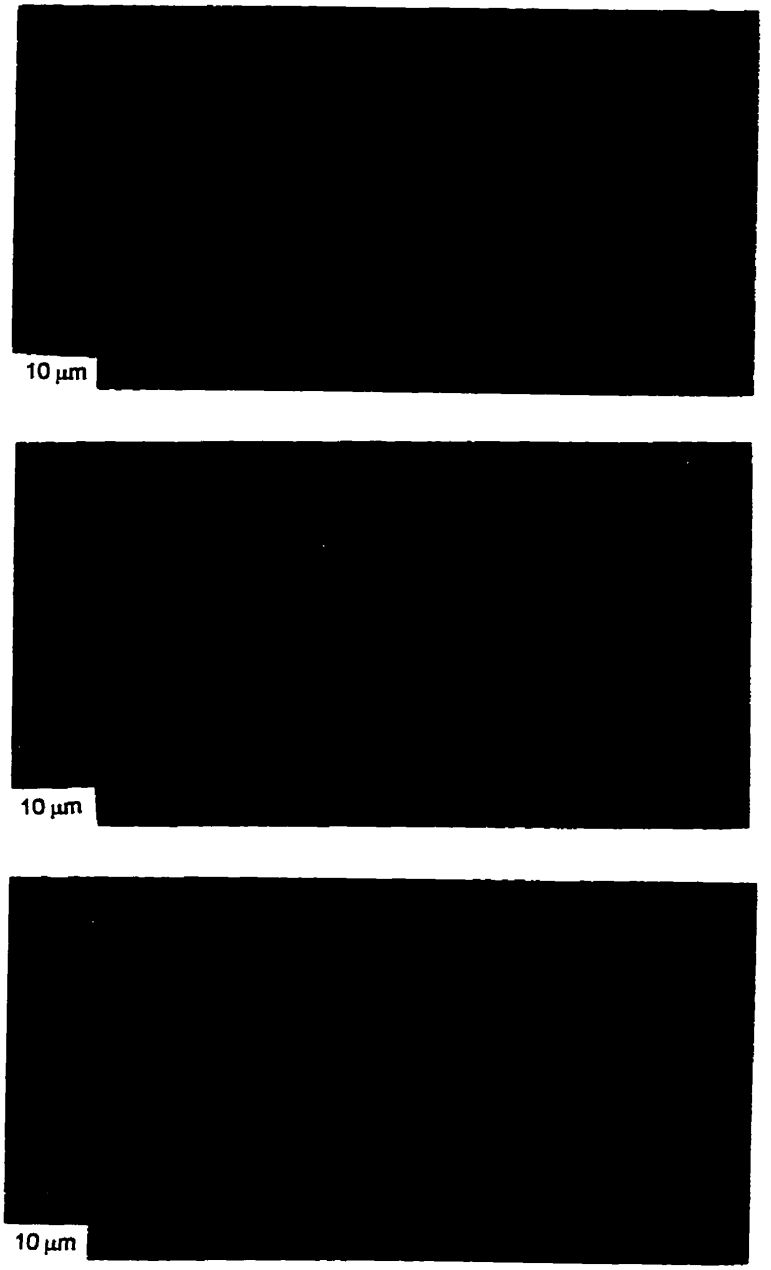


Figure 3.1 Micrographs (1000x) illustrating cell morphology and Gram reaction of the most prominent bacteria from each culture: a) Bonne Bay, b) Come by Chance, c) Port aux Basques

which were 1.84 μm x 0.46 μm in size were found for the Port aux Basques sample (Figure 3.1c). Results from these and other tests used in this study are presented below (Table 3.1).

Table 3.1 Characterization of the representative bacterial samples using classical microbiological techniques

TEST	Bonne Bay	Come by Chance	Port aux Basques
Gram Reaction	Gram Negative	Gram Negative	Gram Negative
Cell Shape	Rod	Rod	Rod
Cell Size	1.76 μm x 0.44 μm	1.79 μm x 0.45 μm	1.84 μm x 0.46 μm
Motility	Motile	Motile	Motile
Oxidase	Positive	Positive	Positive
Catalase	Positive	Positive	Positive
O-F Test	Oxidative	Oxidative	Oxidative
Na^+ Requirement	Required for growth	Required for growth	Na^+ enhanced growth

Physiological tests are of primary importance (Smibert and Krieg, 1994) and the results may help to further narrow the field of possibilities. The Bonne Bay and Come by Chance isolates were found to be oxidase positive, catalase positive and they oxidized sugars in the O-F test. Results from the sodium requirement test show that the Bonne Bay and Come by Chance isolates require sodium in the medium in order for growth to occur (Table 3.2). The isolate from the Port aux Basques culture was also oxidase positive and catalase positive and it also oxidized sugars. This isolate may not have a sodium requirement like

the other two but its growth was greatly enhanced by the presence of sodium in the medium. The results of the sodium requirement test therefore suggest that these isolates are all marine bacteria (Farmer and Hickman-Brenner, 1991). Matching the results of these tests with possible results of known genera (Holt and Krieg, 1984) indicated that the isolates could belong to the genera *Pseudomonas, Zooglea, Azotobacter, or Azomonas.*

Table 3.2 Optical density measurements at 600 nm of three 72 hour cultures of the representative bacterial samples in growth medium containing either 0 mM or 300 mM of NaCl.

	0 mM NaCl			300 mM NaCl		
SOURCE	Tube 1	Tube 2	Tube 3	Tube 1	Tube 2	Tube 3
Bonne Bay	0.020	0.042	0.017	0.151	0.186	0.160
Come by Chance	0.019	0.023	0.017	0.169	0.172	0.198
Port aux Basques	0.082	0.093	0.122	0.273	0.268	0.264

3.1.2 Biolog results

In order to further characterize and hopefully identify the isolates, the Biolog MicroStation System was employed. This system has been used in several studies investigating the bioremediation of environmental contaminants. LaJoie *et al.* (1992) used Biolog GN MicroPlates to identify isolates from soils

that had been exposed to detergents or hydrocarbons. Fredrickson *et al.* (1991) examined variations in the diversity and distribution of microbial populations of a subsurface geologic formation by determining the types of organic substrates commonly metabolized by bacteria. They used Biolog GN MicroPlates to obtain a metabolic profile for individual isolates. Garland and Mills (1991) incubated whole environmental samples to characterize and classify heterotrophic microbial communities using the Biolog system. These studies show that the Biolog system can be used for more than just identifying a particular strain and that the information obtained from an isolate or a microbial community is itself very valuable.

In this study the Biolog system did not give identifications for any of the three bacterial species based on the substrate utilization profiles currently in its data base. However, examination of the MicroPlate breathprint for each of the cultures revealed the substrates that were utilized by each one at 24, 48 and 96 hours. The substrate utilization profile for the Bonne Bay culture showed that it was utilizing 47 of the 95 substrates after 96 hours (Table 3.3). A closer look at these substrates revealed that this culture was utilizing all classes of substrates except the amines and phosphorylated chemicals. This culture was also utilizing all 4 of the aromatics supplied on the MicroPlate; urocanic acid, inosine, uridine, and thymidine.

Table 3.3 Representative bacterial responses to the 95 Biolog GN substrates after 96 hours

Biolog GN Substrate	Bonne Bay	Come by Chance	Port aux Basques
Polymers			
A2 (α-cyclodextrin)	-	-	-
A3 (dextrin)	{+}	-	+
A4 (glycogen)	-	-	-
A5 (tween 40)	+	-	+
A6 (tween 80)	+	-	+
Carbohydrates			
A7 (N-acetyl-D-galactosamine)	-	-	-
A8 (N-acetyl-D-glucosamine)	-	-	-
A9 (adonitol)	{+}	+	{+}
A10 (L-arabinose)	+	+	+
A11 (D-arabitol)	+	+	+
A12 (cellobiose)	{+}	+	{+}
B1 (i-erythritol)	-	-	-
B2 (D-fructose)	+	+	+
B3 (L-fucose)	-	-	-
B4 (D-galactose)	+	+	+
B5 (gentiobiose)	-	-	-
B6 (α-D-glucose)	+	+	+
B7 (m-inositol)	+	+	{+}
B8 (α-D-lactose)	-	-	-
B9 (lactulose)	-	-	-
B10 (maltose)	-	-	-
B11 (D-mannitol)	+	+	+
B12 (D-mannose)	+	+	+
C1 (D-melibiose)	-	-	{+}
C2 (β-methyl-D-glucoside)	-	-	-
C3 (D-psicose)	-	-	-
C4 (D-raffinose)	-	-	-
C5 (L-rhamnose)	-	-	-
C6 (D-sorbitol)	+	+	+
C7 (sucrose)	-	-	-
C8 (D-trehalose)	-	-	-
C9 (turanose)	{+}	-	-
C10 (xylitol)	{+}	+	+
Methyl esters			
C11 (methyl pyruvate)	+	+	+
C12 (monomethylsuccinate)	+	+	+
Carboxylic acids			
D1 (acetic acid)	+	+	+
D2 (cis-aconitic acid)	+	+	+
D3 (citric acid)	+	+	+
D4 (formic acid)	-	-	-
D5 (D-galactonic acid lactone)	-	-	-
D6 (D-galacturonic acid)	-	-	-
D7 (D-gluconic acid)	-	+	{+}
D8 (D-glucosaminic acid)	-	-	-
D9 (D-glucuronic acid)	{+}	-	+
D10 (α-hydroxybutyric acid)	+	+	+
D11 (β-hydroxybutyric acid)	+	+	+
D12 (γ-hydroxybutyric acid)	{+}	+	+
E1 (p-hydroxyphenylacetic acid)	-	-	-
E2 (itaconic acid)	-	-	-
E3 (α-ketobutyric acid)	-	-	{+}
E4 (α-ketoglutaric acid)	+	+	+
E5 (α-ketovaleric acid)	-	-	-
E6 (D,L-lactic acid)	+	+	+
E7 (malonic acid)	+	+	+
E8 (propionic acid)	+	+	+
E9 (quinic acid)	+	+	+
E10 (D-saccharic acid)	+	+	+
E11 (sebacic acid)	-	-	-
E12 (succinic acid)	+	+	+
Brominated chemicals			
F1 (bromosuccinic acid)	+	+	+
Amines			
F2 (succinamic acid)	+	+	+
F3 (glucuronamide)	-	-	-
F4 (alaninamide)	+	+	+
Amino acids and derivatives			
F5 (D-alanine)	-	-	+
F6 (L-alanine)	-	+	+
F7 (L-alanylglycine)	-	+	-
F8 (L-asparagine)	+	+	+
F9 (L-aspartic acid)	+	+	+
F10 (L-glutamic acid)	+	+	+
F11 (glycyl-L-aspartic acid)	-	-	-
F12 (glycyl-L-glutamic acid)	-	+	+
G1 (L-histidine)	-	-	+
G2 (hydroxy-L-proline)	{+}	+	+
G3 (L-leucine)	-	-	-
G4 (L-ornithine)	-	-	-
G5 (L-phenylalanine)	-	-	-
G6 (L-proline)	+	+	+
G7 (L-pyroglutamic acid)	+	+	{+}
G8 (D-serine)	-	-	-
G9 (L-serine)	-	-	+
G10 (L-threonine)	+	-	+
G11 (D,L-carnitine)	-	-	-
G12 (γ-aminobutyric acid)	-	-	+
Aromatic chemicals			
H1 (urocanic acid)	+	+	{+}
H2 (inosine)	+	-	-
H3 (uridine)	+	-	-
H4 (thymidine)	{+}	+	{+}
Amines			
H5 (phenylethylamine)	-	-	-
H6 (putrescine)	-	-	-
H7 (2-aminoethanol)	-	-	-
Alcohols			
H8 (2,3-butanediol)	-	-	-
H9 (glycerol)	+	+	+
Phosphorylated chemicals			
H10 (D,L-α-glycerolphosphate)	-	-	-
H11 (glucose-1-phosphate)	-	-	-
H12 (glucose-6-phosphate)	-	-	-

\+ Positive
{+} Weakly Positive
\- Negative

The substrate utilization profile of the Come by Chance culture showed that it utilized 43 of the 95 available substrates from all classes of compounds except the amines, phosphorylated chemicals and polymers. This culture was only able to use urocanic acid and thymidine as growth substrates from the four available aromatic chemicals.

The culture from the Port aux Basques sample was the most versatile, utilizing 53 of the 95 substrates on the GN MicroPlate. As with the other two cultures it was unable to grow with amines and phosphorylated chemicals and, similar to the Come by Chance culture, it was unable to use inosine or uridine, both aromatic chemicals, as growth substrates.

Several alterations to the Biolog protocol (Klausner, 1988; D'Amato *et al.*, 1991; Miller and Rhoden, 1991; Klinger *et al.*, 1992) may account for why the Biolog system did not match the breathprints of the three isolates with information currently in its database. The recommended growth media is Biolog Universal Growth Medium with 5% sheep blood, Tryptic Soy Agar with 5% sheep blood, or RZA Agar for oligotrophic environmental species, and 30 °C is the recommended incubation temperature. In this study, Colwell's marine medium was used instead of those recommended because they would not support growth. Also an incubation temperature of 25 °C replaced 30 °C, a temperature that prevented growth of the bacteria studied. Furthermore, because of the slow-growing nature of these isolates, readings were taken at 24, 48 and 96

hours rather than at 4 and 24 hours. These differences may have prevented an acceptable identification from being obtained.

This system may not always result in an acceptable identification but understanding the metabolic capabilities of unknown strains yields information not previously available. Such information is still very useful as it may lead to the characterization and subsequent description of new species (Noble, 1995). Furthermore, the substrate utilization profiles obtained for the three isolates provide useful information which may be used in future investigations. If an investigator were to create a personal database of breathprints of reference strains, using media and methods more suitable for environmental bacteria, then it may be possible to identify isolates such as these in the future. This would contribute greatly to our knowledge of the metabolic profiles of environmental bacteria and could make studies between regions more meaningful than they are now (Noble, 1995).

3.2 Bacterial Degradation of the Heterocycles

3.2.1 Benzothiophene

Benzothiophene degradation and bacterial growth of each culture was recorded at 2 incubation temperatures (Figures 3.2, 3.3 and 3.4). Bacterial growth was recorded as μg of dry weight of cells/mL and # of colony forming units/mL. These measurements were determined from the optical density (OD_{600nm}) values and consequently both show the same trend when graphically illustrated. Therefore, only the values for dry weight of cells (μg/mL) are graphically represented but all values obtained are included in Table 3.4.

At 25 °C, the Bonne Bay culture had begun to degrade BT by day 2 but at 4 °C this did not occur until day 8 (Figure 3.2). Only 11% of the original BT remained after 12 days in the culture incubated at 25 °C while at 4 °C, 68% remained after 14 days. The total ion chromatograms (TIC) of the Bonne Bay culture incubated at 25 °C for day 0 and day 12 (Figure 3.5) displays a peak for BT (8-9 minutes), 1-MN (12.3 minutes) and the internal standard, DMBP (21 minutes). The disappearance of 1-MN is evident by day 12, while BT was reduced in abundance from 100% at day 0 to 11% at day 12. No metabolites were detected after microbial degradation. Similar results were obtained for this culture at 4 °C and also for the other 2 cultures at both temperature regimes. The uninoculated controls showed that no other carbon sources were present in detectable levels in the media and no photodegradation products appeared

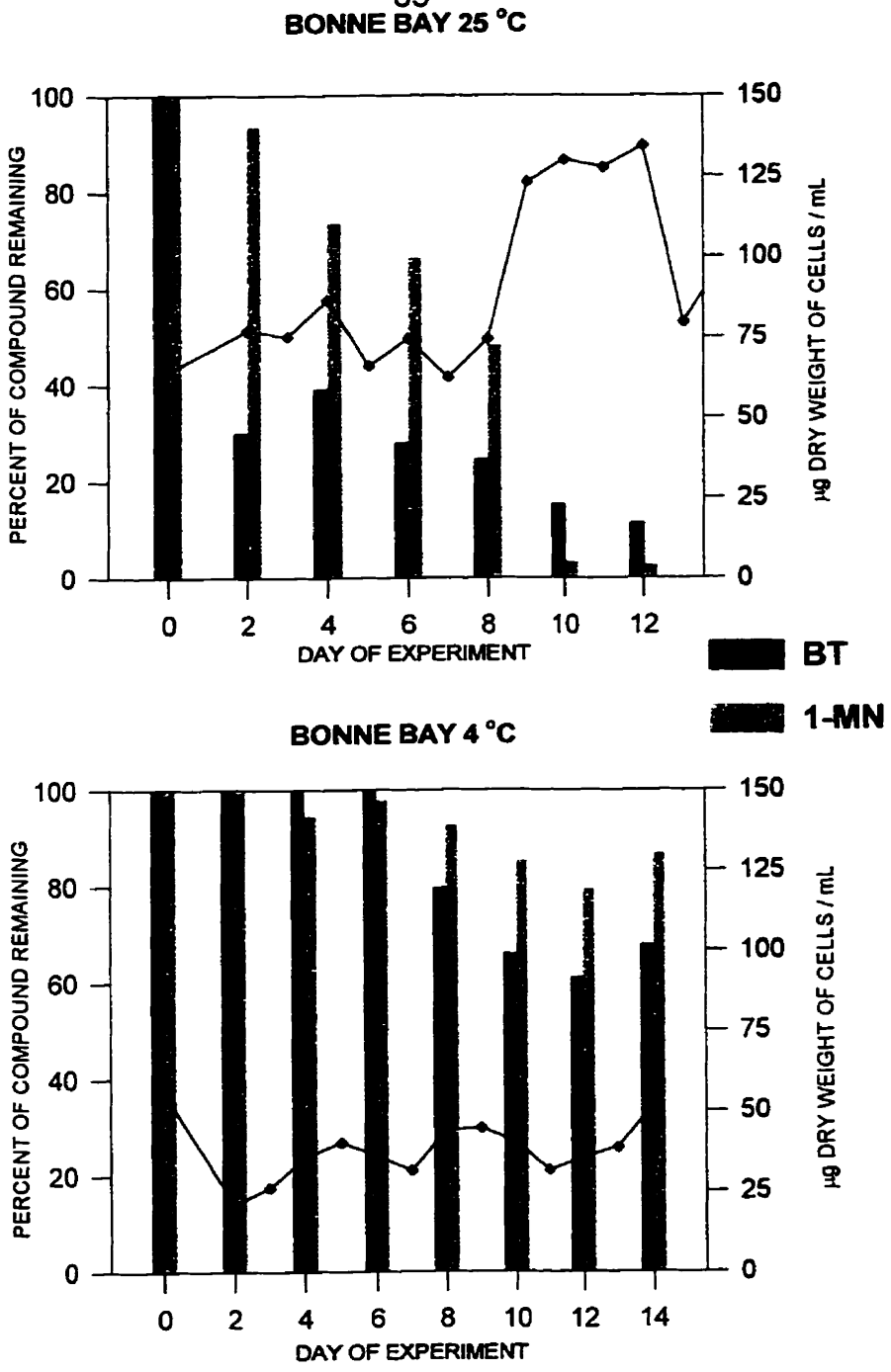


Figure 3.2 Time course of the degradation of 1-MN and BT and the resulting growth of the Bonne Bay bacterial consortium (µg dry weight of cells / mL; ± 8.66) incubated at 25 °C and 4 °C.

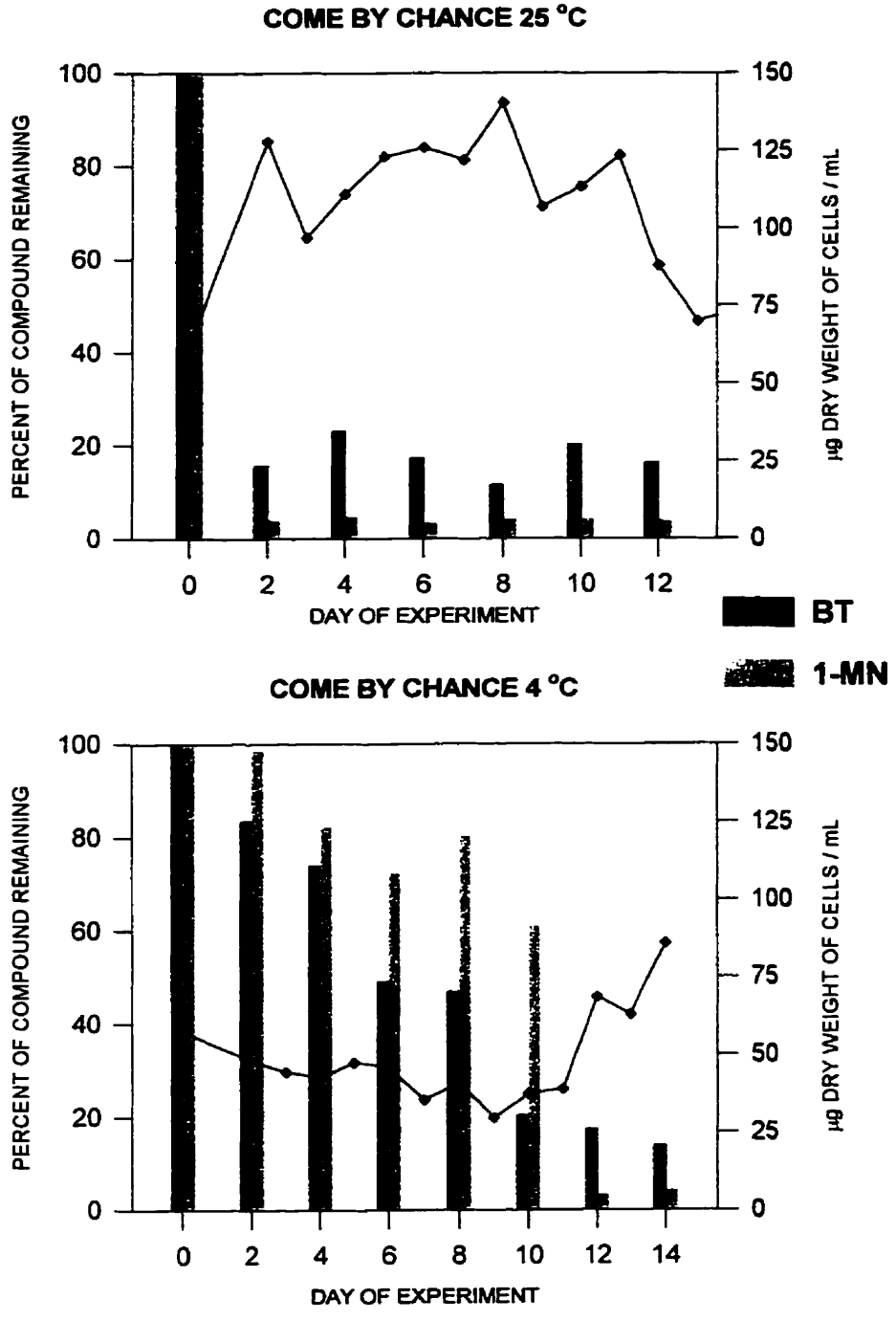


Figure 3.3 Time course of the degradation of 1-MN and BT and the resulting growth of the Come by Chance bacterial consortium (µg dry weight of cells / mL; ± 2.89) incubated at 25 °C and 4 °C.

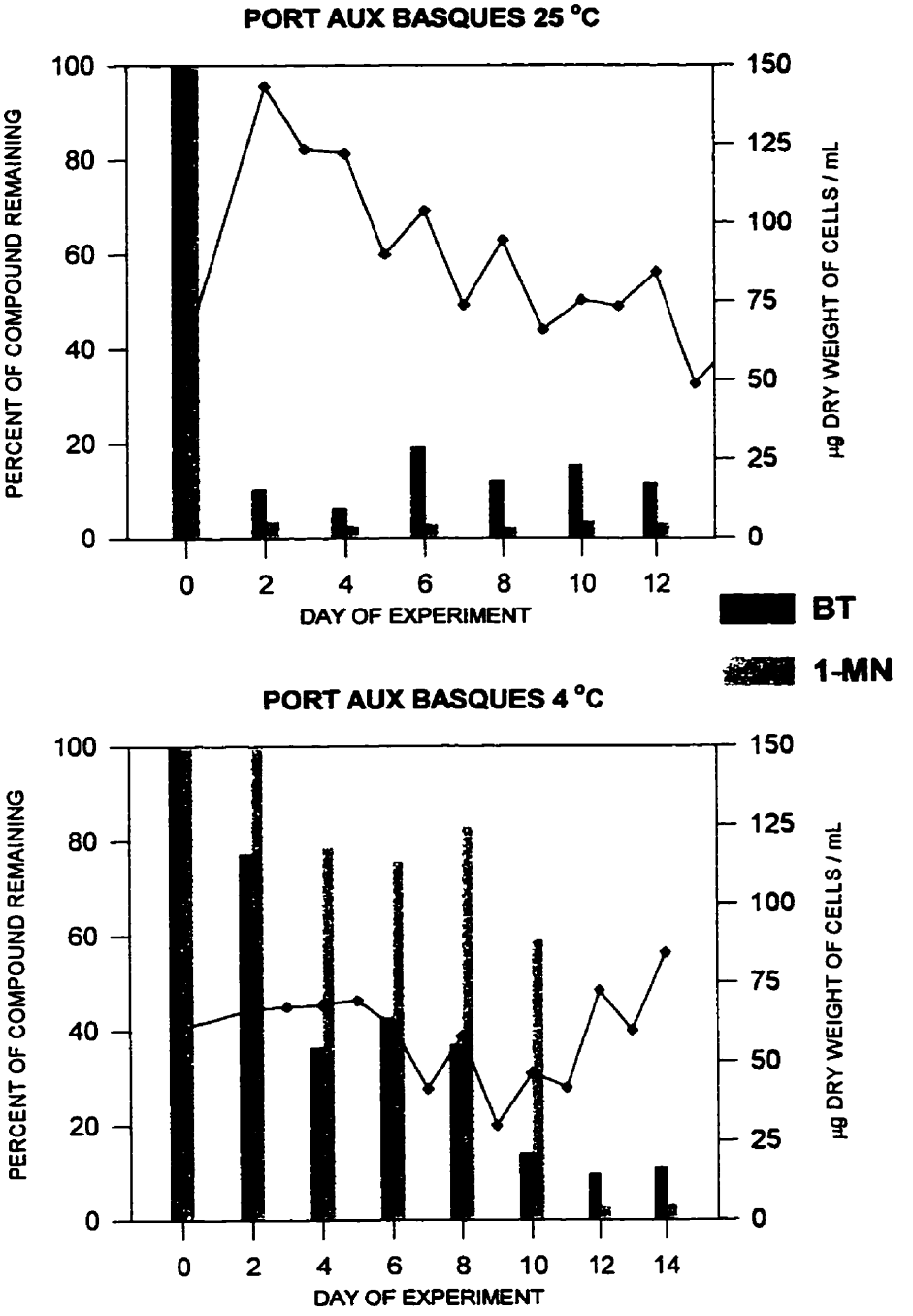


Figure 3.4 Time course of the degradation of 1-MN and BT and the resulting growth of the Port aux Basques bacterial consortium (µg dry weight of cells / mL; ± 2.89) incubated at 25 °C and 4 °C.

Table 3.4 Numerical characterization of the BT(%), 1-MN(%), dry weight (μg/mL), and colony forming units/mL (CFU/mL) levels throughout the experimental duration

	BONNE BAY 25 °C				BONNE BAY 4 °C			
DAY	BT	1-MN	DRY WT.	CFU / mL	BT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	64.000	1.4×10^8	100.000	100.000	56.000	1.22×10^8
1								
2	30.000	93.500	77.000	1.55×10^8	100.000	100.000	21.000	4.5×10^7
3			75.000	1.64×10^8			28.000	6.6×10^7
4	39.000	73.500	86.500	1.9×10^8	100.000	94.400	35.500	7.65×10^7
5			66.000	1.44×10^8			40.000	8.75×10^7
6	27.800	86.200	74.500	1.63×10^8	100.000	97.600	36.000	7.8×10^7
7			62.500	1.345×10^8			31.500	6.8×10^7
8	24.500	48.200	74.500	1.63×10^8	79.560	92.600	44.000	9.6×10^7
9			123.000	2.715×10^8			44.500	9.65×10^7
10	15.200	3.200	130.000	2.87×10^8	65.840	85.200	40.000	8.75×10^7
11			127.500	2.82×10^8			31.500	6.8×10^7
12	11.300	2.600	134.500	2.96×10^8	60.920	79.400	35.500	7.7×10^7
13			79.500	1.745×10^8			38.500	8.3×10^7
14			99.000	2.175×10^8	67.730	86.800	52.000	1.14×10^8
	COME BY CHANCE 25 °C				COME BY CHANCE 4 °C			
DAY	BT	1-MN	DRY WT.	CFU / mL	BT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	62.500	1.45×10^8	100.000	100.000	57.500	1.33×10^8
1								
2	15.600	3.940	128.000	2.98×10^8	83.500	98.560	48.000	1.12×10^8
3			97.000	2.27×10^8			44.500	1.035×10^8
4	23.000	4.620	111.000	2.58×10^8	73.900	82.300	42.500	9.8×10^7
5			123.000	2.88×10^8			47.500	1.11×10^8
6	17.300	3.350	126.000	2.94×10^8	49.000	72.310	46.000	1.06×10^8
7			122.000	2.85×10^8			35.500	8.3×10^7
8	11.400	3.980	140.500	3.28×10^8	46.800	80.150	41.000	9.5×10^7
9			107.000	2.48×10^8			29.500	6.8×10^7
10	20.100	4.150	113.500	2.65×10^8	20.300	60.990	37.500	8.7×10^7
11			123.500	2.88×10^8			39.000	9.05×10^7
12	16.300	3.780	88.000	2.06×10^8	17.300	3.380	68.500	1.59×10^8
13			70.000	1.63×10^8			63.000	1.465×10^8
14			73.500	1.71×10^8	13.700	4.230	86.000	2.0×10^8
	PORT AUX BASQUES 25 °C				PORT AUX BASQUES 4 °C			
DAY	BT	1-MN	DRY WT.	CFU / mL	BT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	60.500	1.08×10^8	100.000	100.000	60.500	1.08×10^8
1								
2	10.300	3.540	143.500	2.59×10^8	77.160	100.000	66.500	1.18×10^8
3			123.500	2.22×10^8			67.500	1.2×10^8
4	6.300	2.500	122.000	2.19×10^8	36.270	78.700	68.000	1.21×10^8
5			90.000	1.61×10^8			69.500	1.24×10^8
6	19.000	2.760	104.000	1.86×10^8	42.510	75.560	61.500	1.09×10^8
7			74.000	1.32×10^8			41.500	7.3×10^7
8	11.900	2.240	94.500	1.69×10^8	36.730	83.110	58.000	1.03×10^8
9			66.000	1.17×10^8			30.000	5.3×10^7
10	15.300	3.450	75.500	1.35×10^8	13.940	59.080	46.500	8.2×10^7
11			73.500	1.31×10^8			42.000	7.4×10^7
12	11.400	3.020	84.500	1.51×10^8	9.630	2.660	72.500	1.29×10^8
13			49.000	8.7×10^7			60.000	1.07×10^8
14			62.000	1.1×10^8	11.030	3.120	84.500	1.51×10^8

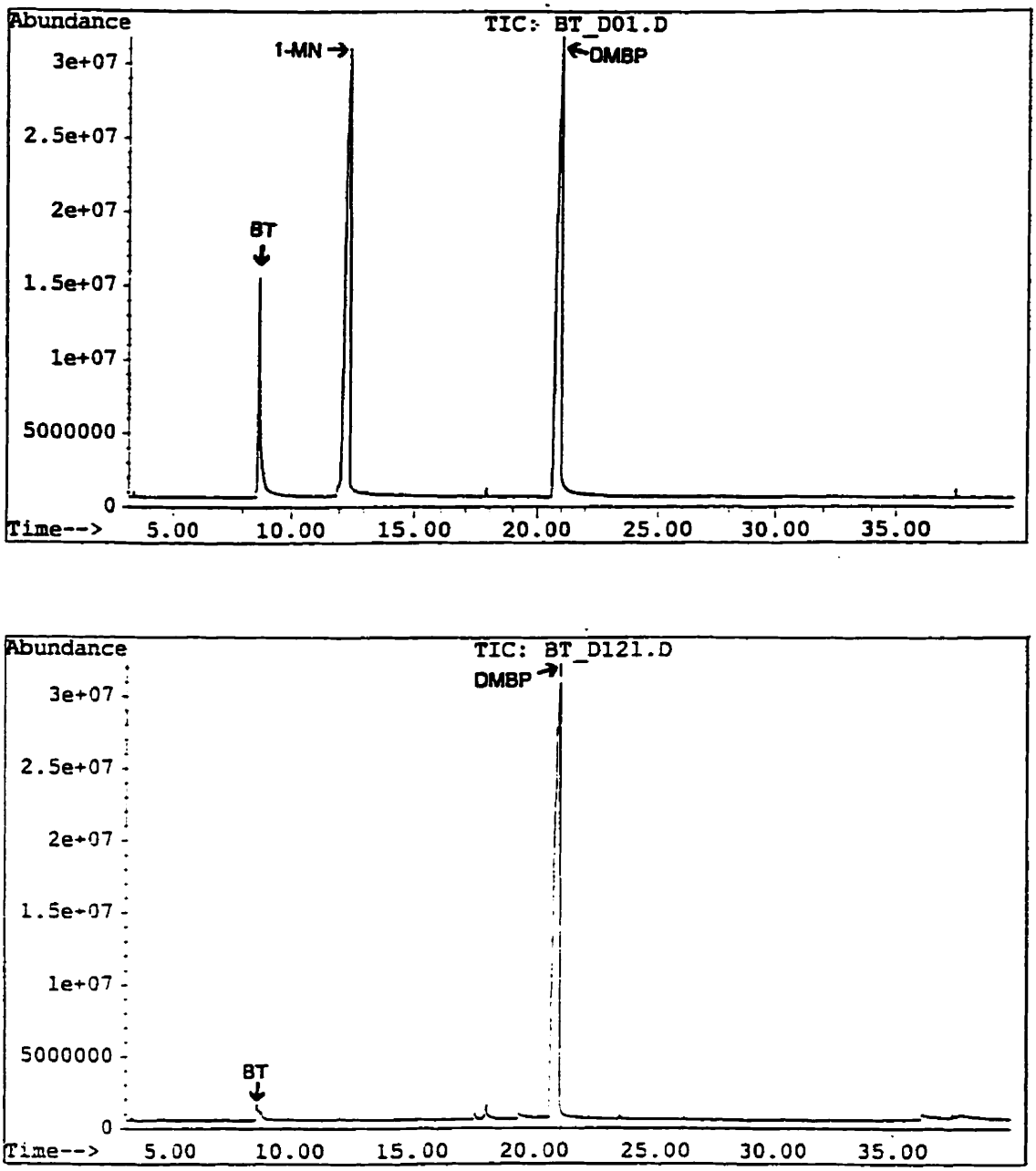


Figure 3.5 Total ion chromatograms of samples from the Bonne Bay consortium incubated at 25 °C showing abundance of BT, 1-MN, and DMBP on the first (top) and final (bottom) days of the experiment.

throughout the experiments with BT and all other compounds being investigated.

The greatest rate of BT degradation at 25 °C was recorded from day 0 to day 2 with 70% degradation in 48 hours or, assuming linearity, 35%/day, which translates into an average of 3.5 mg of BT/day. The greatest rate of bacterial growth was observed from day 8 to day 9 at 48.5 μg dry weight of cells/mL / day (μg/day) and was associated with a significant loss of 1-MN. The greatest rate of growth associated with BT degradation occurred from day 0 to day 2 as an increase from 64 to 77 μg/day which resulted in a growth rate of 6.5 μg/day. At 4 °C, the greatest rate of BT degradation occurred between day 6 and day 8 with 20% degradation in 48 hours which resulted in an average degradation rate of 1.2 mg BT/day. The greatest rate of bacterial growth occurred from day 7 to day 8 yielding a growth rate of 12.5 μg/day (Figure 3.2).

BT degradation had been initiated by the Come by Chance culture by day 2 at both 25 °C and 4 °C (Figure 3.3). In this case, 16% of the original BT concentration remained after 12 days at 25 °C and a similar result of 14% was observed at 4 °C. The greatest rate of BT degradation at 25 °C was observed from day 0 to day 2 with 84% degradation in 48 hours which averages to be 4.2 mg BT/day. This coincided with the greatest observed growth rate of 32.8 μg/day from day 0 to day 2. At 4 °C, the greatest rate of BT degradation was 1.3 mg BT/day from day 8 to day 10 and a rate of 8 μg/day from day 9 to day 10 was the highest bacterial growth rate associated with BT degradation.

The Port aux Basques culture had initiated BT degradation by day 2 at both 25 °C and 4 °C and only 11% of the BT remained at the end of the experiment for each incubation temperature (Figure 3.4). The greatest rate of BT degradation at 25 °C occurred between day 0 and day 2 with an average of 4.5 mg/day. This corresponded to the greatest rate of bacterial growth observed, with an average of 41.5 μg/day. At 4 °C the greatest rate of BT degradation occurred from day 2 to day 4 at an average rate of 2.0 mg/day. The greatest rate of bacterial growth associated with BT degradation at 4 °C was 16.5 μg/day from day 9 to day 10.

Throughout the BT experiment the amount of bacterial growth reported for each culture varied significantly relative to the different incubation temperature. In the 25 °C incubations, all three cultures displayed increases in growth from day 0 to day 2 while a loss of cells was reported for the same period by the Bonne Bay and Come by Chance cultures incubated at 4 °C. Furthermore, the maximum cell growth obtained by each culture was up to 3 times as great at 25 °C than at 4 °C and maximum cell growth was obtained up to 6 times faster at 25 °C than at 4 °C. This is consistent with information presented earlier (Neidhardt *et.al.*, 1990) that shifting the incubation temperature of an exponentially growing culture within the normal temperature range results in a growth rate characteristic of the new temperature. In this case, the temperature was decreased 21 Celsius degrees for the bacterial cultures incubated at 4 °C.

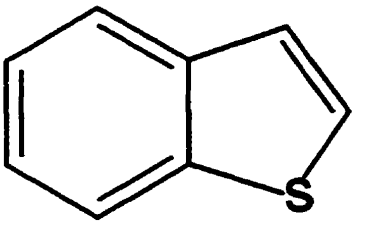
This was expected to cause at least a four fold drop in the growth rate because growth rate generally doubles for every 10 °C increase in temperature (Pelczar *et al.*, 1993). The results show that growth rate is faster, maximum growth is greater, and time to reach maximum growth is shorter at 25 °C than at 4 °C. This implies that degradation of BT by any of these bacterial communities will require more time to commence and will take longer to complete under the cooler field conditions as compared to optimum laboratory conditions.

The degradation of BT by all 3 bacterial communities was very efficient at 25 °C as losses of as much as 90% were reported after only 2 days incubation. The degradation rates reported for the same cultures incubated at 4 °C were significantly slower. The Bonne Bay culture, for example, took 4 times longer to initiate degradation of BT at 4 °C than at 25 °C and when degradation had started, about 3.5 times less BT had been degraded at 4 °C than at 25 °C. For the Come by Chance and Port aux Basques cultures, BT degradation had started at both temperatures by day 2 but the amount degraded by the cultures grown at 25 °C was approximately 4 to 5 times greater than that degraded at 4 °C. This also agrees with information presented earlier that enzyme reactions increase with an increase in temperature, thereby resulting in much faster degradation at 25 °C than at 4 °C.

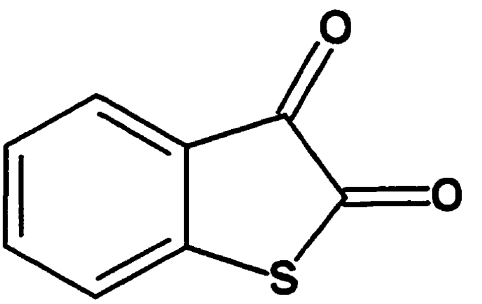
Sagardía *et al.* (1975) reported that the aerobic degradation of benzothiophene (BT) by *Pseudomonas aeruginosa PRG-1* involved a

mechanism whereby oxygen was added directly to the molecule of BT. The products of degradation were unknown but it was suggested that hydroxyl derivatives of one or the other ring were formed, and since these are more soluble they moved into the aqueous phase. Bohonos *et al.* (1977) used GC-MS to examine the metabolites from biodegradation studies on BT. The compounds found were oxidized on the thiophene ring but they could not exclude the possibility of microbial primary oxidation in the benzene ring followed by total mineralization of BT. Fedorak and Grbić-Galić (1991) investigated the aerobic microbial cometabolism of BT and 3-MBT. Their conclusion that the product of BT cometabolism is benzothiophene-2,3-dione (Figure 3.6) supports the findings of Bohonos *et al.* (1977).

For this study, GC-MS was chosen to determine the presence of the first metabolic product produced by the bacterial communities. However, no transformation intermediates or products were present at detectable levels at the time of sampling while the BT was being degraded. In order to enhance the ability to analyze the biodegradation products, the mixture was either acetylated or silylated. The acetylated products react with alcohols to produce acetates, while silylated products react with alcohols, carboxylic acids and amines. These reactions make more volatile derivatives of these compounds as compared to the starting material. A requirement of the chosen analytical method (GC-MS) is to use volatile material that can partition between a stationary phase (the coating



Benzothiophene



Benzothiophene-2,3-dione

Figure 3.6 Oxidation product from the aerobic cometabolism of benzothiophene by *Pseudomonas* sp. BT1. (Fedorak and Grbić-Galić, 1991)

on the column) and a mobile phase (helium gas). Polar compounds such as alcohols and carboxylic acids need to be derivatized and large molecules are also difficult to render volatile. The detection of biodegradation products, however, is limited by the rates of the reactions and the quantity of the material formed. Although several analytical methods are often used together for identification of the products, the complete identification also necessitates the availability of commercial or synthesized standards.

FTIR spectroscopy was used in this study to help verify the identification of any degradation products. FTIR spectroscopy provides information on the presence of functional groups in the sample but it is also limited by the amount of material present in solution. In this experiment, FTIR spectroscopy did not display any bands between 3000 and 3500 cm^{-1} which is the range of absorbance characteristic of alcohols and phenols. This suggests complete mineralization of this compound by each bacterial consortium, as was observed by Bohonos *et al.* (1977). Also, double bonds show an absorbance band at 2730 cm^{-1}, but no absorption band was present to indicate the presence of unsaturations. This observation supports the low abundance of aromatic compounds within the analyzed mixtures, explaining the non-detected products in the GC-MS total ion chromatograms. It is assumed that the BT was degraded into products which were quickly incorporated into the tricarboxylic acid (TCA) cycle but not detected. Therefore all 3 bacterial communities contain a host of enzymes with the capacity to not only mineralize BT but also transform the

metabolic intermediates into products for the tricarboxylic acid (TCA) cycle so efficiently that these compounds were not detected throughout the experiment. Alternatively, the products were too polar to extract with DCM and therefore were further in the metabolic pathway than expected.

3.2.2 3-Methylbenzothiophene

The results of the experiment with 3-MBT are summarized in Table 3.5. At both experimental temperatures, the Bonne Bay culture started to degrade 3-MBT by day 2 (Figure 3.7). The greatest rate of 3-MBT degradation at 25 °C was recorded from day 0 to day 2 with almost 90% degradation in 48 hours and therefore averaging 4.5 μL of 3-MBT/day. The greatest rate of bacterial growth was observed from day 0 to day 1 at 124 μg/day. At 4 °C, the greatest rate of degradation occurred between day 2 and day 4 with 60% degradation, equalling an average of 3 μL of 3-MBT/day. A large growth rate of 53 μg/day coincided with this degradation from day 3 to day 4.

At 25 °C, 6% of the original concentration of 3-MBT remained after 10 days and 9% remained at the end of the 4 °C experiment. The TIC obtained from the extract of this culture at 25 °C is presented for day 0 and day 10 (Figure 3.8). There are 2 overlapping peaks between 12 and 13 minutes, where the first peak at 12.2 minutes represents 3-MBT and the other at 12.4 minutes represents 1-MN. The internal standard, DMBP occurs at 20.7 minutes and a

Table 3.5 Numerical characterization of the 3-MBT (%), 1-MN (%), dry weight (μg/mL) and colony forming units/mL (CFU/mL) levels throughout the experimental duration

	BONNE BAY 25 °C				BONNE BAY 4 °C			
DAY	3-MBT	1-MN	DRY WT.	CFU / mL	3-MBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	42.000	3.8×10^7	100.000	100.000	27.000	2.4×10^7
1			166.000	1.52×10^8			32.000	2.9×10^7
2	10.880	1.000	181.000	1.66×10^8	80.900	85.500	46.000	4.2×10^7
3			116.500	1.095×10^8			59.000	5.4×10^7
4	10.610	1.000	138.000	1.265×10^8	21.400	22.300	112.000	1.025×10^8
5			139.500	1.2758×10^8			172.000	1.58×10^8
6	9.680	1.000	176.000	1.615×10^8	15.000	1.000	221.000	2.03×10^8
7			163.500	1.5×10^8			187.000	1.715×10^8
8	9.180	1.000	129.000	1.18×10^8	9.500	1.000	185.000	1.7×10^8
9			146.000	1.335×10^8			170.000	1.56×10^8
10	6.310	1.000	196.000	1.795×10^8	8.700	1.000	167.000	1.535×10^8
11			132.000	1.21×10^8			162.000	1.485×10^8
	COME BY CHANCE 25 °C				COME BY CHANCE 4 °C			
DAY	3-MBT	1-MN	DRY WT.	CFU / mL	3-MBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	40.000	3.7×10^7	100.000	100.000	33.000	3.0×10^7
1			153.000	1.415×10^8			8.000	2.55×10^7
2	16.460	1.000	145.000	1.345×10^8	100.000	97.300	35.000	3.2×10^7
3			130.000	1.2×10^8			47.000	4.3×10^7
4	11.870	1.000	158.000	1.46×10^8	67.800	74.700	79.000	7.3×10^7
5			234.000	2.17×10^8			98.000	9.05×10^7
6	12.050	1.000	198.000	1.745×10^8	27.500	48.500	104.500	9.65×10^7
7			211.000	1.85×10^8			141.000	1.33×10^8
8	8.550	1.000	114.000	1.055×10^8	11.300	1.000	162.000	1.50×10^8
9			139.000	1.285×10^8			166.000	1.535×10^8
10	7.870	1.000	162.000	1.50×10^8	9.900	1.000	170.000	1.57×10^8
11			132.500	1.22×10^8			152.000	1.41×10^8
	PORT AUX BASQUES 25 °C				PORT AUX BASQUES 4 °C			
DAY	3-MBT	1-MN	DRY WT.	CFU / mL	3-MBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	41.000	5.05×10^7	100.000	100.000	35.000	4.25×10^7
1			150.500	1.86×10^8			26.500	3.2×10^7
2	12.480	1.000	151.000	1.87×10^8	98.900	85.100	35.500	4.35×10^7
3			134.000	1.67×10^8			45.500	5.65×10^7
4	11.760	1.000	157.500	1.95×10^8	64.000	13.200	147.000	1.825×10^8
5			127.000	1.57×10^8			176.000	2.18×10^8
6	11.300	1.000	136.000	1.69×10^8	46.000	1.000	182.000	2.25×10^8
7			148.000	1.84×10^8			125.000	1.55×10^8
8	9.560	1.000	95.000	1.18×10^8	33.100	1.000	172.000	2.13×10^8
9			111.500	1.445×10^8			148.500	1.835×10^8
10	8.390	1.000	103.000	1.27×10^8	32.700	1.000	129.500	1.61×10^8
11			95.000	1.175×10^8			137.500	1.71×10^8

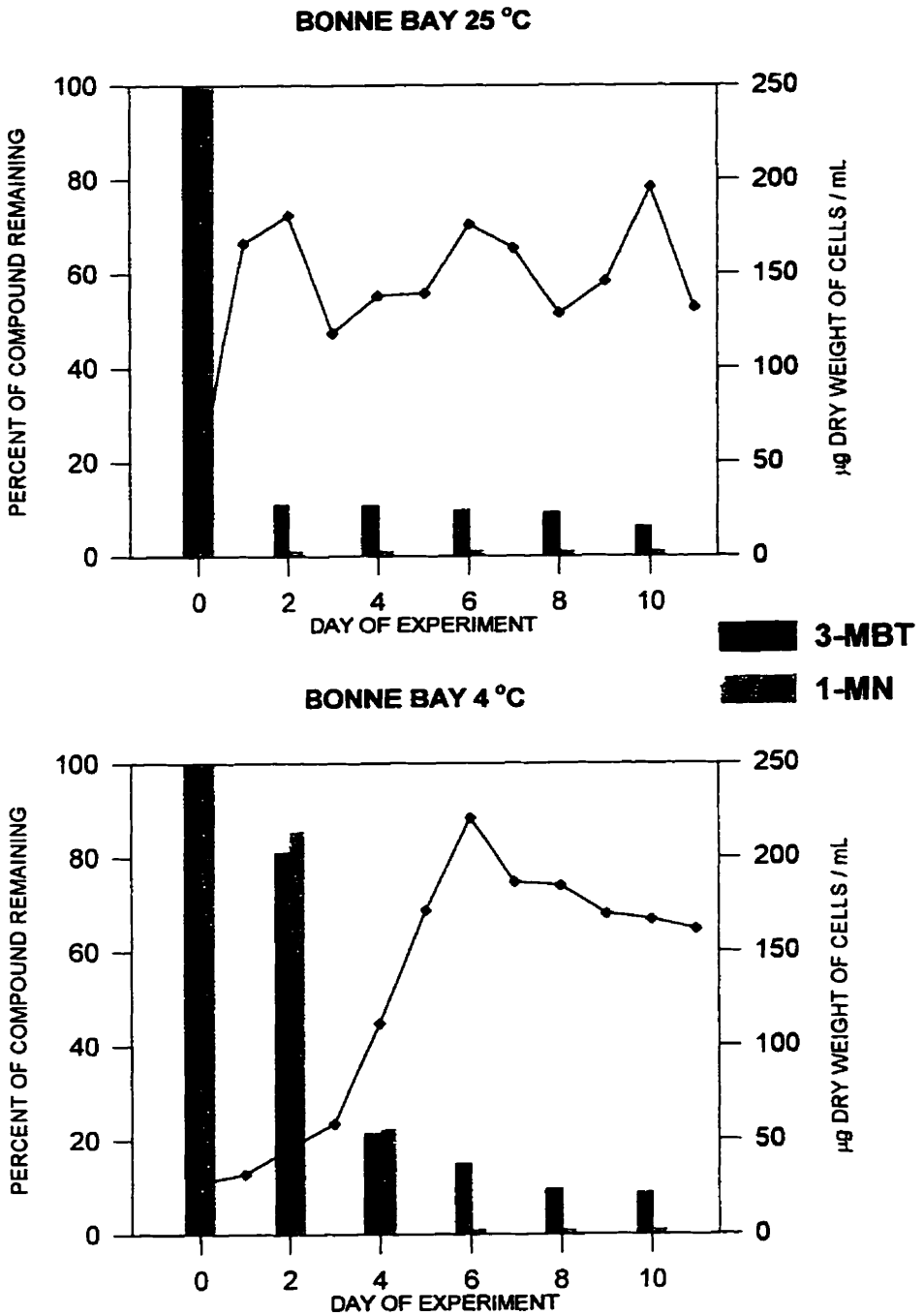


Figure 3.7 Time course of the degradation of 1-MN and 3-MBT and the resulting growth of the Bonne Bay bacterial consortium (µg dry weight of cells / mL; ± 11.55) incubated at 25 °C and 4 °C.

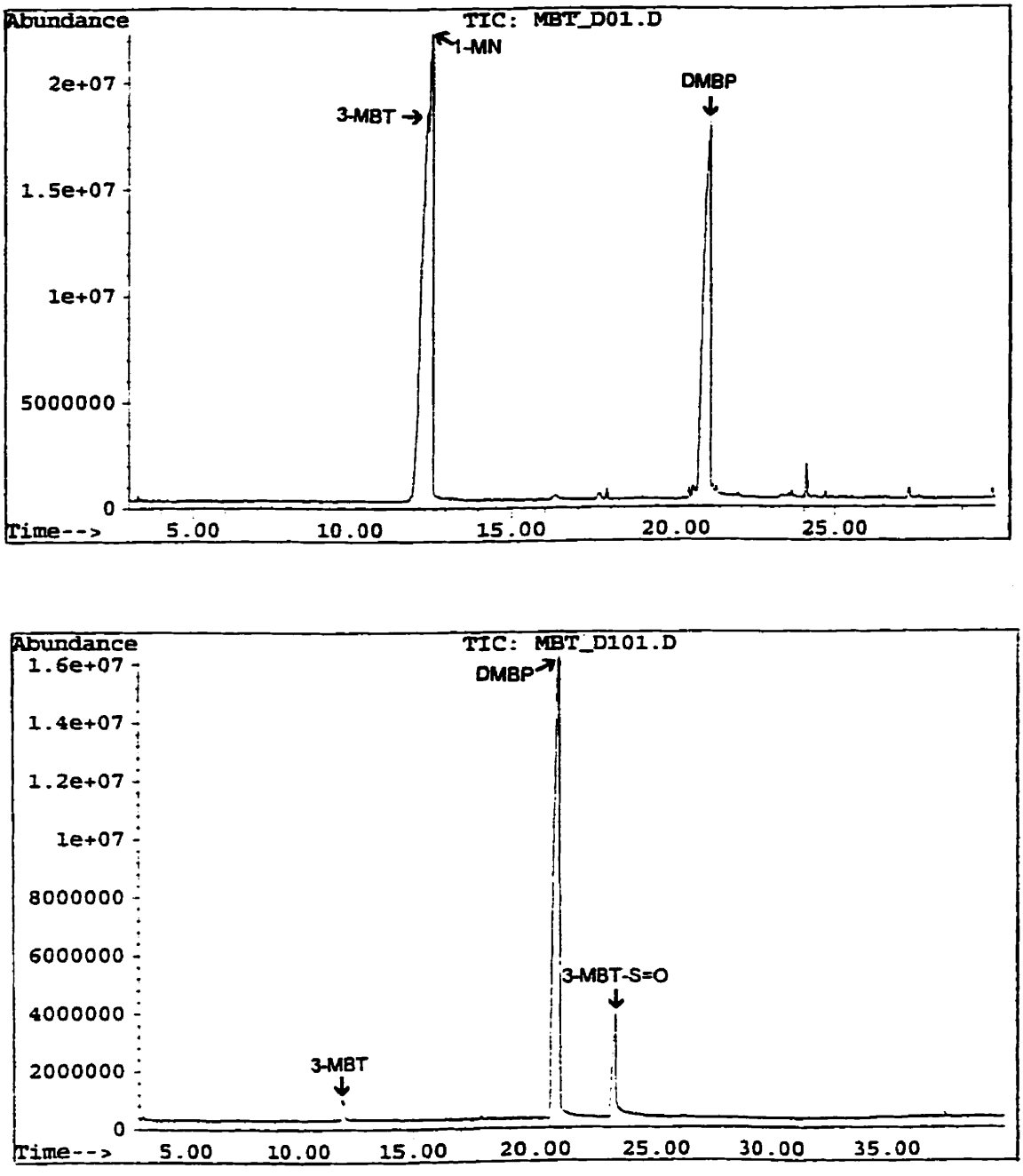


Figure 3.8 Total ion chromatograms of samples from the Bonne Bay consortium incubated at 25 °C showing abundance of 3-MBT, 1-MN and DMBP on the first (top) and final (bottom) days of the experiment.

new compound appeared at 23.2 minutes during GC-MS analysis of the 3-MBT samples. The mass spectrum for this peak (Figure 3.9) shows a molecular ion at *m/z* 164 which would correspond to a metabolite of molecular formula C_9H_8SO which could be assigned to 3-MBT-sulfoxide or an isomer of hydroxy-3-MBT. The fragment ions at *m/z* 135 and 147 correspond to a loss of -CHO and -OH, respectively, as observed with other sulfoxides (Fedorak and Grbić-Galić, 1991).

To verify the identity of the compound as 3-MBT-sulfoxide or hydroxy-3-MBT, the samples were analyzed by FTIR. The FTIR spectrum for the 25 °C Bonne Bay sample from day 4 (Figure 3.10) showed a minor peak at 1093 cm^{-1}, indicating that the product is the sulfoxide of 3-MBT because sulfoxides typically absorb between 1070 and 1130 cm^{-1}. A strong absorbance between 3000 and 3500 cm^{-1}, which was absent in this sample, is characteristic of alcohols and phenols.

Only a minor peak of 3-MBT-sulfoxide was observed during IR analysis so a calibration curve was created using a series of dilutions of dimethylsulfoxide in dichloromethane (Figure 3.11). The area of the peak for each dilution was determined to generate the curve and the area of the peak in Figure 3.10 was similarly calculated to determine the concentration of 3-MBT-sulfoxide in the sample. It was found that 0.124 μL of 3-MBT-sulfoxide was available in the 2 mL sample concentrated for GC-MS analysis. Simple calculations using the relative abundance values from the GC-MS data showed that the concentration of

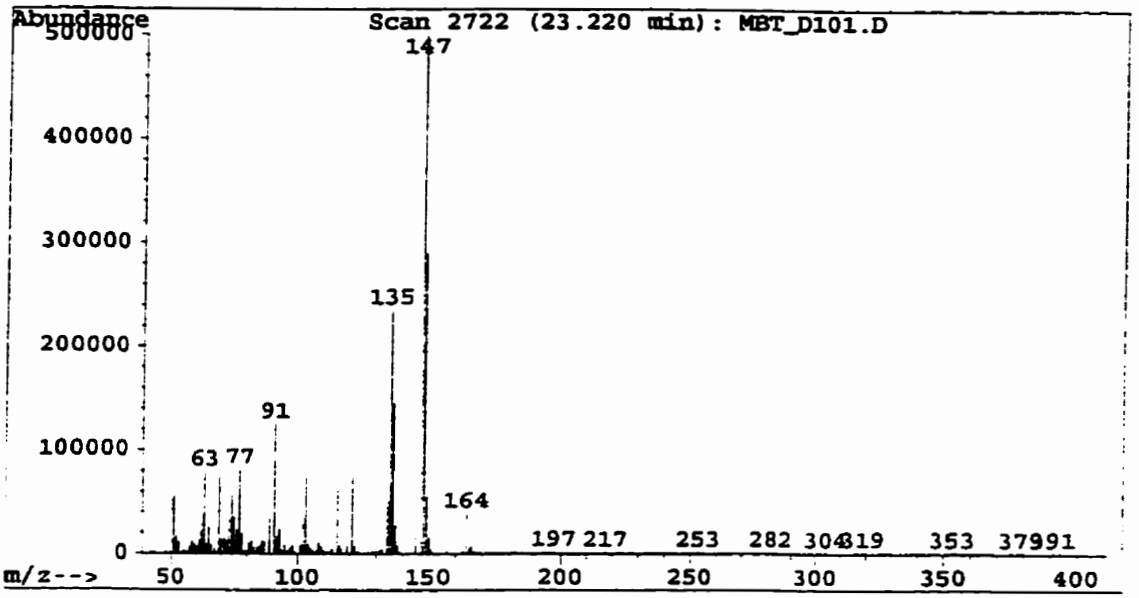


Figure 3.9 Mass spectrum of the metabolite of 3-MBT having a GC retention time of 23.220 minutes.

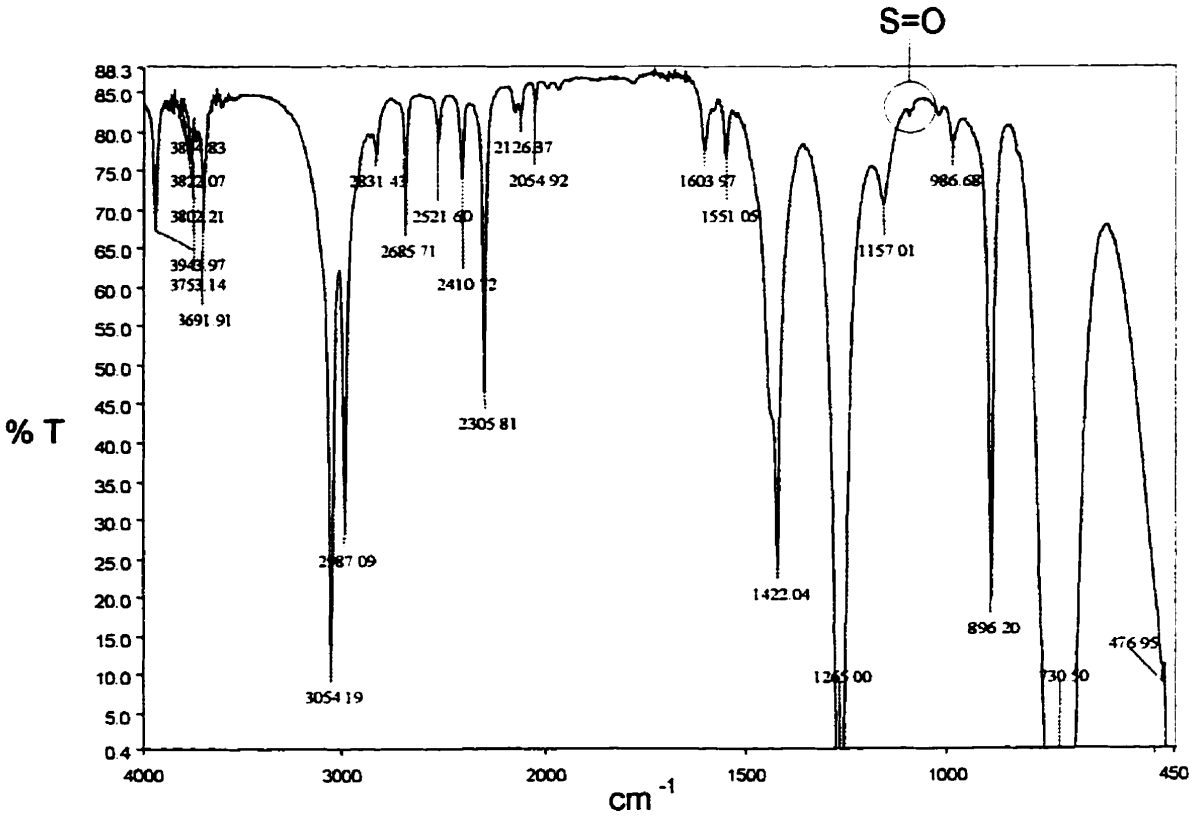


Figure 3.10 FTIR spectrum of the sample obtained on day 4 of the 3-MBT experiment using the Bonne Bay culture incubated at 25 °C. The encircled peak confirms the presence of sulfoxide (S=O) in the sample.

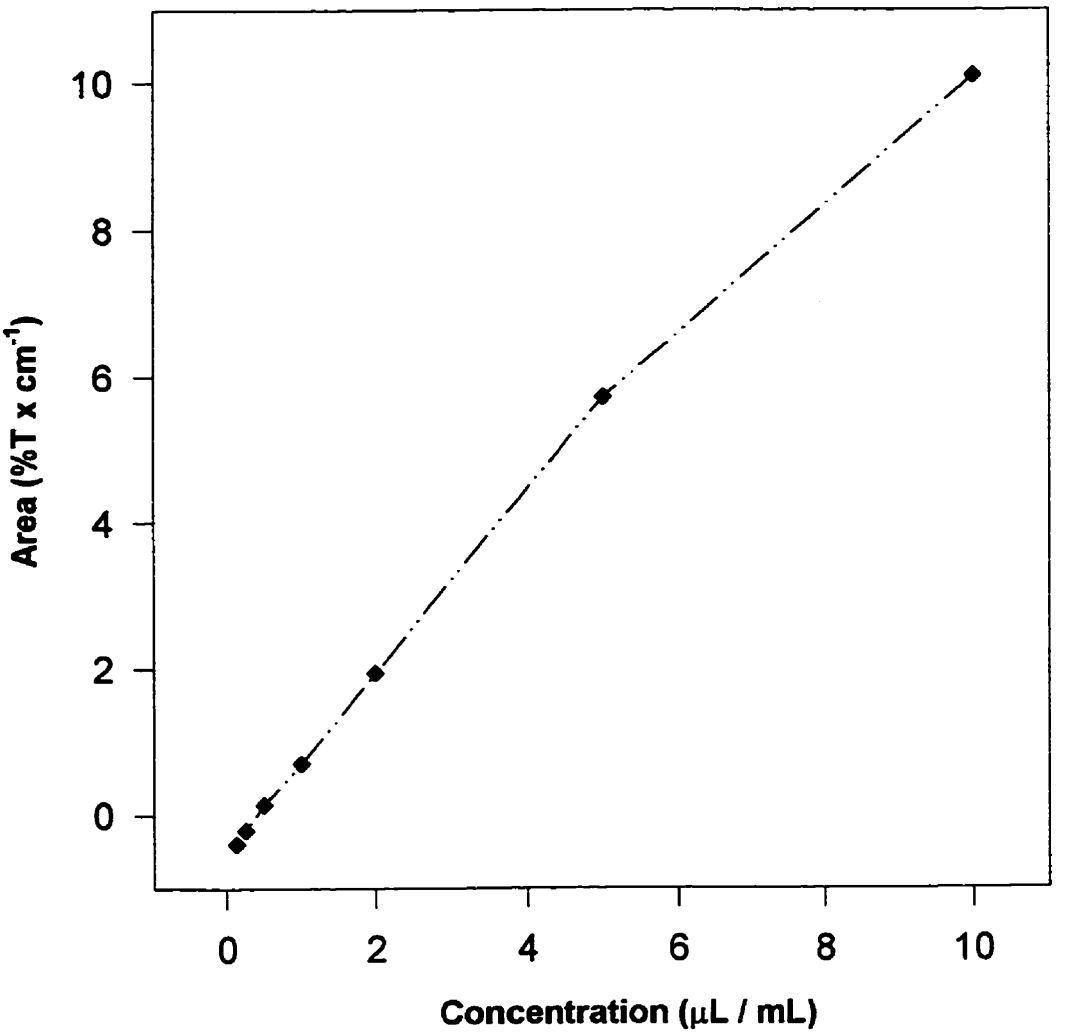


Figure 3.11 Area of IR peak at selected concentrations (µL/mL) of dimethylsulfoxide in dichloromethane. This standard curve was used to determine the concentration of 3-MBT-sufoxide in the sample.

3-MBT-sulfoxide in the sample was 0.121 μL/2mL (0.06 μL/mL). Consequently, of the possible 1μL of 3-MBT available in this sample only 12% was detected as its sulfoxide. The GC-MS and FTIR data for the other communities at each temperature were very similar.

The degradation of 3-MBT was initiated by the Come by Chance culture by day 2 at 25 °C and day 4 at 4 °C (Figure 3.12). Also, 7% of the original concentration of 3-MBT remained after 10 days at 25 °C and 10% remained after the same period at 4 °C incubation. The greatest rate of 3-MBT degradation at 25 °C was observed from day 0 to day 2 with 84% degradation in 48 hours which averages to be 4.2 μL 3-MBT/day. This corresponded to the biggest observed growth rate of 113 μg/day from day 0 to day 1. At 4 °C, the greatest rate of 3-MBT degradation was 2.0 μL/day from day 4 to day 6. However, the highest rate of bacterial growth associated with 3-MBT degradation occurred from day 3 to day 4 at 32 μg/day.

The Port aux Basques culture had initiated 3-MBT degradation at both 25 °C and 4 °C by day 2 and only 8% remained at the end of the 25 °C experiment while 33% remained in the 4 °C setup (Figure 3.13). The greatest rate of 3-MBT degradation at 25 °C occurred between days 0 and 2 at a rate of 4.4 μL/day. This coincided with the largest observed bacterial growth rate of 109.5 μg/day between day 0 and day 1. At 4 °C, the greatest rate of 3-MBT degradation occurred from day 2 to day 4 at an average rate of 1.6 μL 3-MBT/

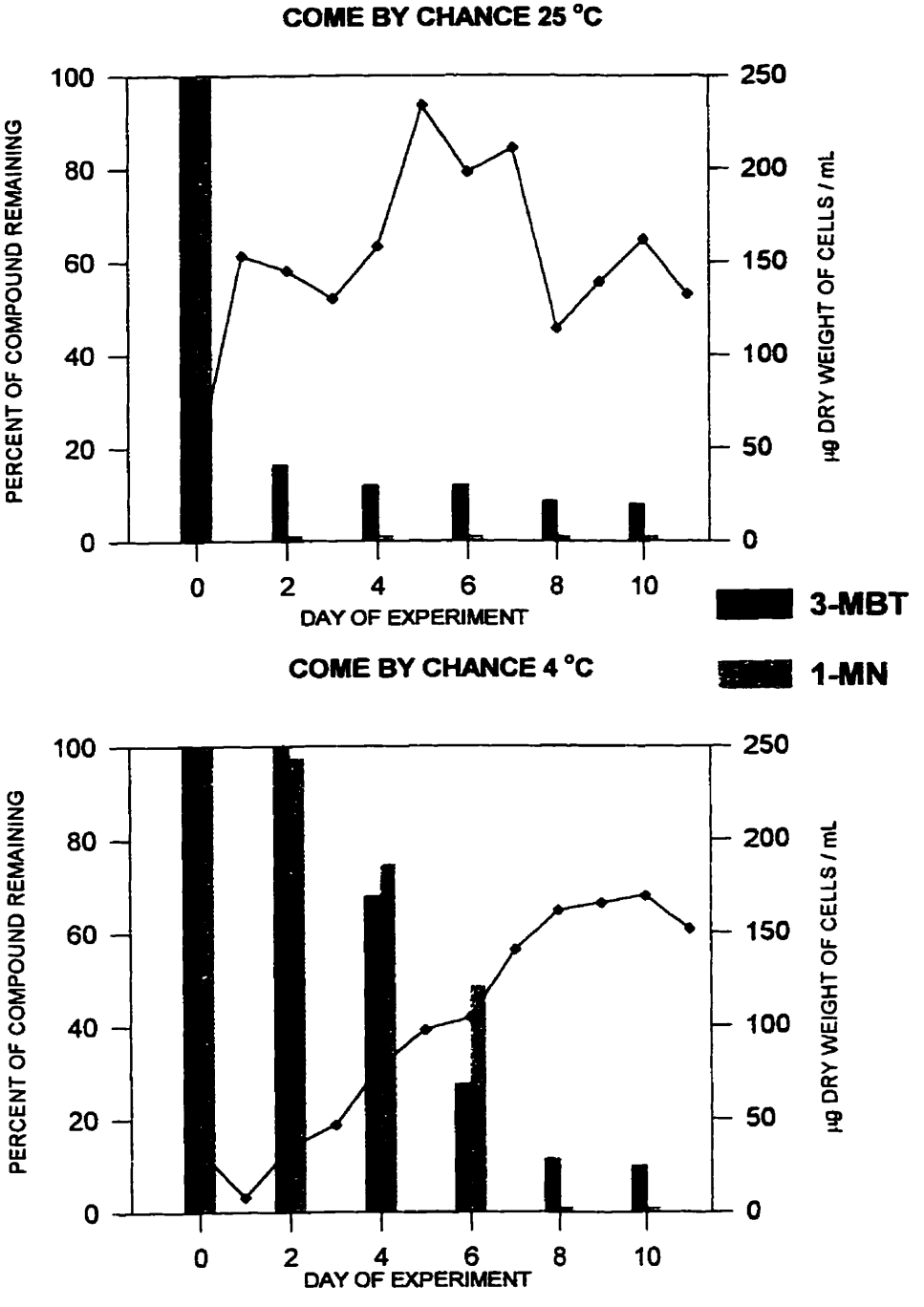


Figure 3.12 Time course of the degradation of 1-MN and 3-MBT and the resulting growth of the Come by Chance bacterial consortium (µg dry weight of cells / mL; ± 2.89) incubated at 25 °C and 4 °C.

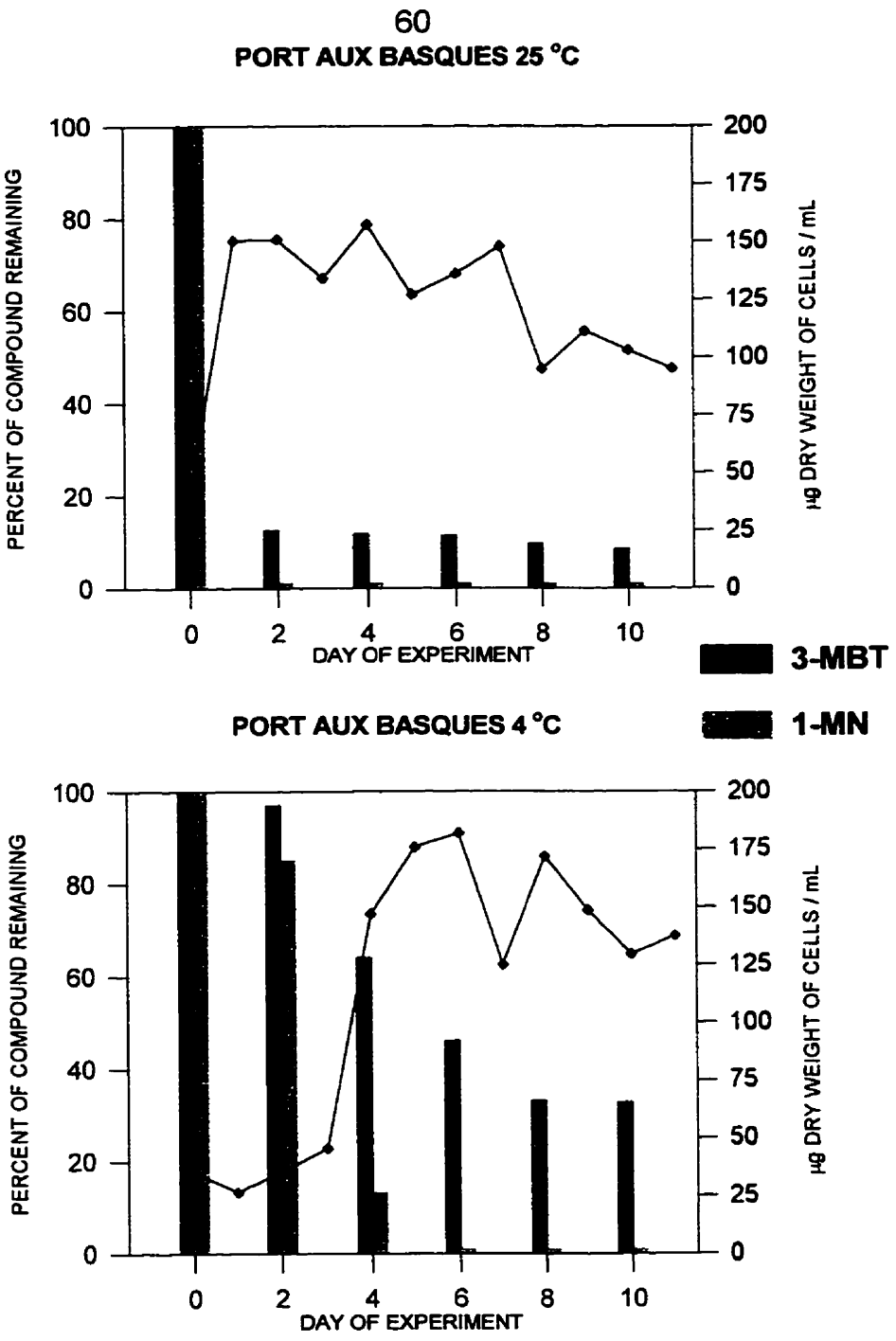


Figure 3.13 Time course of the degradation of 1-MN and 3-MBT and the resulting growth of the Port aux Basques bacterial consortium (µg dry weight of cells / mL; ± 5.77) incubated at 25 °C and 4 °C.

day. The highest rate of growth corresponded with the degradation rate of 101.5 µg/day which was recorded from day 3 to day 4.

With the exception of the Come by Chance culture incubated at 4 °C, all of the cultures started to degrade 3-MBT by day 2. However, the greatest rate of degradation was encountered from day 0 to day 2 at 25 °C incubation for all 3 cultures but at 4 °C, the greatest degradation rate reported for the Bonne Bay and Port aux Basques bacteria was from day 2 to day 4 and from day 4 to day 6 for the Come by Chance bacteria. This implies that bacteria incubated at the lower temperature require 2 to 3 times as much time to reach maximum degradation potential. The maximum bacterial growth rate was observed from day 0 to day 1 for each of the cultures at 25 °C which was 4 to 5 times faster than that recorded at 4 °C.

When compared with the results from the degradation of BT, it was found that degradation of 3-MBT was initiated at about the same time as with the BT in all but one sample, in which case degradation of 3-MBT occurred 3 times faster. However, the proportion of compound degraded by the cultures was greater for 3-MBT than for BT for all three cultures at both incubation temperatures. Consequently, it appears that the methyl-substituted sulfur compound was easier to degrade than the non-substituted compound which makes sense in terms of the inductive effect because the methyl group is an activating group (Morrison and Boyd, 1987). This means that the methyl group releases electrons and neutralizes the positive charge of the ring and so becomes more

positive itself thereby activating the ring and making it more reactive than the non-substituted compound. Furthermore, 3-MBT was in liquid phase and its higher susceptibility to microbial attack was expected because liquid hydrocarbons are more readily metabolized than solid hydrocarbons (Atlas, 1981). This suggests that as the solubility of a compound increases, more molecules will be available to the bacteria for biotransformation.

Fedorak and Grbić-Galić (1991) reported that the cometabolism of 3-MBT results in the formation of its sulfoxide and a small amount of its sulfone (Figure 3.14). In this experiment, the GC-MS results for all 3 bacterial communities showed the appearance of a peak, as 3-MBT was degraded. FTIR analysis confirmed this product to be the sulfoxide of 3-MBT. In all but one of the samples, over 90% of the 3-MBT had been degraded but only 20 to 25% was transformed into 3-MBT-sulfoxide; the other 75 to 80% degradation did not result in detectable transformation products. Therefore, it is assumed that there are at least two enzyme systems at work resulting in 3-MBT degradation through at least two pathways. One pathway results in the formation of 3-MBT-sulfoxide, which was not metabolized further, while the other existing pathway(s) is more prevalent and efficient. The other pathway(s) is assumed to be more prevalent because a greater amount of 3-MBT degradation (75-80%) occurred through this method. It is also more efficient because no intermediates or products accumulated and were detected, therefore suggesting that any intermediates produced were immediately incorporated into the TCA cycle or they were too

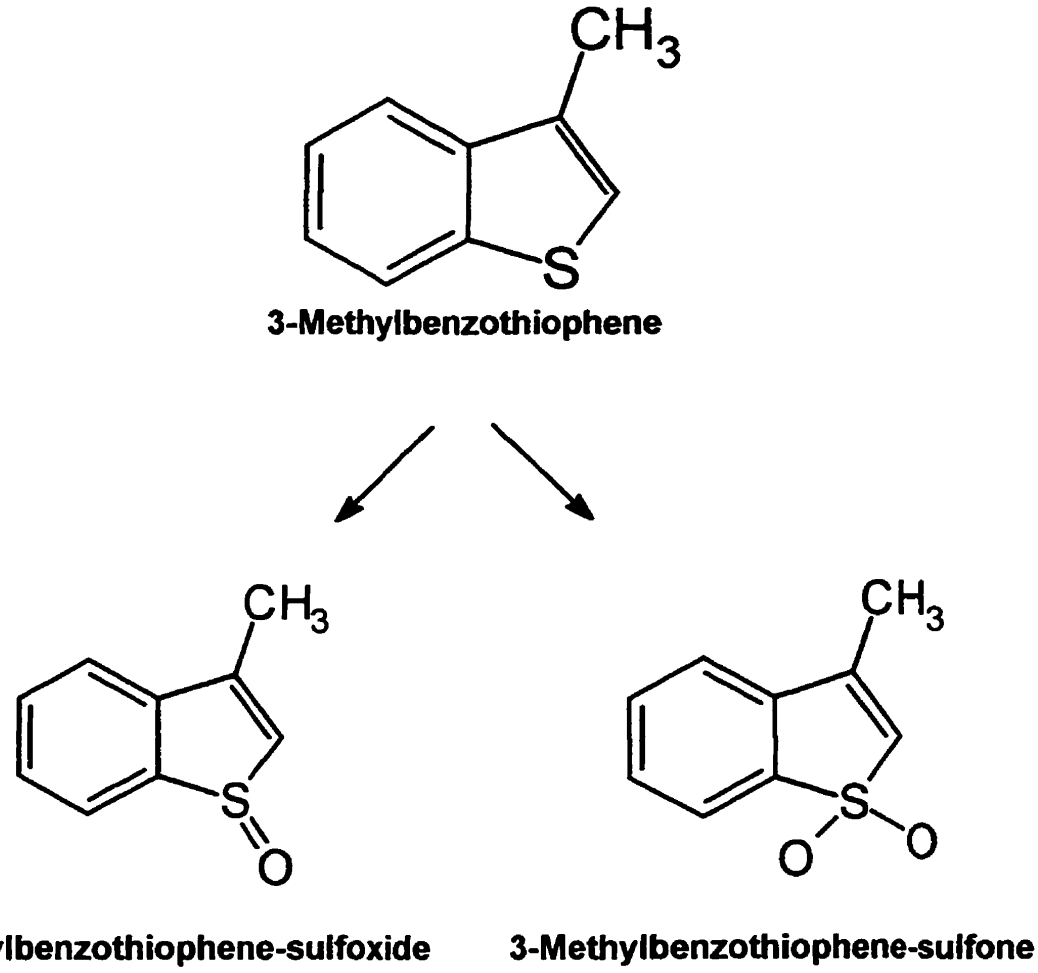


Figure 3.14 Oxidation prroducts from the aerobic cometabolism of 3-methylbenzothiophene by *Pseudomonas* sp. BT1 (Fedorak and Grbić-Galić, 1991).

polar to extract with DCM and to detect.

The formation of 3-MBT-sulfoxide agrees with the findings of Fedorak and Grbić-Galić (1991) and is of great importance environmentally. Sulfoxidation is one of the most important pesticide degradation pathways (Miles, 1991). The sulfoxide product has a much higher water solubility and a lower sorption to soil than the parent compound and therefore has a higher groundwater contamination potential. It also has a slower degradation rate than the parent compound and studies have shown that it persists for a longer period than the parent compound (Lacorte *et al.*, 1996; Minelli *et al.*, 1996). The persistent nature of sulfoxides has resulted in toxicity tests being performed to determine any significant detrimental effects that they have on aquatic biota. Foran *et al.* (1985) studied the acute toxicity of the carbamate insecticide and nematocide aldicarb, and its oxidation products (aldicarb sulfoxide and aldicarb sulfone), to the cladoceran *Daphnia laevis*. They found that aldicarb sulfoxide was similar in toxicity to the parent compound for both juveniles and adults. This indicates that microbial oxidation did not lessen the toxic impact of aldicarb contamination of surface waters. Similar results were obtained in a study using bluegill sunfish (Clarkson 1968, as cited in Day, 1991).

3.2.3 Dibenzothiophene

The results from the experiment with DBT are summarized in Table 3.6. At 25 °C the Bonne Bay culture started to degrade DBT by day 4 but it was not

Table 3.6 Numerical characterization of the DBT (%), 1-MN (%), dry weight (μg/mL) and colony forming units/mL (CFU/mL) levels throughout the experimental duration

	BONNE BAY 25 °C				BONNE BAY 4 °C			
DAY	DBT	1-MN	DRY WT.	CFU / mL	DBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	12.500	5.0×10^6	100.000	100.000	9.500	3.75×10^6
1								
2	100.000	100.000	21.700	8.75×10^6	100.000	100.000	8.000	3.25×10^6
3								
4	27.000	2.400	51.800	2.1×10^7	100.000	7.020	24.000	9.75×10^6
5			65.800	2.675×10^7			32.500	1.325×10^7
6	71.200	3.400	43.800	1.775×10^7	100.000	4.160	33.500	1.35×10^7
7			49.900	2.025×10^7			39.000	1.575×10^7
8	46.200	2.900	71.500	2.9×10^7	100.000	3.520	40.500	1.625×10^7
9			82.900	3.375×10^7			52.000	2.1×10^7
10	39.600	2.900	85.000	3.45×10^7	63.600	2.996	54.000	2.225×10^7
11							46.000	1.875×10^7
12					61.500	3.168	39.000	1.6×10^7
13							53.000	2.15×10^7
14					57.300	3.088	43.200	1.725×10^7
	COME BY CHANCE 25 °C				COME BY CHANCE 4 °C			
DAY	DBT	1-MN	DRY WT.	CFU / mL	DBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	8.500	2.25×10^6	100.000	100.000	9.000	2.65×10^6
1								
2	100.000	100.000	9.600	2.75×10^6	100.000	97.400	8.000	2.275×10^6
3								
4	19.000	28.300	93.500	2.675×10^7	100.000	84.800	27.700	7.85×10^6
5			126.500	3.6×10^7			19.500	5.5×10^6
6	52.000	3.360	54.000	1.505×10^7	100.000	72.500	20.700	6.0×10^6
7			74.000	2.1×10^7			23.500	7.0×10^6
8	17.000	3.040	84.400	2.425×10^7	100.000	65.100	46.500	1.325×10^7
9			89.500	2.56×10^7			70.000	2.0×10^7
10	11.000	3.120	109.000	3.675×10^7	100.000	32.000	83.000	2.375×10^7
11							104.000	2.96×10^7
12					100.000	13.000	96.000	2.75×10^7
13							77.500	2.5×10^7
14					100.000	4.790	97.700	2.785×10^7
	PORT AUX BASQUES 25 °C				PORT AUX BASQUES 4 °C			
DAY	DBT	1-MN	DRY WT.	CFU / mL	DBT	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	12.500	1.05×10^6	100.000	100.000	11.000	9.5×10^5
1								
2	100.000	100.000	50.500	4.125×10^6	100.000	100.000	10.000	8.75×10^5
3								
4	100.000	39.000	166.000	1.36×10^7	100.000	96.000	19.000	1.575×10^6
5			162.000	1.325×10^7			23.000	1.915×10^6
6	62.000	4.100	57.000	4.675×10^6	100.000	78.000	15.500	2.1×10^6
7			96.500	7.85×10^6			68.200	5.575×10^6
8	40.900	3.500	94.500	7.7×10^6	100.000	26.700	78.000	6.375×10^6
9			124.500	1.0125×10^7			113.500	9.25×10^6
10	15.900	3.400	121.000	9.825×10^6	100.000	5.350	138.000	1.125×10^7
11							141.000	1.15×10^7
12					100.000	4.628	149.500	1.22×10^7
13							132.000	1.075×10^7
14					70.870	5.230	138.000	1.12×10^7

until day 10 that degradation had been initiated by this culture when incubated at 4 °C (Figure 3.15). At 25 °C, 40% of the DBT remained after 10 days while 57% remained after 14 days for the 4 °C incubation. The TIC of the extract from the Bonne Bay culture at 25 °C incubation, on the first and final days of the experiment, is presented (Figure 3.16). The peak with a retention time of 24.6 minutes represents DBT and illustrates the degradation of this compound after 10 days. GC-MS analysis did not reveal any transformation products and the results for the other two bacterial cultures at both temperatures were similar to Figure 3.16.

The fastest rate of degradation was from day 2 to day 4 at 73% in 48 hours or 36.5%/day, resulting in an average degradation rate of 3.7 mg DBT/day (Figure 3.15). However, by day 6 the amount of DBT remaining was back up to 71% and then it decreased again. This must be due to the intrinsic variability in the sampling and analysis of cultures, as discussed in the previous chapter, which caused a larger error on day 4. The fastest rate probably occurred from day 6 to day 8 at 2.5 mg/day, which would be consistent with the greatest rate of bacterial growth, which was 21.6 μg/day from day 7 to day 8. At 4 °C, the greatest rate of DBT degradation occurred from day 8 to day 10 at a rate of 1.8 mg/day and the fastest rate of bacterial growth associated with DBT degradation occurred between days 8 and 9 at 11.5 μg/day.

The degradation of DBT had been initiated by the Come by Chance

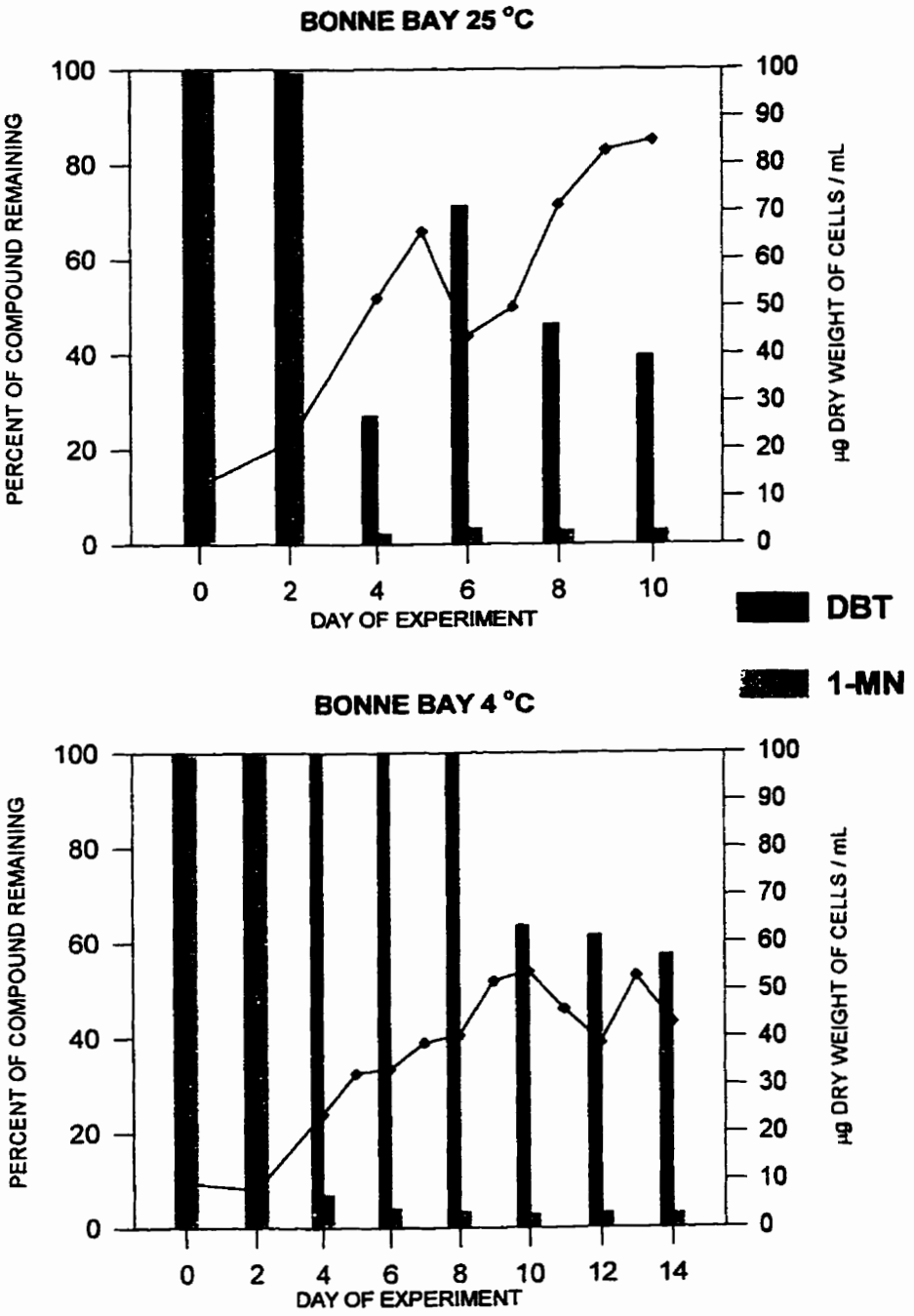


Figure 3.15 Time course of the degradation of 1-MN and DBT and the resulting growth of the Bonne Bay bacterial consortium (µg dry weight of cells / mL; ± 7.64) incubated at 25°C and 4 °C.

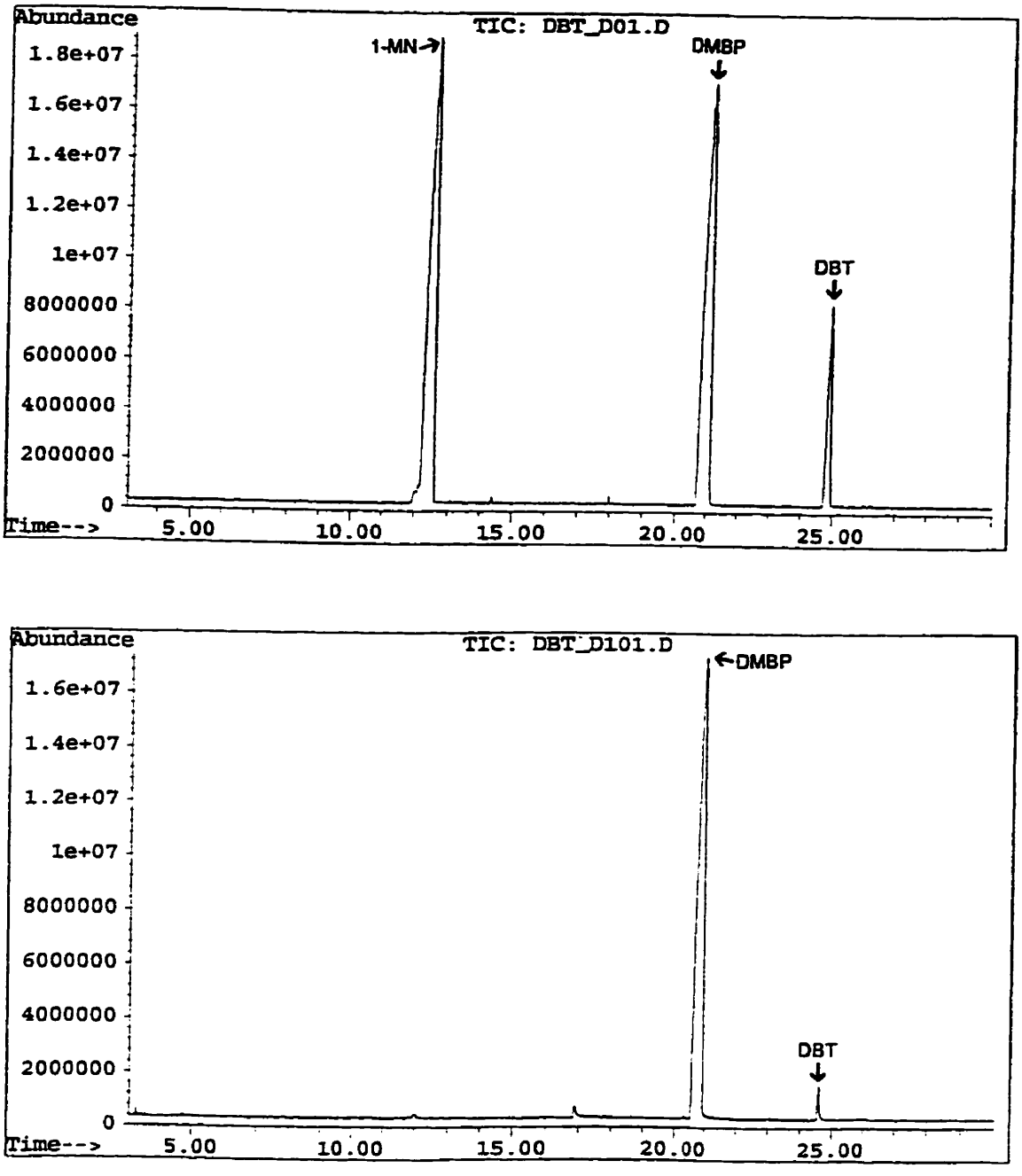


Figure 3.16 Total ion chromatograms of samples from the Bonne Bay culture incubated at 25 °C showing the abundance of DBT, 1-MN and DMBP on the first (top) and final (bottom) days of the experiment.

culture by day 4 at 25 °C but at 4 °C incubation this culture was unable to degrade DBT (Figure 3.17). As with the Bonne Bay culture, there appeared to be sampling error on day 4 for the 25 °C incubated culture and even though the greatest rate of bacterial growth occurred during this period it appears that this growth was associated with 1-MN degradation. Consequently, the greatest rate of DBT degradation is believed to have occurred between days 6 and 8 at an average rate of 18% degradation/day or 1.8 mg DBT/day. The fastest rate of bacterial growth associated with a loss of DBT occurred between day 6 and day 7 with a rate of 20 μg/day.

The Port aux Basques culture had initiated degradation of DBT at 25 °C by day 6 and by day 14 at 4 °C (Figure 3.18). Only 16% of the original concentration of DBT remained at the end of the 25 °C incubation experiment while 71% remained at 4 °C. The greatest rate of DBT degradation at 25 °C for this culture occurred between days 4 and 6 at a rate of 1.9 mg DBT/day. The greatest rate of bacterial growth associated with DBT degradation occurred from day 6 to day 7, the day following the most degradation, at a rate of 39.5 μg/day. At 4 °C, the greatest rate of DBT degradation occurred from day 12 to day 14, which was the only observed loss of DBT. This rate averaged to 15%/day or 1.5 mg DBT/day and the bacterial growth rate associated with this degradation was 6 μg/day.

In the 25 °C setup, DBT degradation was initiated by the Bonne Bay and Come by Chance cultures by day 4 and the Port aux Basques culture on day 6.

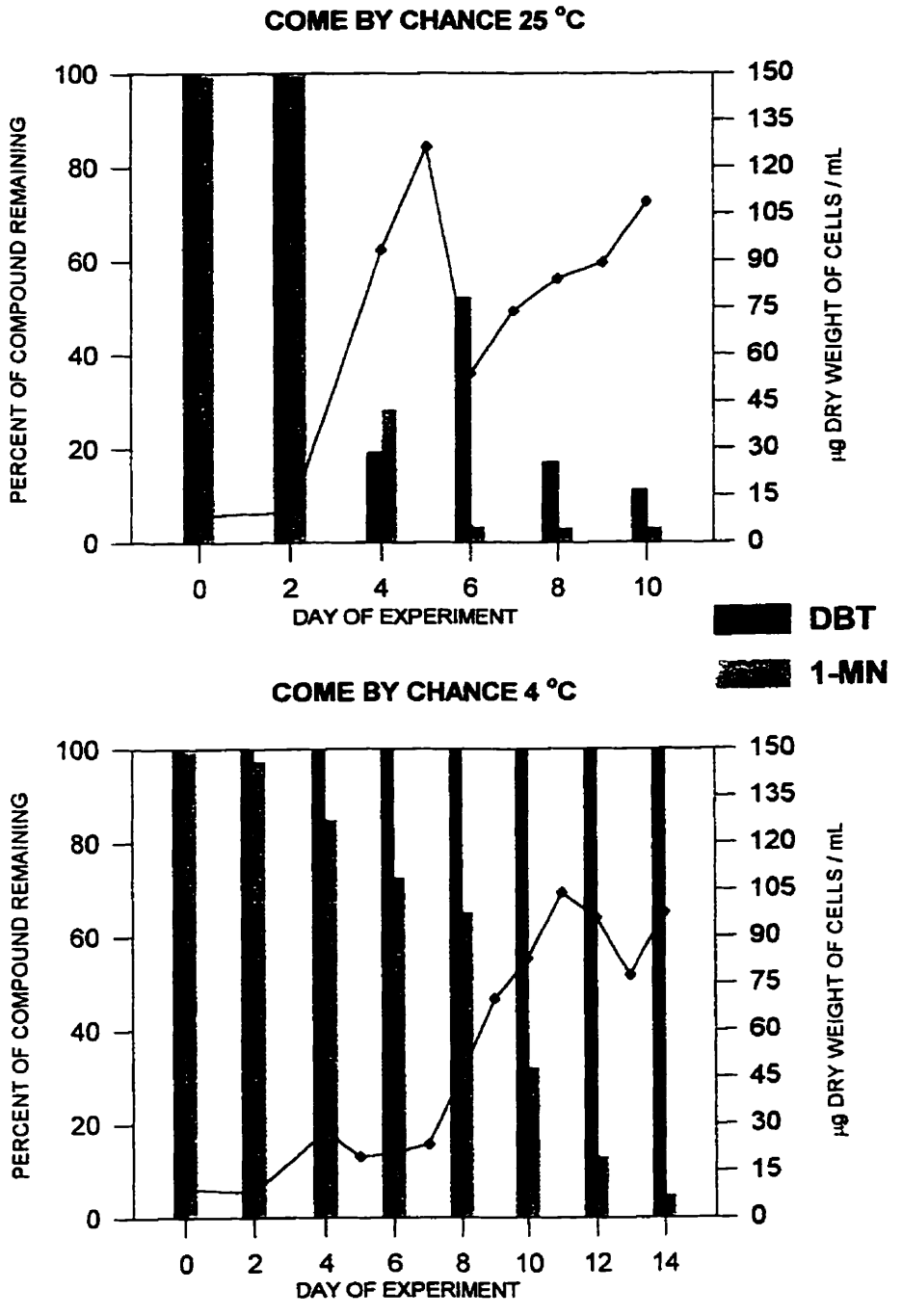


Figure 3.17 Time course of the degradation of 1-MN and DBT and the resulting growth of the Come by Chance bacterial consortium (µg dry weight of cells / mL; ± 5.77) incubated at 25 °C and 4 °C.

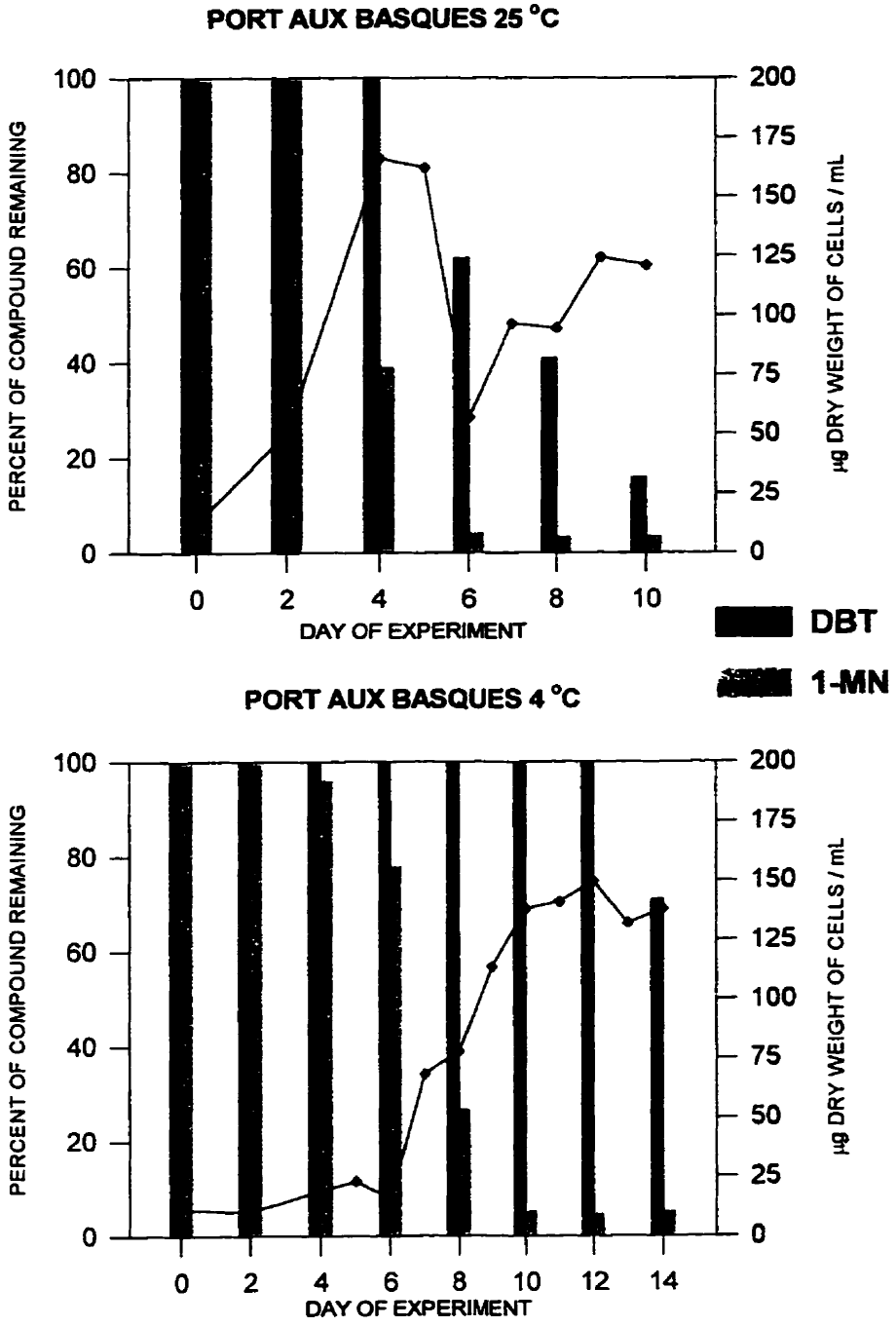


Figure 3.18 Time course of the degradation of 1-MN and DBT and the resulting growth of the Port aux Basques bacterial consortium (µg dry weight of cells / mL; ± 5.77) incubated at 25 °C and 4 °C.

The Bonne Bay culture started to degrade DBT by day 10 at 4 °C but this did not occur until day 14 for the Port aux Basques culture and the Come by Chance culture was unable to degrade any DBT during the 14 day exposure period. These results show that DBT degradation at 4 °C, if it occurred, took more than twice as long to begin than at 25 °C. The results also showed a large decrease in the proportion of DBT remaining from day 2 to day 4, followed by an increase on day 6, for the Bonne Bay and Come by Chance cultures at 25 °C. It can be assumed that this occurred because DBT is in the solid phase and was not completely dissolved in the media at day 0. After degradation was initiated on day 4, the undissolved crystals went into solution and caused an increase in the proportion remaining.

Little, if any, degradation of DBT was achieved by cultures incubated at 4 °C, which suggests that this compound is more difficult to degrade than either BT or 3-MBT. This can probably be attributed to the increased resonance stability imparted by the presence of the second benzene ring which provides more stable bonds within the structure, thereby making it more resistant to bacterial oxidation. The fact that DBT degradation at 25 °C took twice as long as BT or 3-MBT to initiate lends further support to this interpretation and helps explain why DBT has been found to be persistent in the environment (Atlas *et al.*, 1981; Gundlach *et al*, 1983).

Initial studies on the microbial degradation of PASH resulted in the isolation of a number of organisms capable of oxidizing dibenzothiophene (DBT)

during growth in a complex media (Yamada *et al.*,1968 and Nakatani *et al.*, 1968, as cited in Ensley, 1984). Because it is commercially available, DBT is the most widely used compound for studies of PASH biodegradation and for coal and petroleum biodesulfurization (Fedorak, 1990). Cultures of *Pseudomonas* (Kodama *et al.*, 1970, 1973) and *Beijerinckia* (Laborde and Gibson, 1977) have been incubated with DBT and appear to have a common metabolic pathway for DBT oxidation. The two endproducts are 3-hydroxy-2-formylbenzothiophene (HFBT), which is more abundant, and dibenzothiophene sulfoxide (Figure 3.19). A different degradation pathway was proposed by van Afferden *et al.* (1990) who used a pure culture of *Brevibacterium sp. DO* which utilizes DBT as the sole source of carbon, sulfur and energy for growth. They used GC-MS and ultraviolet (UV) spectroscopy to identify the metabolites as dibenzothiophene-5-oxide, dibenzo-thiophene-5-dioxide, and benzoate (Figure 3.20). Most recent investigations with DBT have focussed on the desulfurization of this compound in an attempt to develop the technology to selectively remove sulfur from petroleun prior to refining. The desulfurization end-products include 2-hydroxy-biphenyl (Omori *et al.*, 1992; Oshiro *et al.*, 1995) and biphenyl (Armstrong *et al.*, 1995). However, biodesulfurization was not an objective of this study and therefore does not warrant further discussion.

In this experiment, no DBT transformation products could be detected by GC-MS, presumably for the same reasons as provided for BT and 3-MBT.

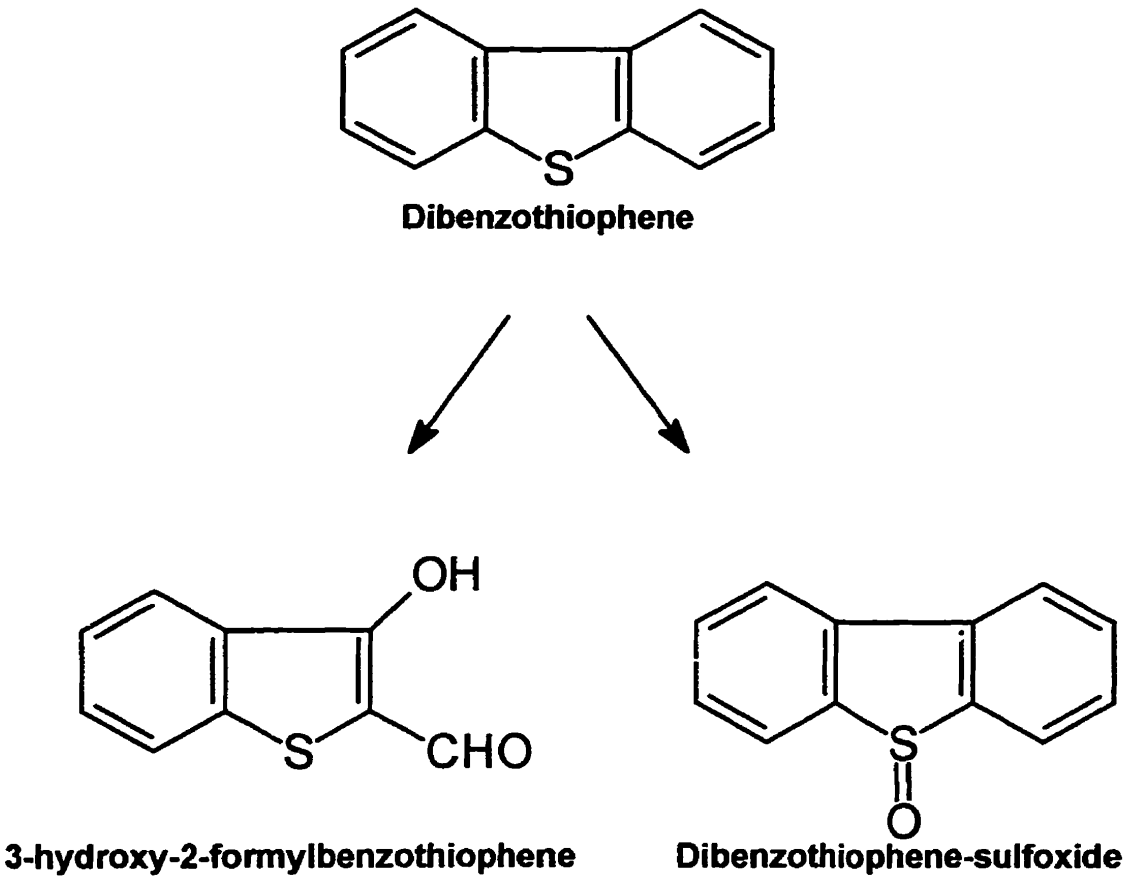


Figure 3.19 Oxidation products from the aerobic cometabolism of dibenzothiophene as proposed by Kodama *et al.*, 1970, 1973, and Laborde and Gibson, 1977).

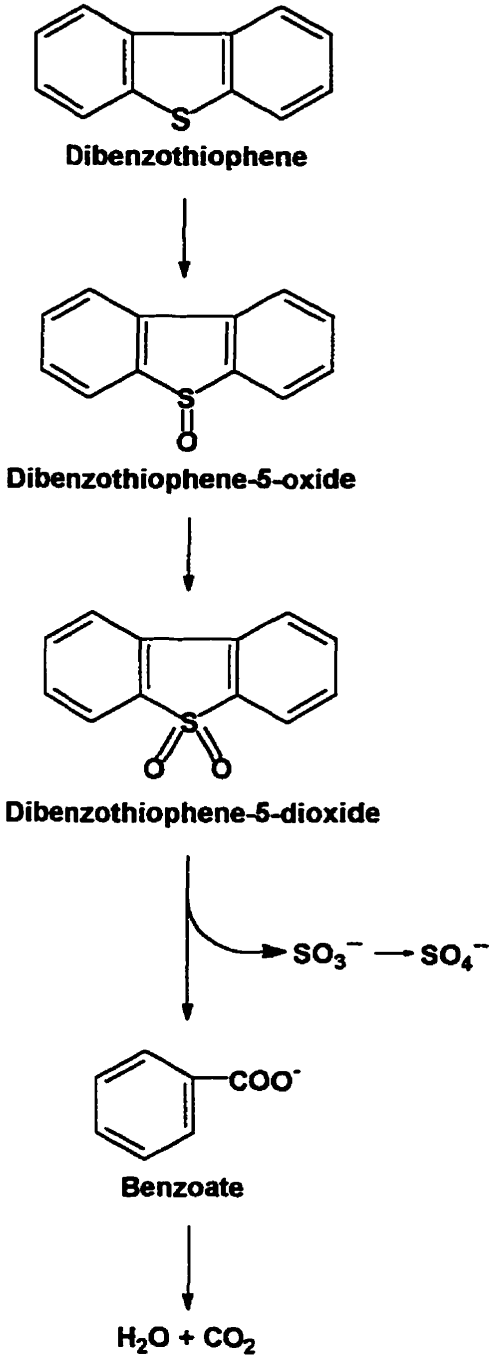


Figure 3.20 Proposed pathway for degradation of dibenzothiophene by *Brevibacterium* sp. DO (van Afferden *et al.*, 1990).

3.2.4 Carbazole

The results from the experiment with CARB are summarized in Table 3.7. The Bonne Bay culture had started to degrade CARB by day 10 at both incubation temperatures (Figure 3.21). At 25 °C, 72% of the CARB remained after 12 days while 79% remained after 14 days for the 4 °C incubation. The TIC shown is for day 0 and day 12 of the experiment with the Bonne Bay culture at 25 °C (Figure 3.22). The peak with a retention time of 27.3 minutes represents CARB and illustrates the loss in abundance of this compound after 12 days. The TIC shows that no other transformation products were detectable. The data for the other bacterial communities at both temperatures are similar to this sample.

The fastest rate of degradation occurred from day 8 to day 10 with a loss of 18%, resulting in an average degradation rate of 9%/day or 0.9 mg CARB/day (Figure 3.21). The fastest bacterial growth rate associated with CARB degradation concurs with the fastest degradation rate as an increase of 42 μg/day was reported from day 9 to day 10. At 4 °C, the greatest rate of CARB degradation occurred between days 8 and 10 but the percentage remaining increased again by day 12. The largest amount of degradation probably occurred from day 12 to day 14 at a rate of 6%/day or 0.6 mg CARB/day. The growth rate associated with this degradation, from day 13 to day 14, was 14 μg/day but the fastest growth rate was recorded between days 8 and 9 at a rate of 22 μg/day.

Table 3.7 Numerical characterization of the CARB (%), 1-MN (%), dry weight (μg/mL) and colony forming units/mL (CFU/mL) levels throughout the experimental duration

	BONNE BAY 25 °C				BONNE BAY 4 °C			
DAY	CARB	1-MN	DRY WT.	CFU / mL	CARB	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	29.000	2.1×10^7	100.000	100.000	13.000	9.0×10^6
1			89.500	5.0×10^7			65.000	4.65×10^7
2	100.000	5.650	101.000	7.35×10^7	100.000	33.600	113.000	8.2×10^7
3			142.000	1.03×10^8			111.000	8.05×10^7
4	100.000	2.160	171.000	1.25×10^8	100.000	2.410	132.000	9.55×10^7
5			212.000	1.54×10^8			134.000	9.7×10^7
6	100.000	1.620	226.000	1.64×10^8	100.000	2.110	138.000	1.00×10^8
7			230.000	1.67×10^8			123.000	8.9×10^7
8	100.000	2.520	205.000	1.49×10^8	100.000	2.170	148.000	1.085×10^8
9			232.000	1.68×10^8			170.000	1.235×10^8
10	82.500	2.100	274.000	1.86×10^8	77.800	2.030	173.000	1.26×10^8
11			252.000	1.83×10^8			183.000	1.335×10^8
12	71.900	2.140	288.000	2.09×10^8	91.100	2.170	165.000	1.2×10^8
13			180.000	1.31×10^8			146.000	1.06×10^8
14			281.000	2.05×10^8	78.500	2.120	160.000	1.165×10^8
	COME BY CHANCE 25 °C				COME BY CHANCE 4 °C			
DAY	CARB	1-MN	DRY WT.	CFU / mL	CARB	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	135.000	1.69×10^8	100.000	100.000	133.000	1.66×10^8
1			284.000	3.6×10^8			240.000	3.04×10^8
2	100.000	3.140	326.000	4.06×10^8	100.000	2.370	277.000	3.51×10^8
3			348.000	4.38×10^8			293.000	3.7×10^8
4	100.000	3.050	344.000	4.34×10^8	100.000	3.240	392.000	4.92×10^8
5			293.000	3.7×10^8			320.000	4.02×10^8
6	100.000	3.180	420.000	5.28×10^8	100.000	2.510	257.000	3.25×10^8
7			384.000	4.82×10^8			359.000	4.5×10^8
8	100.000	2.700	374.000	4.7×10^8	97.500	3.040	434.000	5.48×10^8
9			422.000	5.3×10^8			451.000	5.68×10^8
10	40.200	2.860	428.000	5.4×10^8	35.000	2.520	478.000	6.0×10^8
11			374.000	4.7×10^8			316.000	3.98×10^8
12	10.700	3.150	341.000	4.28×10^8	46.300	3.400	348.000	4.36×10^8
13			412.000	5.19×10^8			301.000	3.8×10^8
14			444.000	5.59×10^8	56.400	3.160	295.000	3.72×10^8
	PORT AUX BASQUES 25 °C				PORT AUX BASQUES 4 °C			
DAY	CARB	1-MN	DRY WT.	CFU / mL	CARB	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	91.000	1.14×10^8	100.000	100.000	75.000	9.4×10^7
1			93.000	1.16×10^8			81.000	1.01×10^8
2	100.000	2.190	234.000	2.97×10^8	100.000	5.220	138.000	1.75×10^8
3			236.000	3.0×10^8			163.000	2.07×10^8
4	100.000	2.680	288.000	3.64×10^8	100.000	2.800	247.000	3.14×10^8
5			251.000	3.18×10^8			151.000	1.915×10^8
6	100.000	2.430	244.000	3.09×10^8	100.000	2.330	265.000	3.34×10^8
7			258.000	3.27×10^8			255.000	3.23×10^8
8	100.000	2.190	266.000	3.36×10^8	63.230	2.430	209.000	2.64×10^8
9			258.000	3.27×10^8			263.000	3.33×10^8
10	84.600	3.040	264.000	3.34×10^8	83.500	2.440	282.000	3.58×10^8
11			211.000	2.67×10^8			234.000	2.97×10^8
12	51.000	2.750	206.000	2.6×10^8	71.000	2.430	230.000	2.92×10^8
13			175.000	2.21×10^8			234.500	2.98×10^8
14			206.000	2.6×10^8	84.000	3.080	247.000	3.14×10^8

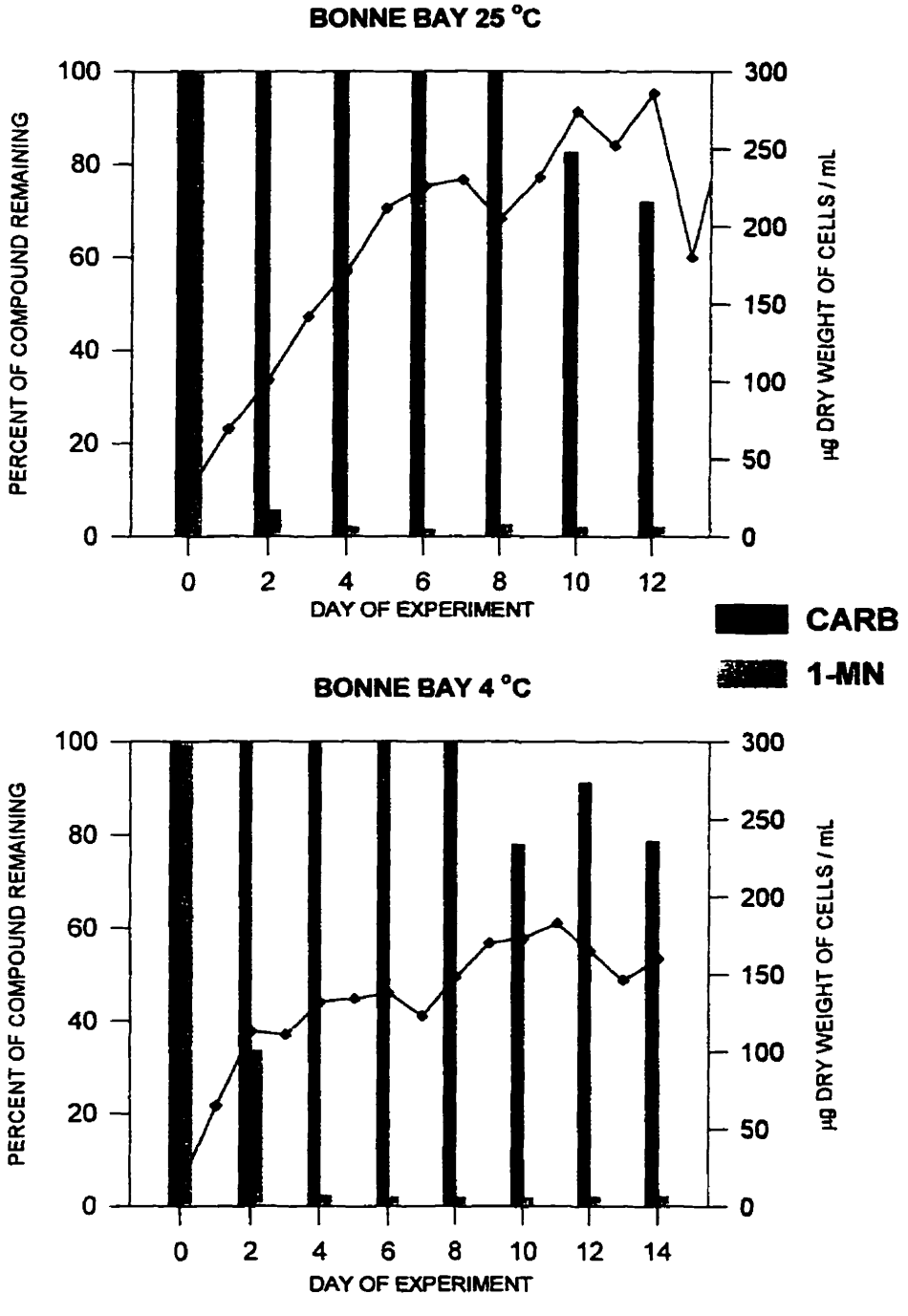


Figure 3.21 Time course of the degradation of 1-MN and CARB and the resulting growth of the Bonne Bay bacterial consortium (µg dry weight of cells/ mL; ± 24.67) incubated at 25 °C and 4 °C.

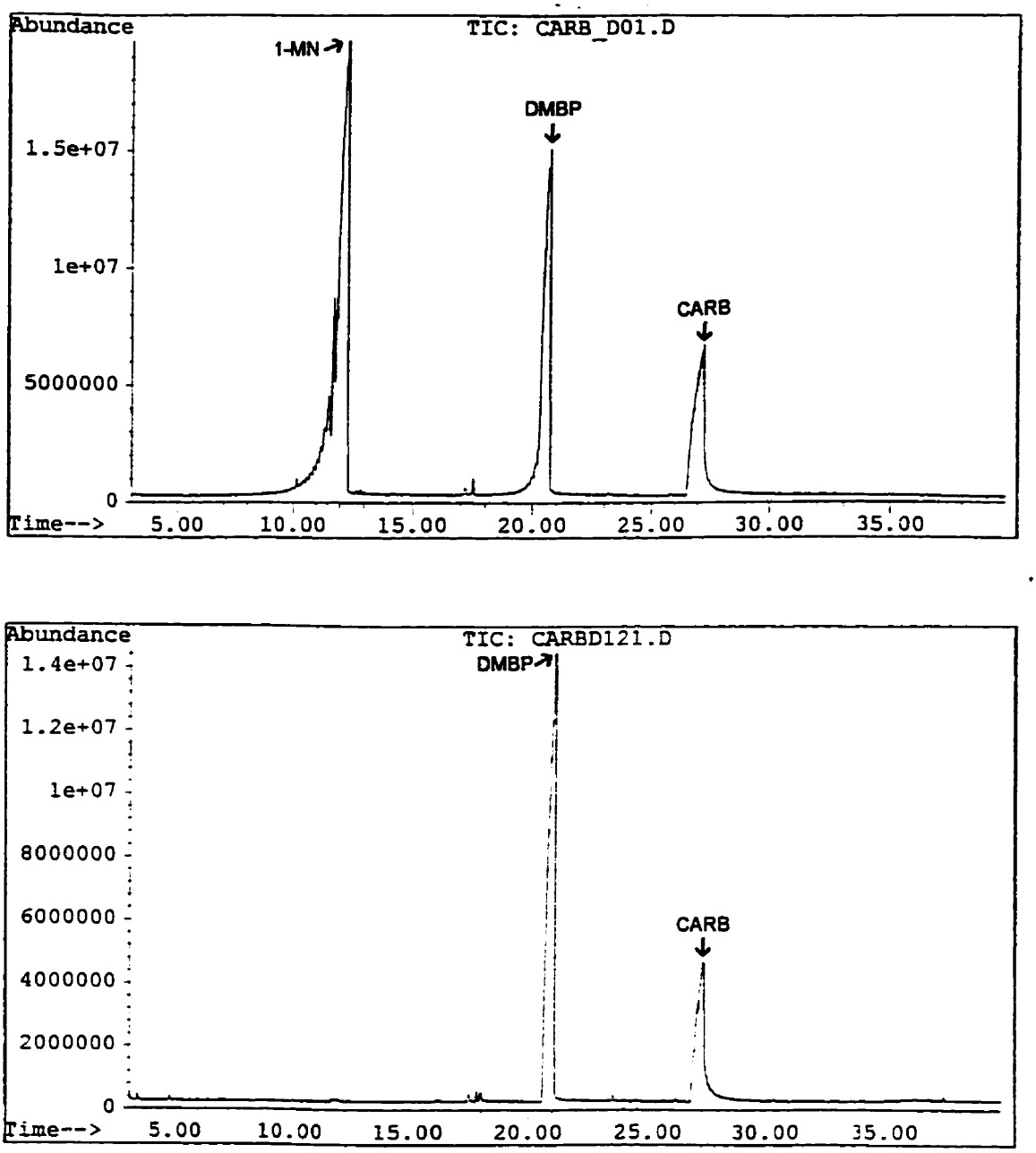


Figure 3.22 Total ion chromatograms of samples from the Bonne Bay culture incubated at 25 °C showing the abundance of CARB, 1-MN and DMBP on the first (top) and final (bottom) days of the experiment.

Degradation of CARB was initiated by the Come by Chance culture by day 10 at 25 °C, but by day 8 at 4 °C (Figure 3.23). Only 11% of the original concentration of CARB remained after 12 days in the 25 °C experiment. At 4 °C, 56% remained after day 14 but this value had increased from 46% at day 12 and 35% at day 10. At 25 °C, the greatest rate of CARB degradation occurred between day 8 and day 10 at a rate of 59.2% in 48 hours or 29.6%/day resulting in an average degradation rate of 3 mg CARB/day. The fastest rate of bacterial growth associated with CARB degradation occurred between days 8 and 9 at a rate of 48 μg/day which agreed with the fastest degradation rate. At 4 °C, the fastest rate of bacterial growth occurred from day 6 to day 7 at a rate 102 μg/day and this was associated with the initial degradation of CARB from 100 to 97.5%.

Similar to the Come by Chance culture, the Port aux Basques culture had initiated degradation of CARB by day 10 at 25 °C but by day 8 at 4 °C (Figure 3.24). Only 51% of the CARB remained after 12 days in the 25 °C experiment, while at 4 °C this value fluctuated between 63% and 84% from day 8 to day 14. The greatest rate of CARB degradation at 25 °C for this culture was between days 10 and 12 at an average rate of 1.7 mg/day. A small growth rate occurred between days 9 and 10 at a rate of 6 μg/day but a constant decline in growth ensued. At 4 °C, the greatest rate of CARB degradation occurred from day 6 to day 8 but this coincided with a large drop in bacterial mass. The greatest rate of cell growth was recorded from day 8 to day 9 at 54 μg/day which was associated

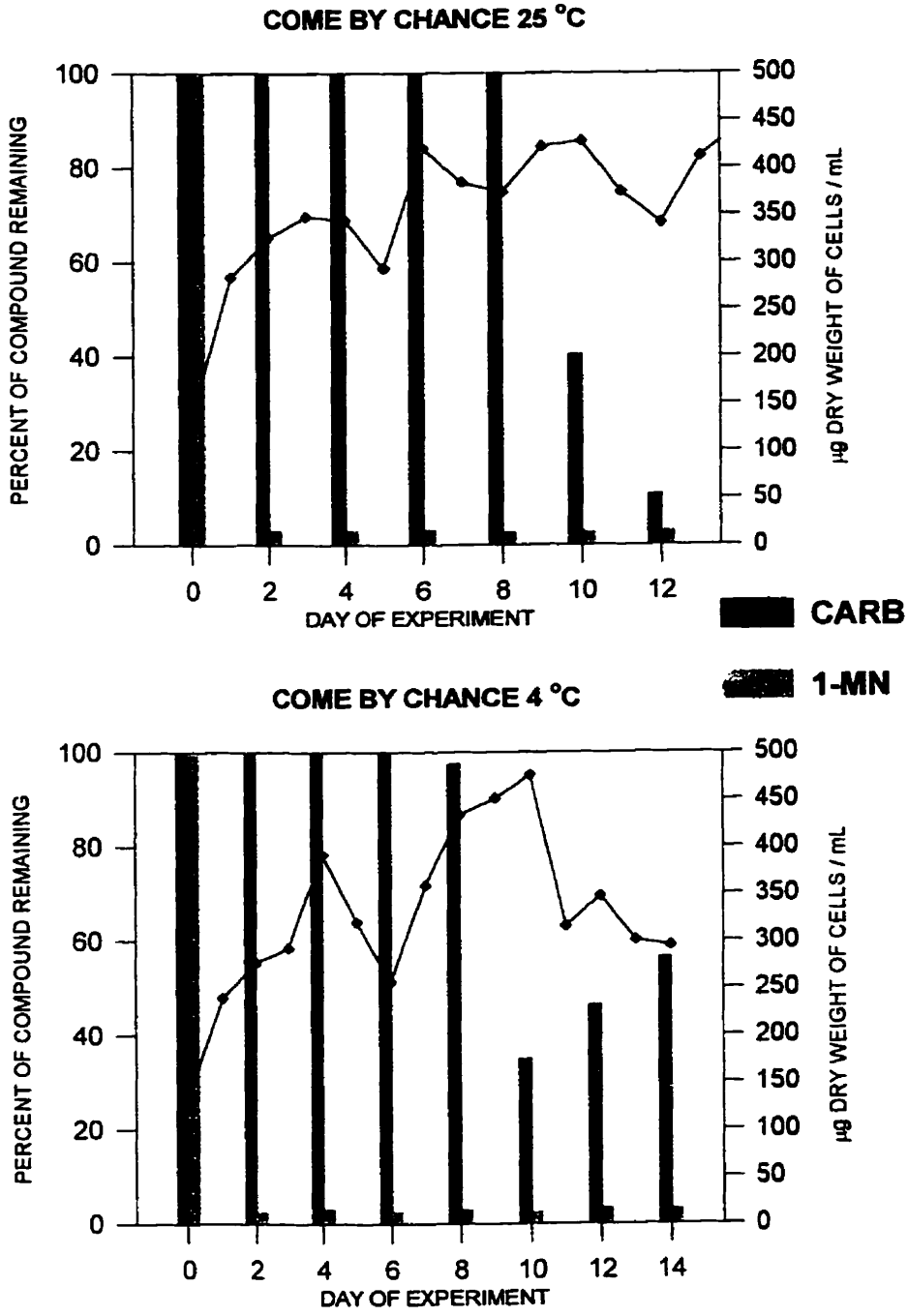


Figure 3.23 Time course of the degradation of 1-MN and CARB and the resulting growth of the Come by Chance bacterial consortium (µg dry weight of cells / mL; ± 10.41) incubated at 25 °C and 4 °C.

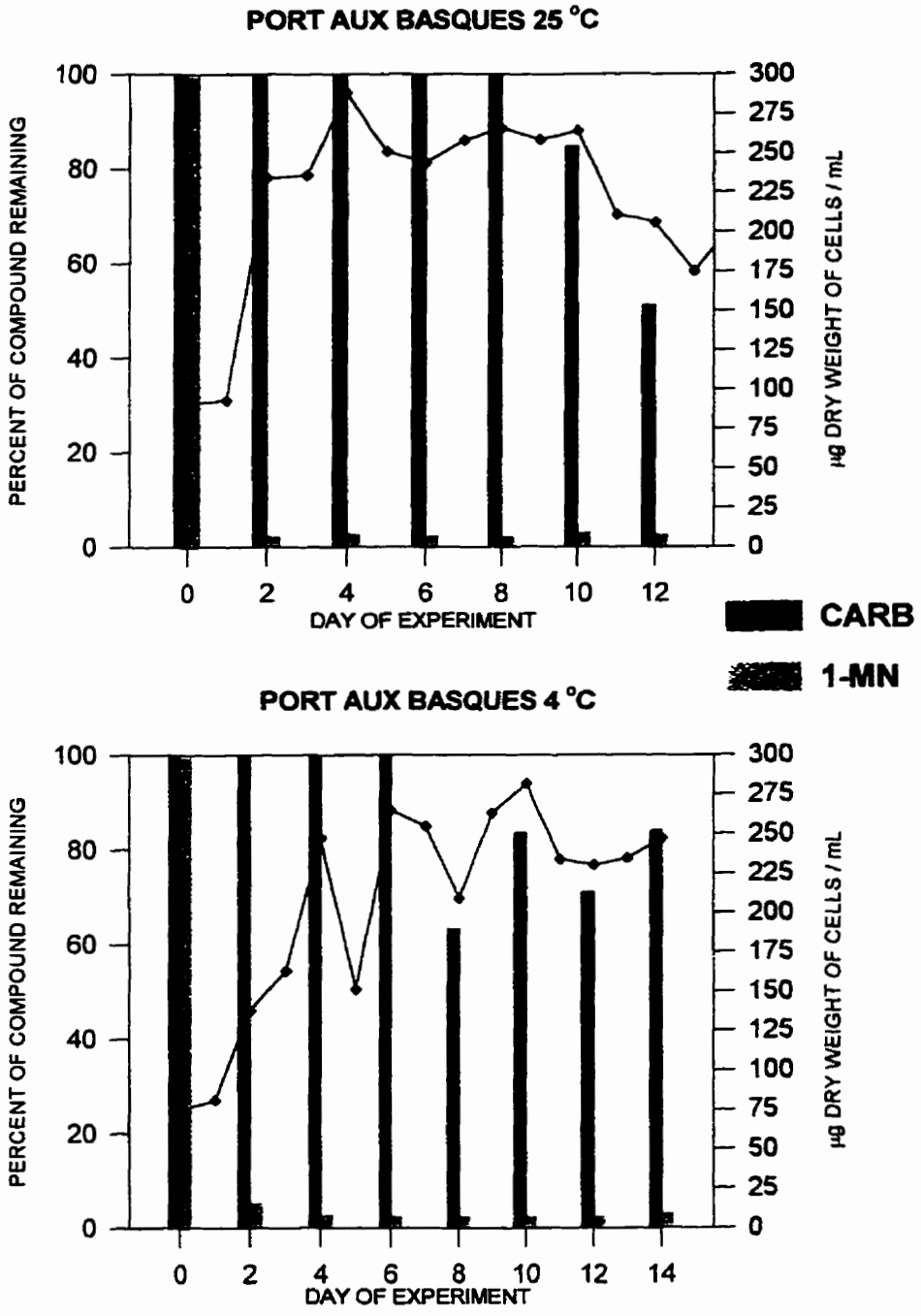


Figure 3.24 Time course of the degradation of 1-MN and CARB and the resulting growth of the Port aux Basques bacterial consortium (µg dry weight of cells / mL; ± 7.64) incubated at 25 °C and 4 °C.

with an increase in the amount of CARB remaining in the media.

In the 25 °C experiment, CARB degradation was initiated by all 3 cultures by day 10. It was also on day 10 that the Bonne Bay culture started to degrade CARB at 4 °C, while the Come by Chance and Port aux Basques cultures commenced degradation on day 8 at this temperature. Once initiated, however, the rate of CARB degradation at 25 °C was at least twice as fast as that reported at 4 °C. The fact that it took 8 to 10 days to begin CARB degradation lends support to the concept that the 3-ringed structure is more stable and much more recalcitrant to microbial attack than the 2-ringed structures.

Studies on the microbial degradation of CARB used bacteria isolated from soil (Grosser *et al.*, 1991), groundwater (Mueller *et al.*, 1991) and water from lakes, streams and sewage plants (Bohonos *et al.*, 1977). These CARB-degrading bacteria have been found to belong to the genera *Pseudomonas* (Finnerty *et al.*, 1983; Foght and Westlake, 1988; Ouchiyama *et al.*, 1993; Resnick *et al.*, 1993; Hisatsuka and Sato, 1994; Kimura and Omori, 1995 ; Gieg *et al.*, 1996; Shotbolt-Brown *et al.*, 1996), *Xanthomonas* (Grosser *et al.*, 1991; Shotbolt-Brown *et al.*, 1996) and *Burkholderia* (Shotbolt-Brown *et al.*, 1996).

Studies to determine the pathways for the bacterial degradation of CARB have been undertaken by several investigators. Resnick *et al.* (1993) reported that CARB oxidation by naphthalene 1,2-dioxygenase from *Pseudomonas sp. NCIB 9816-4* and biphenyl dioxygenase from *Beijerinckia sp. B8/36* yielded 3-

hydroxycarbazole. They proposed that the initial dioxygenation of CARB yielded an unstable *cis* carbazole-3,4-dihydrodiol which lost water and formed 3-hydroxycarbazole (Figure 3.25). However, no CARB dihydrodiol was detected so they could not eliminate the possibility of direct monooxygenation.

Ouchiyama *et al.* (1993) isolated two *Pseudomonas spp.* (CA6 and CA10) that assimilated CARB as the sole source of carbon and nitrogen. Using HPLC and GC-MS they determined that anthranilic acid and catechol were the main degradation products of CARB biodegradation. They reported that CARB was dioxygenated at the angular position yielding a dihydroxylated intermediate which was spontaneously converted to 2'-aminobiphenyl-2,3-diol. An extradiol dioxygenase then attacked the hydroxylated ring at the *meta* position and hydrolysis of this product gave anthranilic acid, which was converted to catechol and further metabolized by the β-ketoadipate pathway (Figure 3.26).

In a more recent study Kimura *et al.* (1996) described the isolation and characterization of transposon Tn5 mutants of *Pseudomonas sp. CA10* deficient in CARB catabolism, and the cloning of the genes involved in CARB catabolism. They concluded that the CARB catabolic pathway comprises at least two different gene clusters; an upper pathway (from CARB to anthranilic acid) cluster and a lower pathway (β-ketoadipate pathway) gene cluster. They suggested that a detailed sequence analysis of these gene clusters may elucidate a mechanism allowing recalcitrant compounds to undergo microbial degradation.

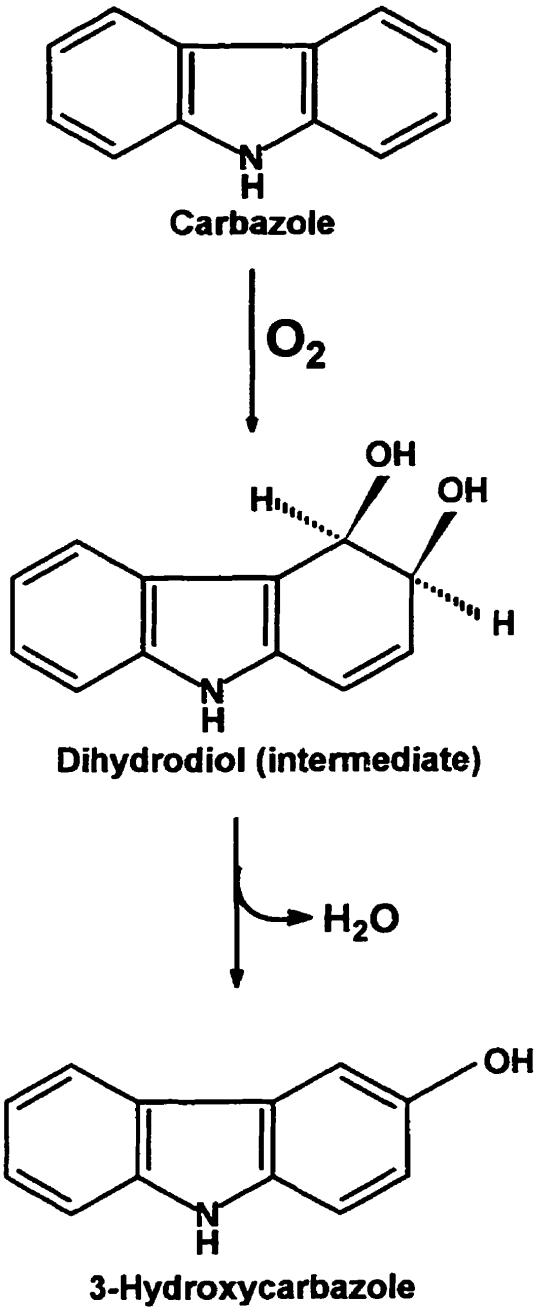


Figure 3.25 Reaction for the formation of 3-hydroxycarbazole by naphthalene dioxygenase and biphenyl dioxygenase as proposed by Resnick *et al.*, 1993.

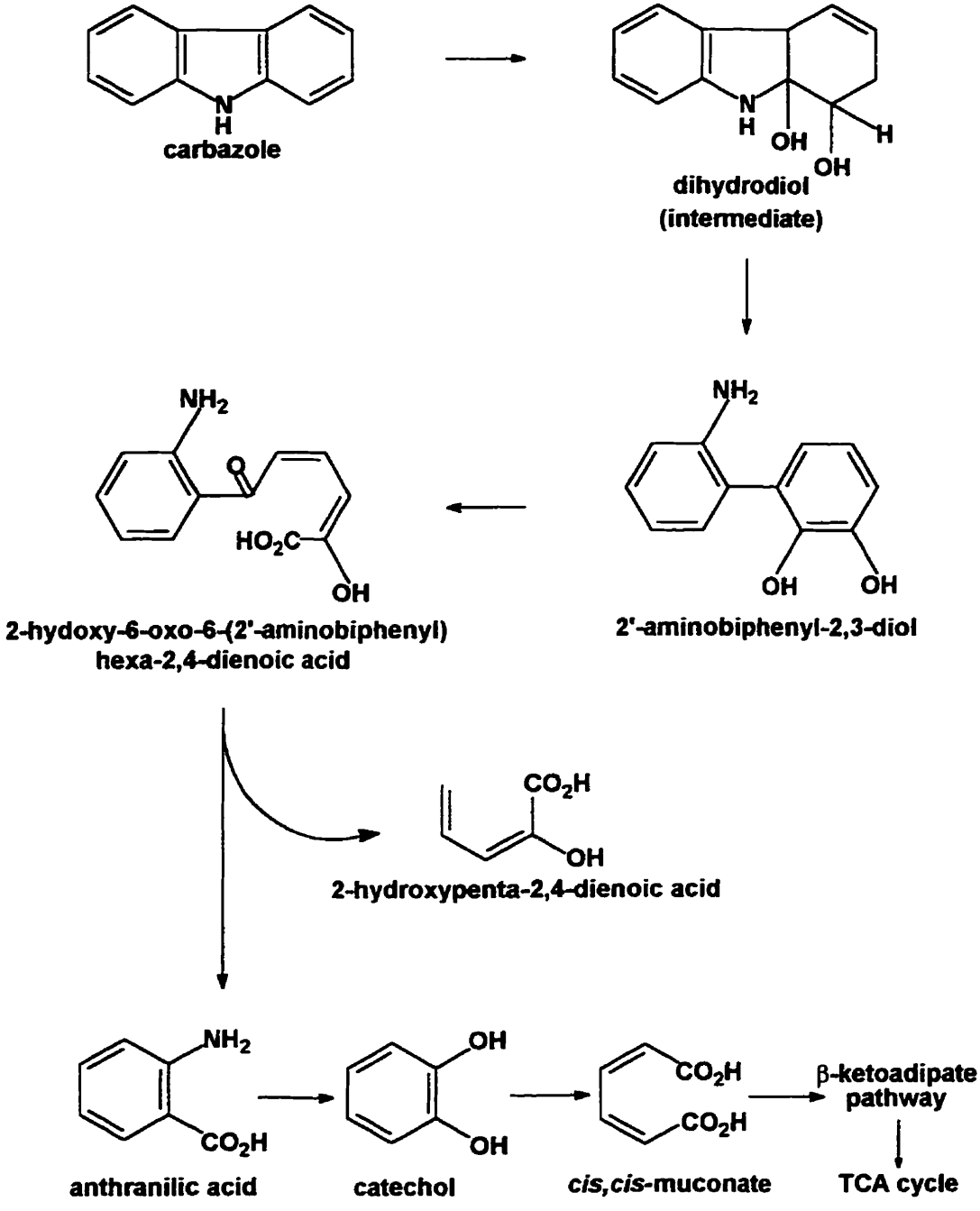


Figure 3.26 Pathway of carbazole degradation by *Pseudomonas sp. CA10* as proposed by Kimura *et al.* (1996).

Gieg *et al.* (1996) used a CARB degrading bacterium designated *Pseudomonas sp. LD2*, isolated from creosote-contaminated soil, to describe some of the metabolic characteristics and to identify some metabolites of CARB degradation. Similar to Ouchiyama *et al.* (1993) they identified anthranilic acid and catechol as intermediates. They also observed 10 additional nitrogen-containing metabolites and conclusively identified 4 of them as indole-3-acetic acid, 5-(2-aminophenyl)-5-oxopenteanoic acid, and the cyclicized product of 5-(2-aminophenyl)-3-oxopent-3-enoic acid and 6-(2-amino-phenyl)-2-hydroxy-6-oxohexa-2,4-dienioc acid. Their results support the hypothesis that CARB is initially oxidized by angular dioxygenation.

In this experiment, no transformation products were detected by GC-MS and FTIR as the CARB was degraded, for the same reasons as discussed previously for the PASH.

3.2.5 Dibenzofuran

The results from the experiment with DBF are summarized in Table 3.8. At 25 °C the Bonne Bay culture had started to degrade DBF by day 2 but when incubated at 4 °C, degradation was not initiated by this culture until day 6 (Figure 3.27). At 25 °C, 25% of the DBF remained after 12 days while 73% remained after 14 days at 4 °C. The TIC of the 25 °C culture is presented for the first and final days of the experiment (Figure 3.28). There was a 75% loss of DBF

Table 3.8 Numerical characterization of the DBF (%), 1-MN (%), dry weight (μg/mL) and colony forming units/mL (CFU/mL) levels throughout the experimental duration

	BONNE BAY 25 °C				BONNE BAY 4 °C			
DAY	DBF	1-MN	DRY WT.	CFU / mL	DBF	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	15.500	2.7×10^7	100.000	100.000	55.000	9.7×10^7
1			202.000	3.59×10^8			51.500	9.0×10^7
2	61.200	3.770	259.000	4.6×10^8	100.000	86.200	81.000	1.42×10^8
3			224.000	4.0×10^8			60.000	1.05×10^8
4	41.400	3.350	265.000	4.7×10^8	100.000	12.200	110.500	1.93×10^8
5			280.000	4.965×10^8			177.000	3.16×10^8
6	42.300	4.670	279.000	4.92×10^8	75.200	3.700	188.000	3.33×10^8
7			222.000	3.96×10^8			162.000	2.89×10^8
8	39.300	4.040	219.000	3.86×10^8	63.600	2.050	186.000	3.31×10^8
9			232.000	4.12×10^8			190.000	3.37×10^8
10	27.500	3.530	255.000	4.52×10^8	70.100	2.870	173.000	3.08×10^8
11			216.000	3.82×10^8			178.500	3.18×10^8
12	24.800	2.900	240.000	4.24×10^8	50.720	3.240	141.500	2.51×10^8
13			231.000	4.10×10^7			138.500	2.45×10^8
14					73.050	2.950	161.000	2.86×10^8
	COME BY CHANCE 25 °C				COME BY CHANCE 4 °C			
DAY	DBF	1-MN	DRY WT.	CFU / mL	DBF	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	11.000	2.0×10^7	100.000	100.000	28.500	5.2×10^7
1			93.500	1.71×10^8			17.500	3.2×10^7
2	100.000	3.720	105.500	1.92×10^8	100.000	100.000	30.000	5.5×10^7
3			108.500	1.99×10^8			33.500	6.1×10^7
4	100.000	4.080	119.500	2.19×10^8	88.800	5.200	57.000	1.05×10^8
5			139.000	2.73×10^8			80.500	1.48×10^8
6	47.800	4.220	130.500	2.4×10^8	88.400	4.300	81.500	1.49×10^8
7			88.000	1.61×10^8			67.000	1.22×10^8
8	45.400	1.940	108.000	1.98×10^8	86.700	5.200	82.000	1.5×10^8
9			107.500	1.97×10^8			82.500	1.51×10^8
10	32.800	4.320	109.500	2.01×10^8	85.300	4.300	98.500	1.81×10^8
11			106.500	1.94×10^8			113.000	2.06×10^8
12	20.700	3.340	104.500	1.91×10^8	77.000	4.300	77.500	1.42×10^8
13			106.000	1.94×10^8			78.500	1.44×10^8
14					81.600	3.300	80.500	1.48×19^8
	PORT AUX BASQUES 25 °C				PORT AUX BASQUES 4 °C			
DAY	DBF	1-MN	DRY WT.	CFU / mL	DBF	1-MN	DRY WT.	CFU / mL
0	100.000	100.000	20.500	3.6×10^7	100.000	100.000	32.500	4.8×10^7
1			160.000	2.87×10^8			25.500	4.3×10^7
2	37.330	5.400	112.500	2.1×10^8	100.000	85.720	41.000	7.3×10^7
3			114.500	2.05×10^8			44.500	8.0×10^7
4	9.820	5.000	164.500	2.95×10^8	100.000	46.730	78.000	1.39×10^8
5			203.500	3.66×10^8			97.000	1.75×10^8
6	8.280	4.600	207.000	3.72×10^8	57.980	3.780	99.000	1.82×10^8
7			165.000	2.97×10^8			93.000	1.67×10^8
8	4.460	3.300	174.000	3.12×10^8	66.140	3.570	118.000	2.12×10^8
9			170.500	3.07×10^8			117.500	2.1×10^8
10	4.670	3.400	189.000	3.42×10^8	69.160	4.860	145.500	2.61×10^8
11			170.500	3.07×10^8			140.000	2.51×10^8
12	6.240	3.770	170.000	3.06×10^8	62.410	3.420	121.000	2.17×10^8
13			147.000	2.64×10^8			103.000	1.84×10^8
14					45.360	3.020	134.500	2.41×10^8

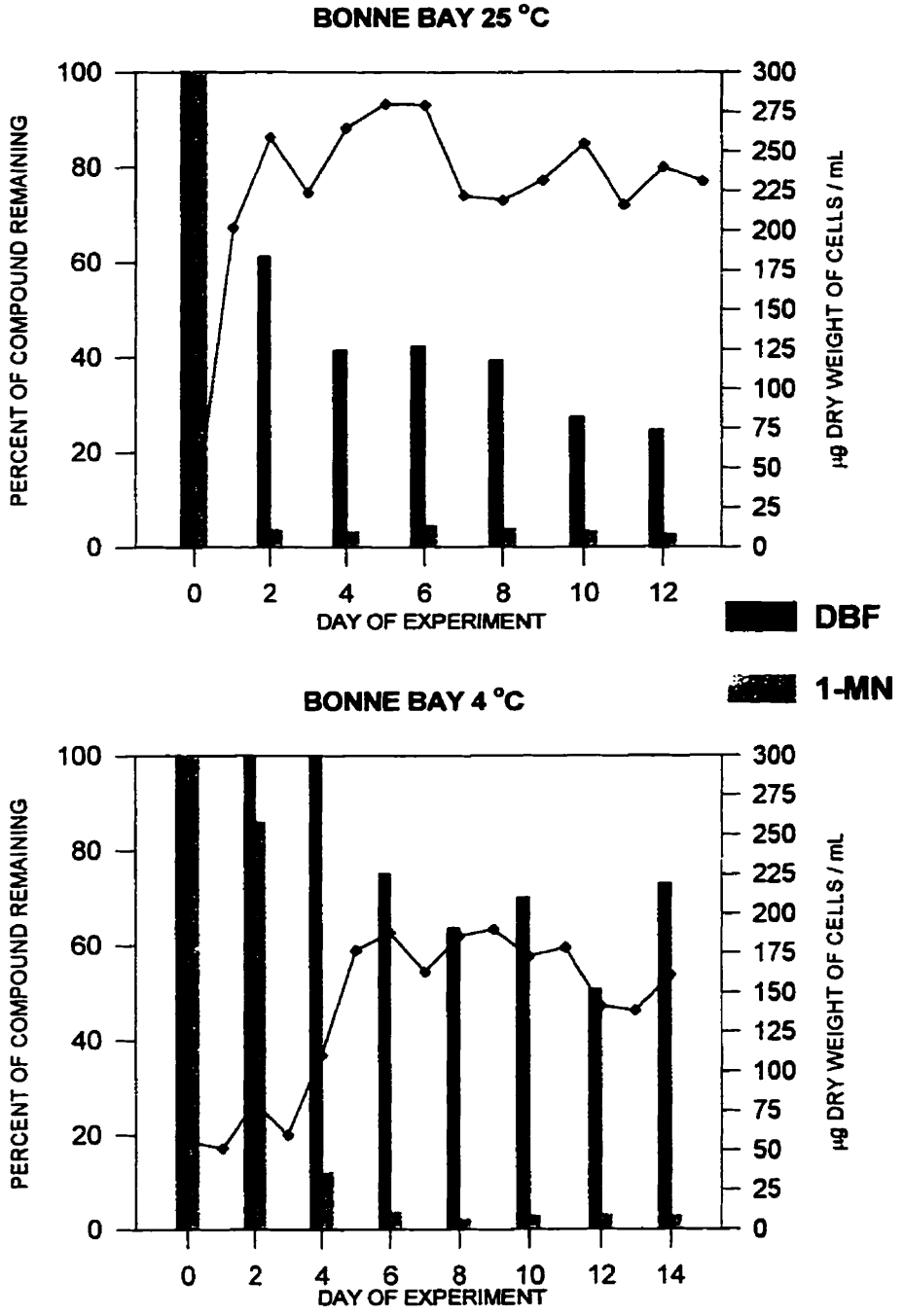


Figure 3.27 Time course of the degradation of 1-MN and DBF and the resulting growth of the Bonne Bay bacterial consortium (µg dry weight of cells / mL; ± 16.07) incubated at 25 °C and 4 °C.

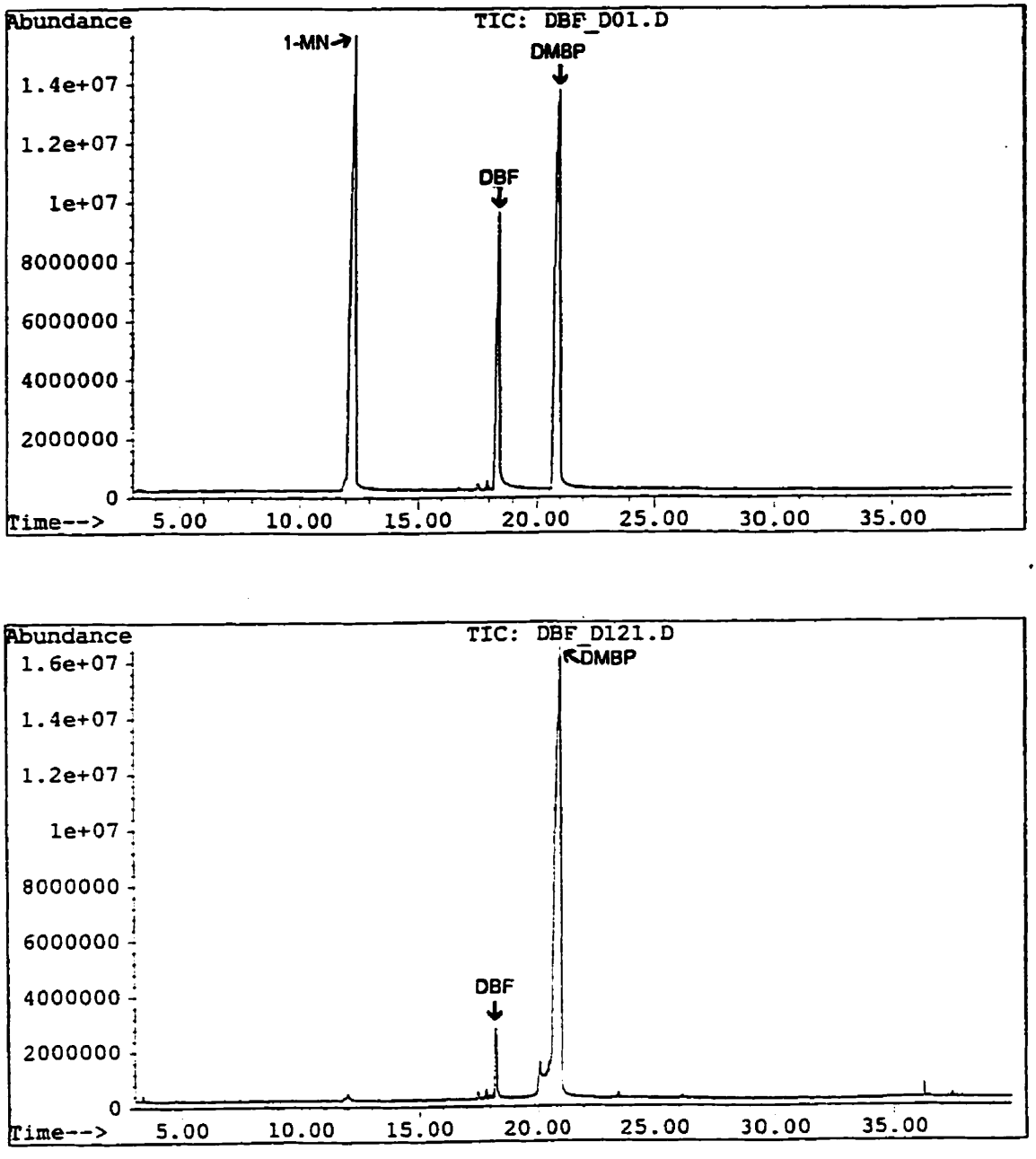


Figure 3.28 Total ion chromatograms of samples from the Bonne Bay culture incubated at 25 °C showing the abundance of DBF, 1-MN and DMBP on the first (top) and final (bottom) days of the experiment.

(retention time: 18.4 min.) from day 0 to day 12 and no transformation products accumulated from day 0 to day 12. The GC-MS results of the other 2 bacterial cultures at both 25 °C and 4 °C are similar to Figure 3.28.

The fastest rate of DBF degradation at 25 °C occurred from day 0 to day 2 at 39% in 48 hours, which translates to 19%/day or 1.9 mg DBF/day (Figure 3.27). This concurred with the fastest rate of bacterial growth which was recorded from day 0 to day 1 at a rate of 187 μg/day. At 4 °C, the greatest rate of DBF degradation occurred from day 4 to day 6 at a rate of 12%/day or 1.2 mg/day and the fastest bacterial growth rate coincided with this as 66.5 μg/day was recorded between day 4 and day 5.

DBF degradation was initiated by the Come by Chance culture by day 6 at 25 °C and day 4 at 4 °C (Figure 3.29). Only 21% of the original concentration of DBF remained after day 12 for the 25 °C incubated culture while 82% remained for the 4 °C incubated culture after 14 days. At 25 °C, the greatest rate of DBF degradation occurred between day 4 and day 6 at a rate of 52% in 48 hours or an average of 2.6 mg DBF/day. A bacterial growth rate of 19.5 μg/day from day 4 to day 5 accompanied this degradation rate. At 4 °C, the fastest rate of bacterial growth occurred from day 3 to day 5 at a rate of 23.5 μg/day and this coincided with the initial degradation of DBF and also the greatest rate of DBF degradation at 0.56 mg/day from day 2 to day 4.

The Port aux Basques culture had initiated degradation of DBF by day 2

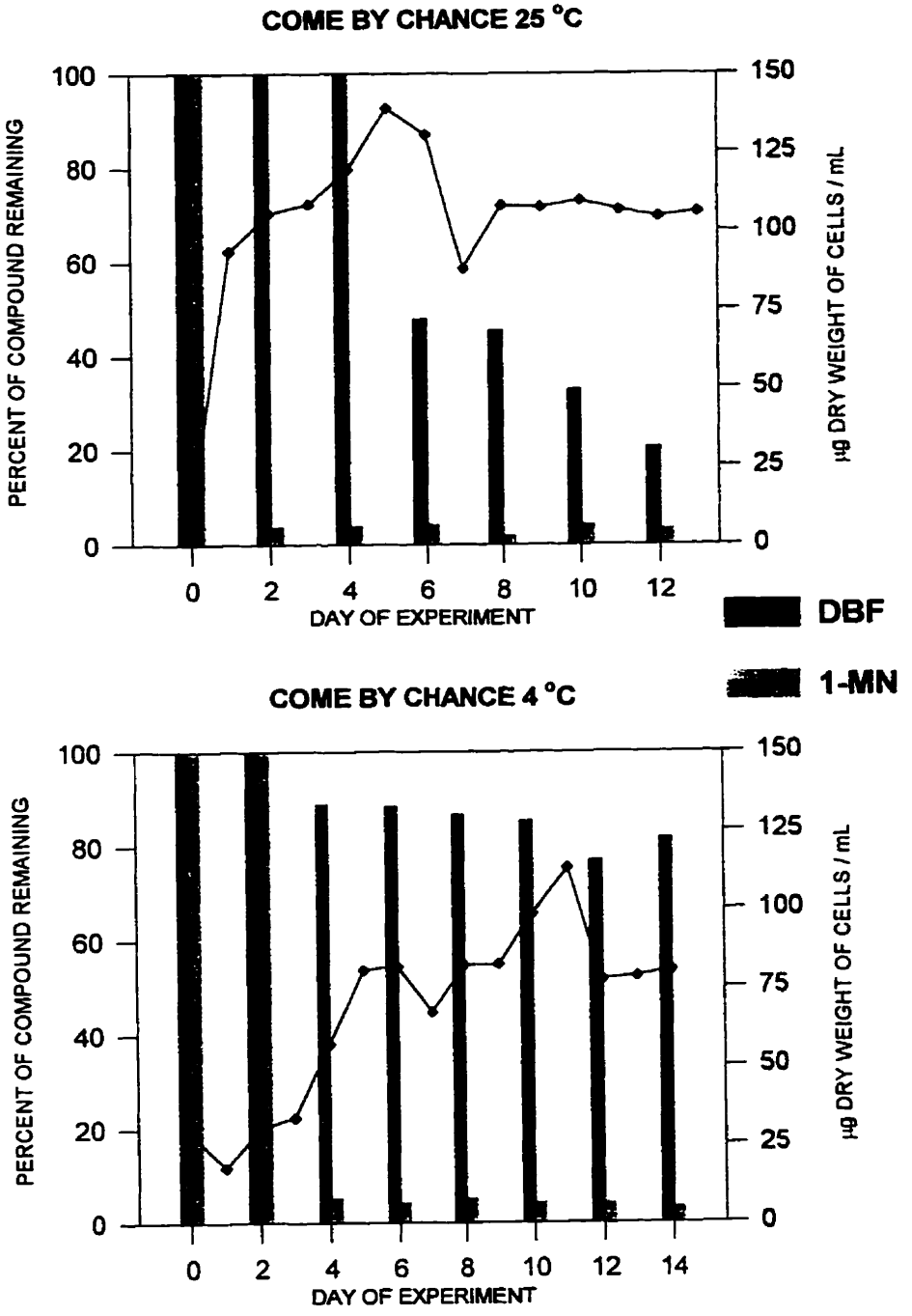


Figure 3.29 Time course of the degradation of 1-MN and DBF and the resulting growth of the Come by Chance bacterial consortium (µg dry weight of cells/ mL; ± 8.66) incubated at 25 °C and 4 °C.

at 25 °C and day 6 at 4 °C (Figure 3.30). At 25 °C, 6% of the original concentration of DBF remained after day 12 while 45% remained after 14 days in the 4 °C setup. The greatest rate of DBF degradation at 25 °C for this culture occurred between day 0 and day 2 at a rate of 63% in 48 hours or 3.1 mg DBF/day and was accompanied by a growth rate of 82.5 μg/day from day 0 to day 1 which was the highest rate observed for this culture at this temperature. At 4 °C, the greatest rate of DBF degradation occurred from day 4 to day 6 at 42% or 2.1 mg DBF/day. However, the percent of DBF remaining increases from day 6 to day 8 and from day 8 to day 10. The highest rate of bacterial growth associated with DBF degradation at 4 °C occurred between days 13 and 14 at a rate of 31.5 μg/day which did not correspond to the fastest degradation rate.

When the cultures were incubated with DBF the Bonne Bay and Port aux Basques cultures had initiated degradation by day 2 at 25 °C and day 6 at 4 °C. This shows that degradation occurred 3 times faster at 25 °C than at 4 °C. Results were much different for the Come by Chance culture which had initiated DBF degradation at 4 °C by day 4 but this did not occur until day 6 when incubated at 25 °C. Once degradation had started, however, the rate of degradation was more than 4 times faster at 25 °C than at 4 °C. In addition, the time to reach fastest bacterial growth rate was 4 times faster at 25 °C for the Bonne Bay and Come by Chance cultures and 13 times faster at 25 °C for the

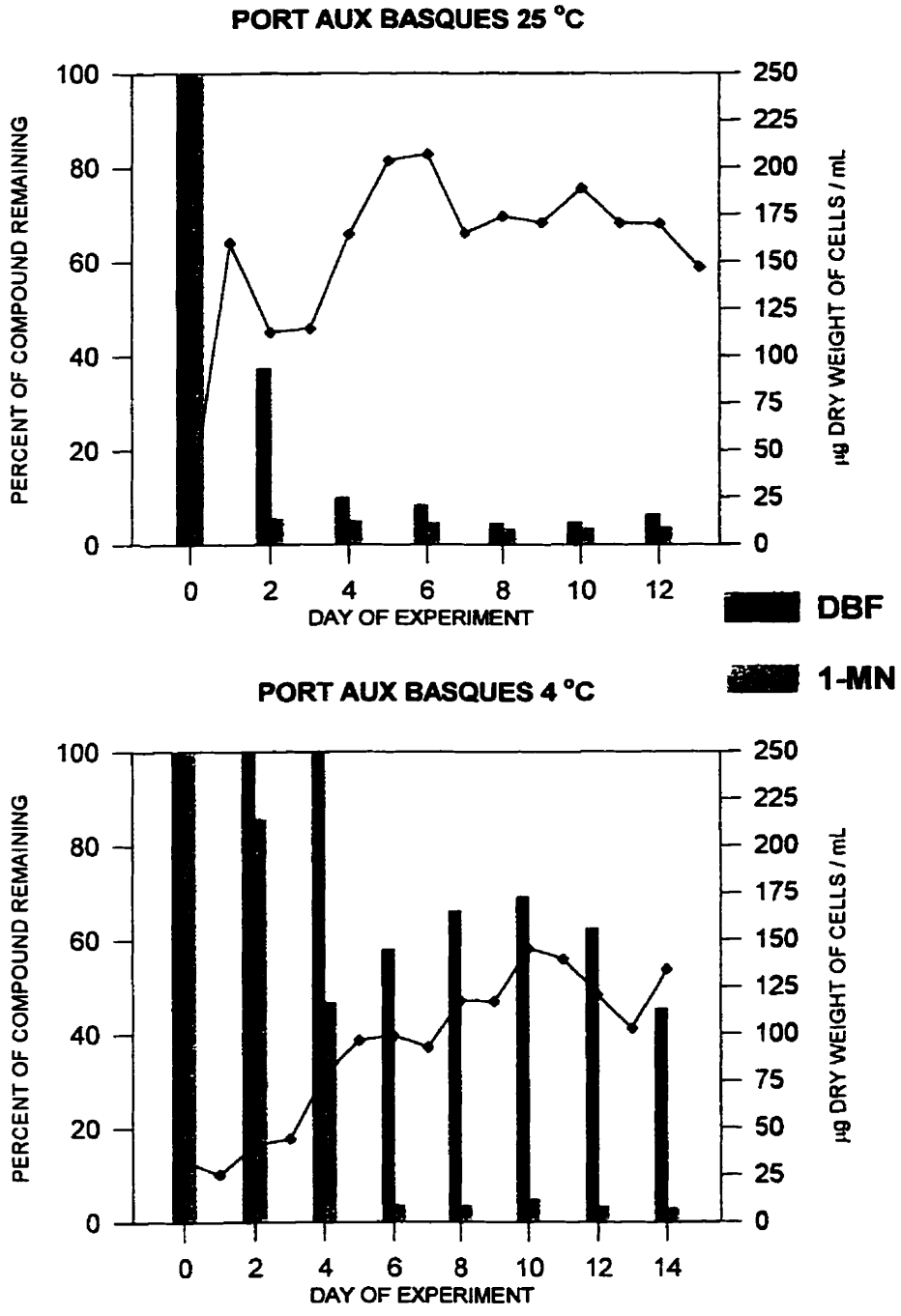


Figure 3.30 Time course of the degradation of 1-MN and DBF and the resulting growth of the Port aux Basques bacterial consortium (µg dry weight of cells/ mL; ± 11.55) incubated at 25 °C and 4 °C.

Port aux Basques culture. Consequently, the apparent faster degradation at 4 °C may be the result of the variability in extraction efficiency, or other such variation, as proposed previously.

The microbial degradation of DBF has been studied and degradation pathways elucidated. Cerniglia *et al.* (1979) investigated bacterial and fungal oxidation of DBF. Both *Cunninghamella elegans* and a mutant strain of *Beijerinckia* (B8/36) oxidized DBF to 2,3-dihydroxy-2,3-dihydrodibenzofuran (Figure 3.31). When formed through bacterial oxidation by *Beijerinckia B8/36* this metabolite was unstable and was rapidly converted into a mixture of 2- and 3-hydroxydibenzofuran under aerobic conditions. This did not occur with the fungal metabolite which therefore suggested that *Beijerinckia B8/36* and *C. elegans* form the *cis*- and *trans*- isomers of 2,3-dihydroxy-2,3-dihydrodibenzofuran, respectively. *Beijerinckia B8/36* also produced a second unstable dihydrodiol identified as 1,2-dihydroxy-1,2-dihydrodibenzofuran which gave 2-hydroxydibenzofuran as the major dehydration product. The results suggested that bacteria and fungi utilize different mechanisms to initiate the oxidation of DBF.

More recently, Engesser *et al.* (1989) used two DBF degrading bacteria, *Brevibacterium sp. DPO1361* and *DPO220*, which used fluorene as a sole source of carbon and energy. The cells transformed fluorene into several metabolites but one indicated the presence of a novel dioxygenase which

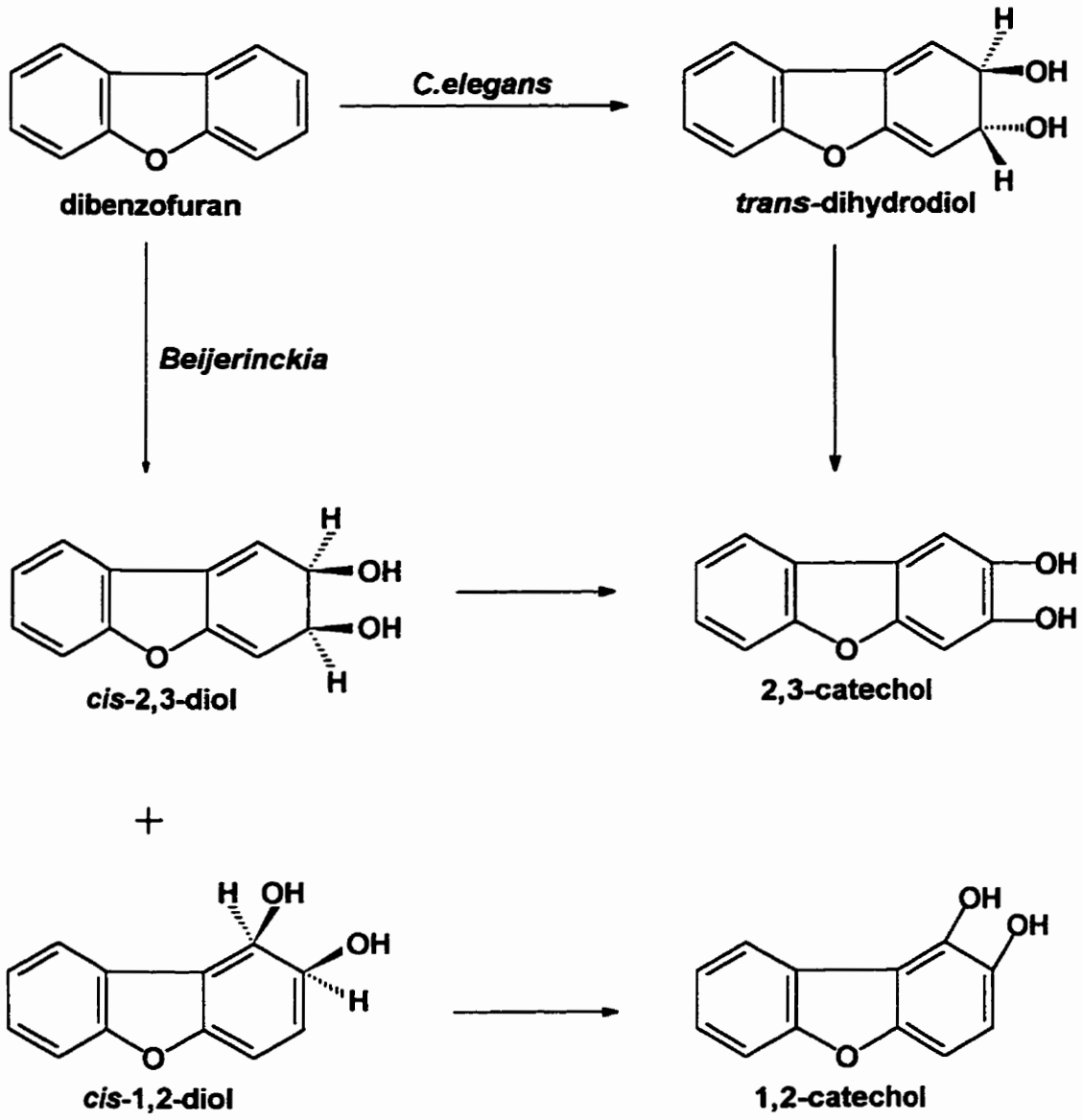


Figure 3.31 Reaction sequences for the initial reaction in the metabolism of dibenzofuran by *Beijerinckia* and *C.elegans* as proposed by Cerniglia *et al.* (1979).

attacked polynuclear aromatic systems in the unusual angular position. They proposed that DBF was likewise degraded via angular dioxygenation so that one aryl oxygen ether bond was transformed to a hemiacetal. The product of the first enzymatic reaction in the degradation sequence was therefore proposed to be 2,2',3-trihydroxybiphenyl.

In a subsequent investigation using the *Brevibacterium sp.DPO1361*, Strubel *et al.* (1991) determined that the 2,2',3-trihydroxybiphenyl was subject to *meta* ring cleavage. This yielded 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid which was hydrolyzed to 2-oxo-4-pentenoate and salicylate by 2-hydroxy-6-oxo-6-phenyl-2,4-hexadienoic acid hydrolase (Figure 3.32).

Similarly, a *Pseudomonas sp. HH69*, isolated by Fortnagel *et al.* (1990) converted DBF to 2,2',3-trihydroxybiphenyl and salicylic acid, again proving the presence of novel dioxygenation at the 4,4a position of DBF. The same pathway was again elucidated in an experiment by Monna *et al.* (1993) using *Staphylococcus auriculans DBF63* and also by Bertini *et al.* (1995) using *Sphingomonas sp. RWI*.

Using *Sphingomonas sp. HH69*, Harms *et al.* (1995) investigated the metabolism of 11 substituted dibenzofurans and illustrated the degradative pathways leading from the substrates to carbon dioxide and water. Strain HH69 utilized 7 of the 11 substituted dibenzofurans tested as the sole sources of carbon and energy. The 4 substrates which did not serve as carbon sources for

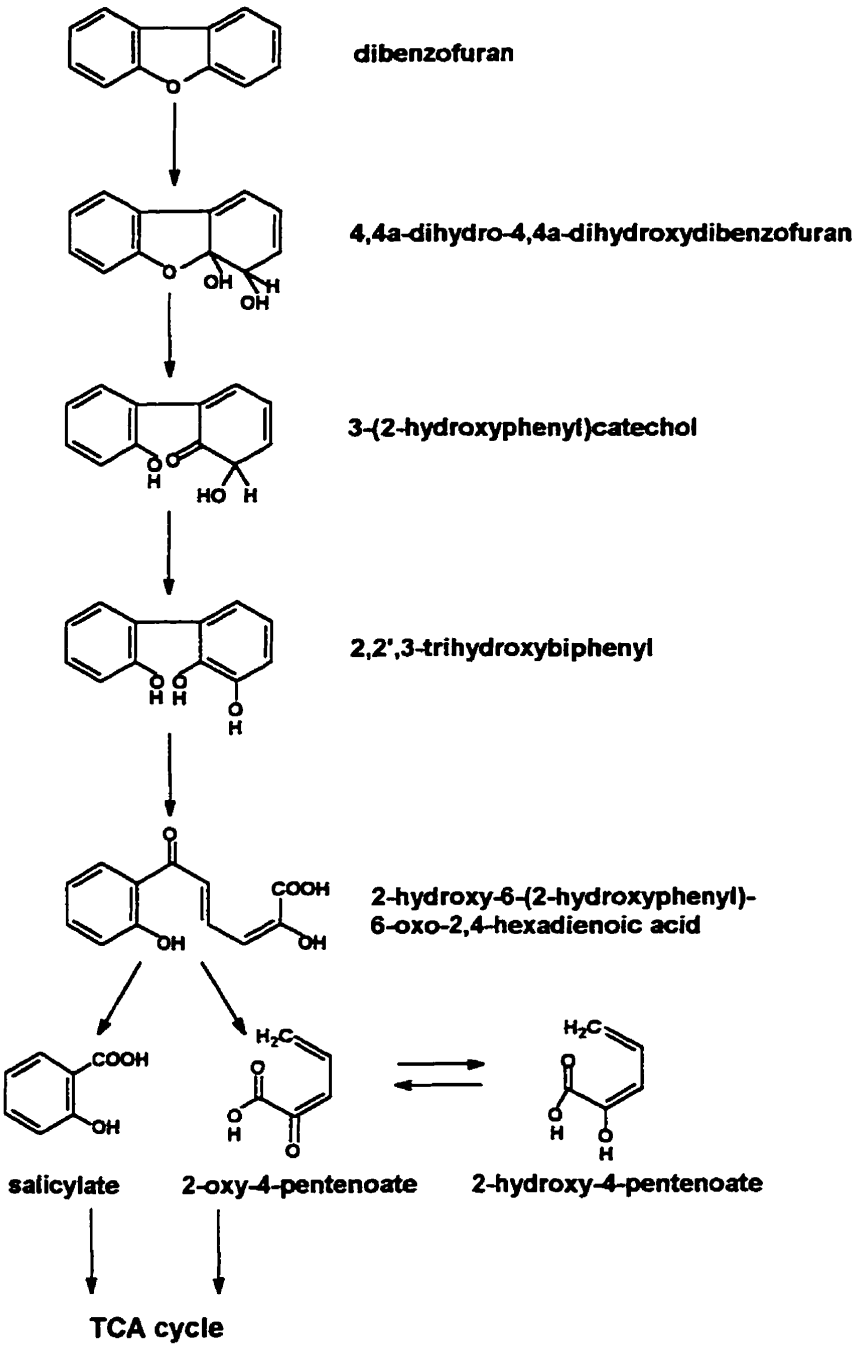


Figure 3.32 Degradation pathway for dibenzofuran as proposed by Strubel *et al.* (1991).

the strain were converted to salicylic acid derivatives that could not be utilized by the bacteria.

Like BT, DBT and CARB, the GC-MS and FTIR results did not reveal the presence of transformation products as DBF was degraded, probably for the same reasons as discussed earlier for these compounds.

Chapter IV: Conclusion

The graphs depicting the rate and extent of degradation observed for the 5 compounds suggest that the 2-ringed structures are more susceptible to microbial degradation than the 3-ringed compounds. Consequently, a two sample test of means was performed to determine if this was correct. This technique tests the hypothesis that the means of two populations are equal when the samples are small and the population standard deviations are unknown (assuming that the population standard deviations are equal) (Mansfield, 1986). In an attempt to define degradation as a function of time and quantity, the values employed for this test and all subsequent statistics were obtained by dividing the proportion of compound degraded by the end of each experiment, by the number of days required to initiate degradation. Therefore, in the case of the Bonne Bay sample in the BT experiment at 25 °C , a value of 44.35 was obtained by dividing 88.7% degradation by 2 (degradation initiated by day 2).

The test of means resulted in a t value of 4.382 for cultures incubated at 25 °C and 5.698 for the same incubated at 4 °C. The actual t value at 95% confidence ($t_{.025}$) and 13 degrees of freedom is 2.160 which is well below the values obtained. As a result, the means are significantly different and therefore, it is concluded that 2-ringed heterocycles are significantly more susceptible to microbial degradation than are the 3-ringed heterocycles studied, at both

incubation temperatures. This is likely the result of the extra stability provided by the second benzene ring, as explained previously.

The Bonne Bay bacterial consortium degraded 3-MBT more quickly and efficiently than BT while the Come by Chance consortium initiated BT degradation at 4 °C faster than 3-MBT at 4 °C, but there was more than twice as much BT remaining after day 10 of the experiment at both temperatures. The Port aux Basques consortium, however, seemed to degrade BT more efficiently than 3-MBT despite initiating degradation of both compounds on the same day, at each incubation temperature. A two sample test of means was performed to determine if BT degradation by all 3 cultures was equal to 3-MBT degradation at both temperature regimes. The result was a t value of 1.074 for cultures incubated at 25 °C and 0.868 for the same at 4 °C. The actual $t_{.025}$ for 4 degrees of freedom is 2.776 and because the calculated values are less than this value, it is concluded that the observed differences in the degradation of BT and 3-MBT are not statistically significant.

The water solubility of DBT is 1.0 mg/L, it is 6.5 mg/L for DBF, and 1.2 mg/L for CARB. A concentration of 10 mg/200mL (50 mg/L) was used in the present experiments so the compounds did not dissolve in the MSM when added. Consequently, the bacteria were not initially exposed to all of the compound added, and it is assumed that as some of the compound was degraded, more dissolved and became more available for microbial attack. The

concentration of chemicals in contaminated soils has been reported to be as high as 97mg/kg of soil (Erickson *et al.*, 1993) so the high concentrations of heterocyclic compounds used in this study better represent acute exposure of bacterial communities to S-, N- and O-heterocycles as a result of environmental contamination through oil or chemical spills.

Statistical analyses were also conducted to determine if either of DBT, CARB or DBF was degraded faster or to a greater extent than another. A one way analysis of variance (ANOVA) was the test employed for this purpose. An ANOVA is a technique designed to divide the total variation in a set of data in order to split up this total variation into component parts, each of which can be ascribed to a particular source (Mansfield, 1986). The F value obtained for experiments at 25 °C was 4.985 while 2.454 was the F value obtained for those at 4 °C. The actual $F_{.05}$ value at 2 and 6 degrees of freedom is 5.14 which is greater than the calculated values which means that the level of degradation is not significantly different for either DBT, CARB or DBT, at both 25 °C and 4 °C.

In order to determine if any one culture displayed a greater ability to degrade the heterocycles studied an ANOVA was performed for each incubation temperature. The F values obtained were 0.0471 for cultures incubated at 25 °C and 0.0794 for those at 4 °C. The actual $F_{.05}$ for 2 and 12 degrees of freedom is 3.89 which is much greater than the calculated values. Consequently, no one culture demonstrated a significantly greater ability to degrade the 5 heterocycles

studied.

Initially, it was expected that the Come by Chance and Port aux Basques consortia would degrade the heterocycles of study better than the consortium from Bonne Bay because the sediments from which they were isolated exhibited previous hydrocarbon contamination and past studies have reported that environments that have not been exposed to a contaminant do not harbour the bacteria capable of degrading the contaminant (Furukawa *et al.*, 1979; Fulthorpe and Wyndham, 1989; Hickey *et al.*, 1993; Brunsbach and Reineke, 1993; Theim *et al.*, 1994). However, a recent study by Fulthorpe *et al.* (1996) has shown that microbial populations from undisturbed and uncontaminated soils have the ability to mineralize 3-chlorobenzoate (3-CBA) and 2,4-dichlorophenoxyacetate (2,4-D). This suggests that 3-CBA and 2,4-D degrading bacteria are ubiquitous, even in pristine areas where one would not expect them to have a selective advantage. The Bonne Bay sediments, though probably not pristine, were collected from a beach in Gros Morne National Park and did not exhibit any visible signs of major PAH contamination. The bacterial community isolated from this beach sediment not only showed the ability to degrade all of the heterocycles used, but it also degraded these compounds at a rate that was not significantly different from the communities isolated from visibly contaminated sediments. This suggests that S-,N-, and O-heterocycle-degrading bacteria are also very widespread in areas where they are not selectively advantageous.

Furthermore, bacteria from uncontaminated areas have the ability to degrade these compounds as well as bacterial communities from visibly contaminated areas.

Finally, in an attempt to determine if the incubation temperature had a significant effect on the level of degradation of the heterocycles studied, a two sample test of means was conducted and a t value of 2.23 was obtained. The actual $t_{.025}$ for 28 degrees of freedom is 2.048 which is less than that calculated. Therefore, the observed decrease in level of degradation (3 to 5 times) with a decrease in temperature from 25 to 4 °C, is statistically significant.

Previous investigations have identified the biotransformation products of the heterocycles studied. Even though this study also attempted to identify these products, the main goal was to compare the rate and extent of biodegradation of these heterocycles, by three different bacterial communities, at an optimum temperature for culture and also at a temperature more typical of a northern environment. Previous results were obtained using a single bacterial species and allowed the isolation and identification of the degradation products. However, in the present study, degradation by the mixed field cultures did not proceed at a rate where the isolation and identification of the products could be achieved, except in the case of 3-MBT, and its sulfoxide.

The results from this study prove that bacterial communities from local marine beach sediments have the ability to degrade some of the S-, N-, and O-

heterocycles commonly found in crude oil. Biodegradation by these bacterial communities is achieved at optimum laboratory temperatures and, more importantly, at a temperature more typical of a northern environment, even though biodegradation is generally 3 to 5 times less at this temperature. One of the five objectives of the study was not completely achieved as the most prevalent bacterium from each bacterial consortium was not identified. However, a metabolic profile resulting from the Biolog analysis did provide some worthwhile information which could be useful to future investigators.

In future studies, an investigation of the variability of members and/ or enzyme systems within bacterial communities could provide helpful information. An investigation to determine if different types of heterocycles require different bacteria for degradation would also complement this study especially considering in this study the Come by Chance culture incubated at 4 °C was unable to degrade DBT while all other heterocycles were degraded to some degree by this culture. Modifications to the method to include other factors (such as nutrient addition) which may improve the ability of bacterial consortia to degrade heterocycles could also be a future consideration. In addition, sampling the seawater and sediment during sample collection, for analysis of PAH and/ or heterocycle content, could provide information to support the enhanced biodegradation of S-, N-, or O-heterocycles by a particular bacterial community.

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IMAGE EVALUATION
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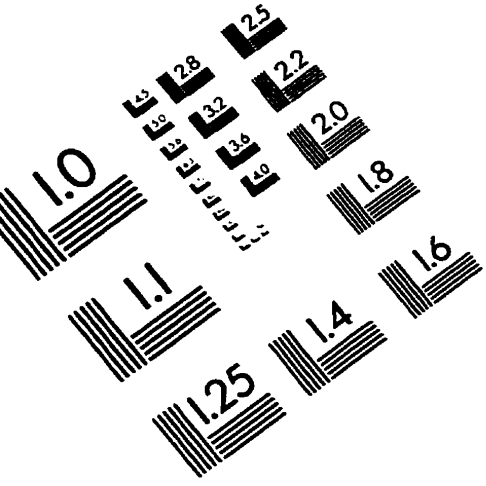

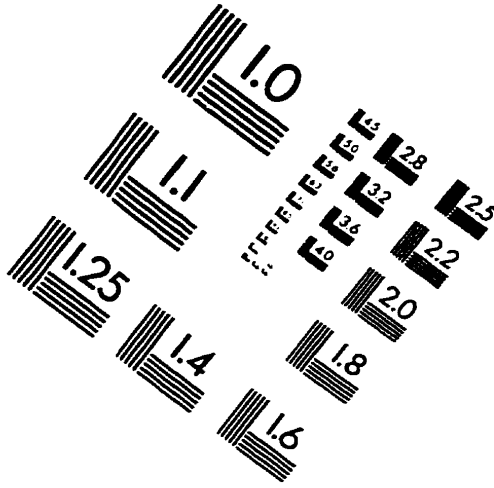

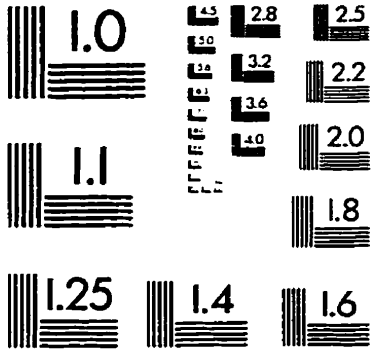

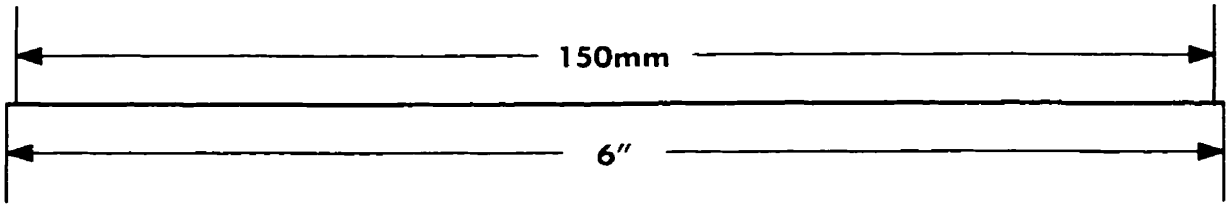

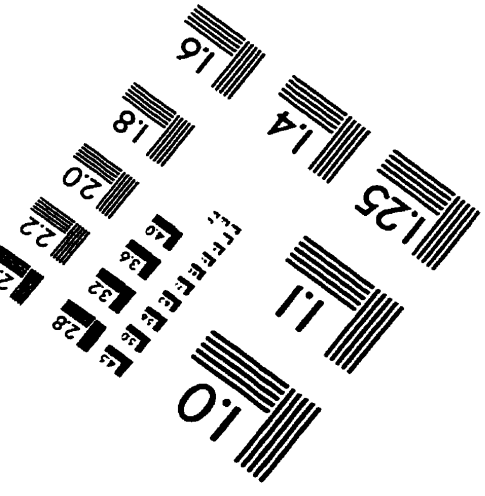

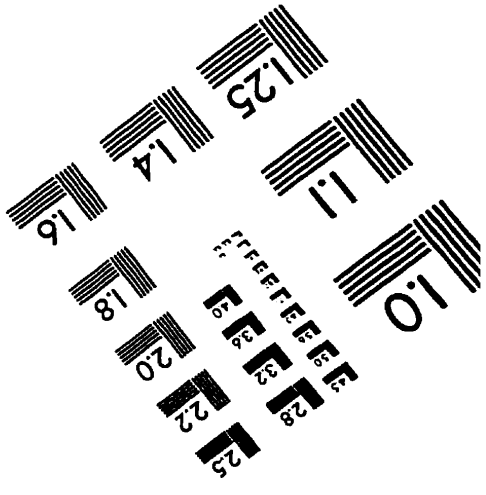

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