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HOWARD RICHARD ROSER JR.

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ELEMENTAL COMPOSITION OF PSEUDOMONAS PUTIDA UNDER COPPER STRESS

University of New Hampshire

Ph.D.

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1979
ELEMENTAL COMPOSITION OF *PSEUDOMONAS PUTIDA*
UNDER COPPER STRESS

by

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B.S. Rensselaer Polytechnic Institute

A THESIS
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In Partial Fulfillment of
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Department of Microbiology
December, 1979
This thesis has been examined and approved.

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Date: December 4, 1979
To my folks for their trust and encouragement.
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ABSTRACT

ELEMENTAL COMPOSITION OF PSEUDOMONAS PUTIDA
UNDER COPPER STRESS

by

HOWARD RICHARD ROSSER, JR.

University of New Hampshire, December, 1979

An investigation to determine the effect of an ecological stress, elevation of the concentration of a metal ion (Cu) in the growth medium, on its uptake and the elemental composition of a marine bacterium, Pseudomonas putida (ATCC 27935) was undertaken.

P. putida was grown in a Na-gluconate-based artificial seawater medium (26.5 ppt) in a chemostat at 25 °C, pH 7.4 D = 0.156 h\(^{-1}\). Where Cu stress was examined, CuCl\(_2\) was added to a final concentration of 5 \(\times 10^{-5}\) M. Under unstressed growth conditions, minimum doubling time was 4.44 h. Under Cu stress conditions, minimum doubling time was 7.22 h, corresponding to a dilution rate in the chemostat of D = 0.096 h\(^{-1}\). Cells were harvested continuously from chemostat effluent by centrifugation at 10,000 x g at 2 °C. Cells were washed in ice-cold, double-distilled, deionozed water prior to analyses. Dried, acid-digested (concentrated, redistilled nitric acid) cells were analyzed by atomic emission and flame and graphite furnace atomic absorption spectrometry.
C, N and H analyses were performed on the dried cellular material.

Under unstressed conditions, protein, RNA and DNA accounted for 51.1, 12.2 and 3.4 %, respectively, of cell dry weight. Under Cu stress protein synthesis was reduced significantly (P < 0.05); RNA and DNA were unaffected (42.5, 12.2 and 2.95 %, respectively). C (46 %), N (12 %) and H (7 %) content of P. putida was unaltered in the presence of elevated concentration of Cu. P content (2.65 %) was increased significantly under conditions of Cu stress (3.14 %). The P increase was proportional to a Mg increase (from 6905 to 8133 ppm) under Cu stress. It is suggested that these elements increase proportionately to phospholipid content of the outer cell wall. Ca (1766 ppm) and Sr (25.9 ppm) contents also are increased proportionately under Cu stress (2489 and 37.9 ppm, respectively), probably due to binding to increased amounts of cell wall material.

Fe (125 ppm) and Zn (49 ppm) contents were unaltered under Cu stress. Cu content (11 ppm) was increased significantly and was inversely proportional to cell density in the chemostat. Likewise, RNA content was inversely proportional to Cu content of the cells indicating an approach of the threshold of Cu toxicity of RNA synthesis. Trace element composition (Mn 3.5 ppm, Ni 3.8 ppm, Cd 2.0 ppm, and Cr 5.8 ppm) was not changed significantly under Cu stress. Pb (3.3 ppm)
was reduced significantly (0.37 ppm), likely due to its adsorption to, rather than transport into the cell.

Whereas Cu stress on the elemental composition of *P. putida* was unable to affect profound elemental alterations, changes in P, Mg, Ca, Sr, Cu and Pb were significant. A 17% average reduction in protein content under Cu stress, also was recorded. The elemental composition of bacterial cells, while under stringent genetic control, responded sufficiently to Cu stress to accommodate the stress.
I. INTRODUCTION

Major elemental constituents of the bacterial cell have been identified (Luria, 1960). Composition of the "ash" fraction, i.e., that fraction of the cell remaining after acid digestion or muffle furnace combustion, is largely unknown. Bacteriologists today are forced to rely largely of data collected decades ago (Dawson, 1919; Roberts, et al, 1955; Rouf, 1964). Much of the available data was obtained under poorly defined conditions of cultivation, sample preparation and analysis making interpretation of the data difficult, and comparisons with results from other investigations tenuous. Important physiological roles and strict requirements have been described for many elements detected in the "ash" fraction (Bowen, 1966; Williams, 1971; Stanier, et al, 1976). The relative concentrations of major and minor components of the ash are of fundamental interest in bacterial cytology and physiology.

Recognition of the toxic properties exhibited by heavy metals has resulted in widespread interest in their occurrence as environmental pollutants (Reish, et al, 1977, 1978; Leland, et al, 1978). Heavy metals reduce bacterial community stability by decreasing bacterial diversity, coincidently increasing numbers of surviving organisms (Starr and Jones, 1957; Guthrie, et al, 1977; Singleton
and Guthrie, 1977). Increased substrate turnover times (Albright and Wilson, 1974; Vaccaro, et al, 1977), decreased substrate mineralisation rates (Goulder, et al, 1979), and increased production of $^{14}$C-intermediate metabolites (Vaccaro, et al, 1977) have been reported, indicating effects on metabolic processes of autochthonous organisms. Given the crucial role that microorganisms play in cycling of C, N, S, P, and other elements through natural systems (Alexander, 1977), disruption of normal metabolic processes as a result of heavy metal stress of the environment is a potential problem warranting careful consideration.

Accumulations of toxic heavy metals have been reported in all levels of the food web, including microorganisms (Baas-Becking, 1959; Nelson and Colwell, 1975; Patrick and Loutit, 1976; Guthrie, et al, 1977; Singleton and Guthrie, 1977), phytoplankton (Riley and Roth, 1971; Knauer and Martin, 1973), molluscs (Segar, et al, 1971), echinoderms and coelenterates (Riley and Segar, 1970), and zooplankton and fish (Hutchinson, et al, 1976). Investigations indicating that bioaccumulation and biomagnification of toxic heavy metals through the food web do (Patrick and Loutit, 1976) and do not (Kneip and Lauer, 1973) occur, were found. Microorganisms have been implicated in the initial metal bioaccumulation process (Tornabene and Edwards, 1972; Doyle, et al, 1975; Sayler, et al, 1975). The potential biomagnification of toxic heavy metals through the food web
resulting from accumulation of high concentrations of heavy metals by bacteria is of interest.

Accumulation of heavy metals by microorganisms also has been suggested as a process resulting in deposition of heavy metals in aquatic sediments (Trudinger and Bubela, 1967; McLerran and Holmes, 1974). Beveridge and Murray (1976) suggested:

Traces of microbes and considerable concentrations of metals have been found in sediments from ancient geological horizons (e.g., the isoprenoid hydrocarbons from the precambrian "Nonesuch" formation and the mineralized remains of microorganisms from the Precambrian Gunflint chert of northern Lake Superior). These are usually closely associated with metal deposits, often of large proportions. The exact manner of deposition of these metals is of open debate and the associated microbial remnants lead one to speculate as to the possibility of their biological origin.

This investigation was undertaken to determine the concentrations of major, minor and trace elements present in a bacterial cell under a precisely defined set of cultural, preparative and analytical conditions. Relative concentrations of the major biomolecules protein, RNA and DNA also were determined. The results obtained under "standard" growth conditions were compared to results obtained under conditions in which a heavy metal stress, an elevated Cu concentration, was included. Relative uptake of
heavy metals by microorganisms and effects of heavy metal stress on cell physiology as indicated by distribution of major biomolecules in the cell were investigated.
Although the importance of metals in bacterial metabolism has long been recognized, articles dealing specifically with elemental composition of microorganisms are not common in the microbiological literature. Articles dealing with mechanisms for transport of metals (Weinberg, 1977), their physiological roles in bacterial cells (Bowen, 1966), transformation of metals by microorganisms (Jernelov and Martin, 1975; Summers and Silver, 1978), and the effects of various heavy metals on the metabolic activities of isolated microorganisms (Cobet, et al., 1970) and on natural microbial populations (Jones, 1973) are available. This review is limited to articles which provide information on the extent to which microorganisms are able to concentrate metals within their cells under a stated set of analytical and cultural conditions. A majority of the early reports in the field are not considered since the importance of clearly defining the cultivation, preparation and analysis of samples was not recognized rendering the results useless for comparison to subsequent investigations. Major ions (alkali and alkaline earth metals) and minor ions (primarily transition elements) are considered separately except where coordinate activities between the metals warrant otherwise. No attempt is made to describe the specific physiological functions of the various metal ions.
Physiological information of this type is readily available elsewhere (Bowen, 1966; Williams, 1971; Weinberg, 1977).

Analysis of microbial cells for content of the alkali metals Na and K has presented problems to many investigators. Ready removal or exchange of these cations by wash procedures designed to eliminate contaminating extracellular materials has resulted in wide variations in reported cellular content. Curran, et al (1943) recognized the need for employing a wash procedure to remove associated cell debris from the material to be analyzed. Examining Bacillus megaterium grown in Brain Heart Infusion (BHI) broth, cell K values of 21,000 ppm after a single wash with fresh BHI broth, and 7000 ppm after 5 consecutive washes were reported. Epstein and Schultz (1965) indicated that cell K concentrations were determined primarily by the osmolality of the cells' milieu and varied greatly depending of the growth medium. Cell washes of lower osmolality than the growth medium resulted in a rapid, massive efflux of K from the cell. Distilled water washes were particularly effective in removing intracellular K and chilling the wash solutions enhanced this effect. Using an osmotically neutral wash, they reported intracellular K values for Escherichia coli grown in glucose/salts medium ranging from 17,900 to 39,900 ppm as the medium was varied from 148 to 938 mOsm. Na values, however, were independent of osmolality of the medium and varied directly with Na content of the growth medium. Thus in the same glucose/salts medium
containing 3286 ppm Na, intracellular Na content ranged from 3860 to 3240 ppm as the osmolality of the medium was varied from 270 to 938 mOsM. Results of analysis of a marine bacterium (*Alteromonas haloplanktis*) by Takacs, et al (1964) corroborated the findings of Epstein and Schultz (1965). Using a radiolabelled excluded polymer to estimate extracellular space within the pellet, and thus extracellular Na and K in the pellet, Takacs et al (1964) were able to use fresh growth medium in their wash procedure. Within experimental error, intra- and extracellular Na values were the same within the range of Na concentrations tested (0 to 1 M; Na = 0.467 M in seawater of 35 ppt salinity). Intracellular K values, however, were approximately twice the extracellular values at the two concentrations tested (0.01 and 0.15 M; K = 0.01 M in seawater of 35 ppt salinity). This ratio was not influenced by Na content of the medium.

Jones, et al (1976), using three chilled 0.5 M ammonium formate washes obtained Na values of 5600 and 255 ppm and K values of 700 and 36 ppm for *Alcaligenes marinus* and *Pseudomonas putida*, strain Roche (ATCC 29735), respectively. The ammonium formate wash resulted in extensive leakage of the major ions from *A. marinus* (Jones, et al, 1979). The unexpectedly low values for major ions in *P. putida* suggested a similar phenomenon in this organism. Using three 200 ml chilled distilled water (DW) washes, Na and K values obtained for *A. marinus* were 3100 and 3090 ppm,
respectively. Using 0.01 M phosphate buffer as a washing solution resulted in slightly higher values.

Rouf (1964), using a DW wash, obtained K values for *E. coli*, *Micrococcus roseus*, *Sphaerotilus natans* and *Bacillus cereus* of 14,200, 11,100, 2000 and 55,200 ppm, respectively, and Na values of 100, 8500, 1000 and 7500 ppm, respectively. Medium values were not reported. Tempest (1969) reported variations in K content of *Enterobacter aerogenes* grown in chemostat culture in glycerol/salts medium under conditions of Mg and K limitation. MgCl₂ or NaCl washes were used. Under conditions of Mg limitation, K content increased from 1200 to 2400 ppm as the dilution rate (d=hl⁻¹) was increased from 0.1 to 0.8. Under conditions of K limitation, K content ranged from 8000 to 16,000 ppm over the same dilution rate range. At a dilution rate of 0.2 h decreasing the temperature from 35 to 25°C resulted in an increase in K content from 10,500 to 14,000 ppm.

Sakharova, et al (1978) reported a different situation for *B. megaterium* cells grown in a citrate-limited chemostat culture and washed with DW. K values ranged from 9000 to 11,000 ppm and were unaffected by changes in growth rate. Na values were in the range of 2000 ppm and were observed to decrease with increasing growth rate. The K content decreased at alkaline and acid pH's and Na content increased with increasing acidity.
Coleman (1974) reported intracellular Na and K values of 506 and 22,800 ppm in unwashed Bacillus amyloliquefaciens cells grown in a complex maltose, casein hydrolysate medium. While 95% of the K was isolated with the intracellular fluids, 80% of the Na was cell envelope bound. These data were at variance with the results of Beveridge and Murray (1976) in which the relative affinities of the two ions for Bacillus subtilis cell wall fragments are 2.4:1 K:Na. K values reported by Coleman (1974) remained constant through a typical growth cycle, while Na values increased during the transition from logarithmic to stationary phases.

Taken in toto, the data suggested that both Na and K exist in osmotically active and readily exchangeable forms in most cells. Failure to recognize this phenomenon was likely the cause of anomalously low values reported by early investigators (Baas-Becking, 1959). Variations in uptake concentrations have been common among different genera under similar cultural conditions. Temperature, pH, and organic and ionic content of the growth medium have a profound influence on ionic content of the organisms.

Considerably more information is available on the cellular content of the alkaline earth metals, Mg and Ca. Knowledge of important metabolic and structural functions served by these ions has resulted in many studies dealing with their uptake in and association with bacterial cells. The involvement of Mg and Ca in maintaining structural...
integrity of bacterial cell walls is well documented (Asbell and Eagon, 1966; Rayman and MacLeod, 1975; Beveridge and Murray, 1976). Coleman (1974) determined that while only 32% of the Mg in \textit{B. amyloliquefaciens} was envelope-bound, 96% of the Ca was envelope-bound. This was interpreted as indicating a primarily structural role for Ca. The bulk of the cellular Mg has been shown to be involved in protein synthesis in microorganisms, and is largely ribosome-associated (Tempest, 1969). Free intracellular Mg concentrations have been estimated to be in the range of 25-100 ppm (Lusk, \textit{et al}, 1968). Beveridge and Murray (1976) showed that cell wall fragments of \textit{B. subtilis} grown in a complex Nutrient Broth/amoeno acid/glucose medium had a high affinity for Mg and Ca; relative binding affinities were 200 ug Mg and 16 ug Ca bound per mg cell wall material. Eagon \textit{et al}, (1965) found slightly lower Mg:Ca ratios in ashed \textit{Pseudomonas aeruginosa} cell wall material from cells grown in a glucose/yeast extract medium. Mg was detected at 2000 ppm while Ca was present at 1500 ppm. In \textit{E. coli} grown in a glucose/histidine medium, Mg was retained completely irrespective of the wash solution used (0.3 M buffered sucrose, 0.3 M NaCl or DW), while Ca was less tightly bound, with only 67% remaining after a DW wash and 46% remaining after the NaCl wash relative to the 0.3 M sucrose wash (Kung, \textit{et al}, 1976). Whole cell Mg content was measured at 2800 ppm and Ca content at 2300 ppm. Mg uptake and exchange in microbial cells has been shown to be an
energy-dependent process (Webb, 1970) mediated by two distinct transport systems (Nelson and Kennedy, 1972; Jasper and Silver, 1977). Ca, however, was generally found to be actively exported by microbial cells (Chipley and Edwards, 1972; Jasper and Silver, 1977). Ca probably entered cells by facilitated diffusion; active export was reduced at low temperature or in the presence of respiratory inhibitors (Silver, et al, 1975). Removal of inhibitors or elevation to normal growth temperatures resulted in instantaneous efflux of Ca. Ca concentrations associated with E. coli were approximately 2000 ppm (Silver, et al, 1975). Intracellular Ca concentrations were only a few percent of the medium values. Mg and Ca values reported by other investigators vary widely, depending on growth and preparative techniques. Curran, et al, (1943) reported Mg and Ca concentrations in B. megaterium grown in BHI broth and washed in fresh medium of 13,000 and 12,000 ppm, respectively. For the same organism grown in a chemostat in a glucose/citrate medium and washed in DW, Sakharova, et al (1978) reported Mg values ranging from 1000 to 8000 ppm and Ca values ranging from 300 to 1800 ppm as the dilution rate (growth rate, u) was varied from 0.1 to 0.8 h⁻¹. An increase of Mg content with increasing u in chemostat culture was observed by Tempest (1969) in work with B. subtilis and E. aerogenes although concentrations were considerably lower. Rouf (1964) reported Mg as 10,400, 19,900, 9500 and 17,200 ppm and Ca as 200, 1000, 2300 and
400 ppm in *E. coli*, *M. roseus*, *S. natans* and *B. cereus*, respectively. Although medium content varied considerably in this investigation, all cells were washed in DW at room temperature. Jones, *et al.*, (1976) reported Mg and Ca values for the two marine microorganisms *A. marinus* and *P. putida* grown in a tryptone/yeast extract seawater medium. Cells washed in chilled 0.5 M ammonium formate showed Mg contents of 1580 and 292 ppm and Ca contents of 790 and 272 ppm, respectively. When *A. marinus* was washed in chilled DW, Mg and Ca concentrations of 3550 and 3090 ppm, respectively, were detected (Jones, *et al.*, 1979). Mg and Ca values for four identified and twelve unidentified marine pure cultures were reported by Jones, *et al.* (1979) using the same tryptone/yeast extract seawater medium and chilled 0.5 M ammonium formate wash. Mg values ranged from 156 to 1362 ppm (excluding values considered disparate by the authors) and Ca values, from 70 to 830 ppm. It is obvious from these results that, though more tightly bound than Na and K, Mg and Ca values were dependent upon a number of cultural and preparative procedures. The need for exact control of pH, medium composition and growth rate in studying major cationic species in microorganisms makes the chemostat an attractive experimental tool for this type of work.

Trace element metabolism in microorganisms has been the subject of many treatises (Bowen, 1966; Weinberg, 1977) and so is not considered in a review of trace element content of microorganisms. Trace metals of biological interest are
considered as a group since many investigators include several or all of these ions in their studies of trace metal/microbial interactions. Data are considered in the general order of adsorption phenomena, trace metal uptake and transport, and trace metal content.

Because of the tendency of transition metals to react with organic ligands to form stable chelates, the phenomenon of adsorption to bacterial cells, as opposed to energy-dependent uptake, is commonly observed. Beveridge and Murray (1976) examined the adsorption of Fe, Mn, Cu, Co, Ni and Sr to B. megaterium cell wall fragments. They found large amounts of Fe and Cu (189.0 and 18.8 ug, respectively, per mg cell wall material), lesser amounts of Ni and Sr (6.2 and 2.15 ug, respectively, per mg cell wall material), and no Co or Mn adsorbed to the cell wall fragments when placed in a solution containing each metal at a concentration of 5 mM. Chipley (1972) found a similar adsorption phenomenon with Salmonella enteritidis cell wall fragments. Although Ca was most strongly bound, Zn>Mn>Fe were also bound. A large proportion of the binding was sensitive to the presence of respiratory inhibitors such as 2,4-dinitrophenol (DNP) and N,N'-dicyclohexylcarbodiimide (DCCD) and thus is assumed to be associated with active transport carrier molecules. Webb (1970) reported a similar cross-reactivity of Ni>Co>Zn with the Mg transport systems of B. megaterium and E. aerogenes, distinguishing between binding to peripheral phosphate and carboxyl groups at 0°C, which was
not DNP inhibited, and binding to Mg carrier molecules which was inhibitor sensitive. McLerran and Holmes (1974) examined the ability of marine bacterial isolates from Corpus Christi harbor to remove radiolabelled Zn and Cd from solution. After 120 h incubation in 2216E broth, cells and precipitate were separated by differential centrifugation, washed in buffered saline solution and the distribution of Zn and Cd examined. Of the 85% of the Zn removed from solution, 20% was "assimilated" or cell-associated, while 80% was precipitated as ZnS or co-precipitated with FeS. Of the 70% of the Cd removed from solution, 10-15% was "assimilated" and 85-90% was precipitated. No attempt was made to distinguish between adsorbed metal and metal transported into the cell in this study of a simulated marine system.

Jones, et al. (1976) described the effect of increasing concentrations of Zn on the growth of A. marinus, a marine isolate, in 0.1% tryptone/yeast extract seawater broth. The effect on levels of Zn on other major and minor ions in the cell were determined. Zn concentration factors were greatest at low (200X at 0.35 ppm) and high (202X at 18.35 ppm) medium levels of Zn. Intermediate levels resulted in smaller concentration factors, with a minimum (48X) at 7 ppm Zn. From a normal cellular Zn concentration of 70.1 ppm, increasing Zn medium levels resulted in increased Zn cellular levels up to 3700 ppm at 18.35 ppm Zn in the medium. It seems likely that at this high level, a majority
of the Zn ions were adsorbed, albeit tightly, to cell wall ligands.

In a similar study of Co uptake by *Proteus vulgaris* and *E. coli*, Neyland, et al (1952) found that both cells took up Co rapidly from a defined glucose/nicotinamide/mineral salts medium; the amount of Co bound was proportional to cell density and Co content of the medium in the range of 1-100 ug/ml. Concentration factors were approximately 200X, i.e., 237X at 1 ppm Co in the medium, 212X at 10 ppm and 153X at 100 ppm. The nature of the uptake, which was assumed to be adsorption due to the short time frame of the experiment (15 min) was apparently different in the two organisms. Co was bound very firmly by *E. coli* and not removed by repeated DW washes or transfer to Co-free medium. However 70-80% of the Co was removed from *P. vulgaris* cells simply by dilution in DW. Uptake was reduced in Meat Extract broth due to the presence of "Co-combining constituents" which competed effectively with the cells for available metal.

Sadler and Trudinger (1967) reported on the binding of Cu to bacterial cells. The amount of Cu bound per unit weight of bacteria depended upon the Cu concentration of the medium and was reduced in the presence of complexing agents. The pattern of Cu-binding immediately after addition of Cu to growing cultures suggested an initial rapid binding of Cu to the bacterial cell wall during which the amounts of Cu bound per cell were 2-3 times higher than normally found in
cells growing at that external Cu concentration, followed by progressive fall in Cu content until a cell Cu concentration was reached which was characteristic for growth in that medium Cu concentration.

Microbial uptake of Pb by *Micrococcus luteus* and an *Azotobacter* sp. has been described by Tornabene and Edwards (1972). Growth in Trypticase-soy broth culture in which PbBr$_3$ crystals in dialysis tubing were suspended, allowed for immobilization of 4.9 and 3.1 x 10$^2$ mg Pb/g dry weight whole cells, *M. luteus* and *Azotobacter* sp., respectively. The Pb (99.9 %) was associated with the cell wall/cell membrane fractions indicating that adsorption was the process of primary importance. Doyle, et al (1975) investigated Cd uptake in *E. coli*, *B. cereus*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Aspergillus niger* in BHI broth containing up to 80 ppm Cd as CdCl$_2$. Only *E. coli* and *B. cereus* grew at the highest Cd concentration. For *E. coli*, concentration factors ranged from 140X (700 ppm cellular Cd at 5 ppm medium Cd) to 122.5X (9800 ppm cell Cd at 80 ppm). For *B. cereus*, concentration factors ranged from 3870 X (38,700 ppm cell Cd at 10 ppm) to 1114X (89,100 ppm at 80 ppm). As in the work of Tornabene and Edwards (1972) a majority of the Cd uptake was the result of adsorption to the cell surface.
Nelson and Colwell (1975) and Sayler, et al (1975) considered the case of Hg uptake and accumulation in marine microorganisms. Using HgCl\textsubscript{2} (6 ppm) and phenylmercuric acetate (3 ppm) in a Casamino acids/yeast extract/glucose medium, resistant organisms were isolated and classified into predominately 8 generic types. Isolates were capable of taking up 6-10% of the Hg present in the growth medium, with sediment isolates being more active. Uptake varied with cell density and Hg content of the growth medium. Again, uptake of the metal was a fast, energy independent adsorption phenomenon. \textit{Pseudomonas} sp.#14 (Sayler, et al., 1975) was characterized as accumulating 12 ug Hg per mg cells per 15 min from a solution containing 6 mg HgCl\textsubscript{2} per liter.

Patrick and Loutit (1976) reported high accumulation of Cr, Cu, Mn, Fe, Pb and Zn by mixed populations of marine isolates or by \textit{Sphaerotilus} mats in Bacto-Sphaerotilus medium amended with 10% soil extract. Selected results are presented in (Table 1.). From these results, and work with other isolates, the authors concluded that the process of metal concentration differed among different bacteria and tended to increase drastically during late log and early stationary phase when energy was not available for cell wall repairs and greater cell surface area was exposed.
A related phenomenon in certain Fe and Mn oxidizing microorganisms involves the accumulation of microscopically visible deposits of ferric and manganic oxides within the sheath of the organisms (Ali and Stokes, 1971, Dondero, 1975). Letunova, et al (1978) reported exceedingly high levels of Mn uptake by microorganisms isolated from the Mn Biogeochemical Province of the Georgian SSR. Mn values for air-dried biomass were reported as high as 220 ppm in a medium with no Mn additions (0.2 ppm Mn background). 2100 ppm Mn was detected in the same organisms grown in the medium amended with 1 ppm Mn indicating a concentration factor of 1750X. At higher medium concentrations cell concentration factors were lower but Mn concentrations as high as 70,000 ppm were reported for some isolates grown in a medium amended with 1000 ppm Mn. Deposition of manganic oxides at the cell surface was likely the basis for these exceedingly high Mn levels.

Kung, et al (1976) reported that Zn in E. coli was tightly bound largely due to its intracellular activities and the crucial role Zn plays in gram negative cell wall stability. Bucheder and Broda (1974) addressed the same phenomenon in E. coli. The energy-independent, quick, easily exchangeable binding they attributed to adsorption; the energy dependent carrier-mediated, slower uptake was described as specific ion transport. Under condition of fermentation or respiration where energy was abundant, transport was far more important than adsorption.
in the uptake of Zn. A relatively high steady state concentration of 500 ppm was observed under starvation conditions. Concentration factors of over 1000X were observed in media with an initial Zn concentration of 10 uM (0.65 ppm). This was considerably greater than the 10X concentration factor expected due to an electrical potential difference across the cell membrane of -25 mV.

Specific transport systems for Fe and Mn have been characterized (Silver, et al, 1970; Neilands, 1974; Weinberg, 1977). Cross-reactivity allowed for active transport of other ions for which specific carrier-mediated systems did not exist. For example, Silver, et al (1975) reported uptake of Sr and Mn by Ca transport systems in B. subtilis and E. coli. Lear and Oppenheimer (1962) have reported that Serratia marinorubra was capable of removing radiolabelled Sr and Y from a Bacto-Peptone seawater medium and concentrating these ions by factors of 1300-1400X for Sr and 30,000-46,000X for Y. Nelson and Kennedy (1972) reported that System I, the nonrepressible Mg transport system of E. coli catalyzed the uptake of Co, Mn and Ni. Silver, et al (1970) reported that Co and Fe was transported by the Mn transport system of E. coli.

Udel'nova, et al (1978) have reported increased uptake of Cr in Chromatium vinosum when grown under photoautotrophic conditions, and increased uptake of Ti when grown under photoheterotrophic conditions, even though
specific transport systems for these ions have not been described. Increased uptake of Mn under photoautotrophic growth may be the reason for the concommitant increase in Cd uptake.

A relatively small number of comprehensive studies exist which address the problem of trace element composition of microorganisms. Curran, et al (1943) examined Fe, Al, Cu and Mn content in vegetative cells and spores of B. megaterium. Cells were grown in an organic-rich medium and washed with fresh medium. Fe, Al, Cu and Mn values for washed cells were 160, 278, 50, and 40 ppm, respectively. Medium values were not listed.

Rouf (1964) presented data on the trace element composition of E. coli, M. roseus, S. natans and B. cereus. A partial list of results appears in Table 2. Poorly defined cultural conditions and inadequate medium analysis take away from the usefulness of the data.

Koval'skii, et al (1965) examined spectrographically the trace element composition for formalin-fixed, DW-washed Bacterium dysenteriae, Bacillus paratyphoid A, Vibrio cholerae and Mycobacterium tuberculosis. Unfortunately, no information on growth conditions was provided, and cell preparations were not washed in all instances, thus making interpretation of the data difficult.
One of the more comprehensive investigations of trace metal composition examined two marine microorganisms, A. marinus and P. putida under standard cultural conditions and under conditions of Zn stress (Jones, et al, 1976). Cells were grown to stationary phase in a yeast extract/tryptone seawater-based medium, harvested by centrifugation, washed in 0.5 M ammonium formate, wet-ashed and analyzed for cation content. Partial results appear below in Table 3. Wide variations in major ion content were evident, and slightly more subtle differences in minor ion content were discernible. The large differences in major ion content likely reflect organizational or structural differences in the two cell walls which react differently to the ammonium formate wash solution. The smaller differences detected in minor ion content may be real, or may simply be within limits of intraexperimental error.

The effect of added Zn on the trace element composition of A. marinus is presented in Table 4 (Jones, et al, 1976). No major alterations in trace element content were evident even when Zn concentrations as high as 18.35 ppm were present in the growth medium. The trace elements in A. marinus were bound tightly and not displaced by other transition elements present at concentrations that were up to several orders of magnitude higher.
The most extensive investigation of elemental composition in microorganisms was by Jones, et al (1979). Under identical cultivation conditions and using a standard sample preparation technique, five standard laboratory organisms (B. megaterium, E. coli (NBC 8114), Pseudomonas fluorescens (NCB 8858), Pseudomonas ovalis (NCB 9229), and S. aureus (NCB 6571)), four identified marine isolates (Alteromonas haloplanktis (NCB 19), Pseudomonas putida (ATCC 29735), Pseudomonas sp. (NCMB 130) and Vibrio fisheri (NCMB 1143), and twelve unidentified marine isolates were analyzed. The effects of DW, 0.5 M ammonium formate, 0.01 M phosphate buffer and 0.01 M phosphate buffer with 10⁻⁴ M EDTA as wash solutions on the ionic content of bacterial cells were addressed. Also described was an experiment in which the entire cultivation/analysis process was repeated a number of times so that an estimation of precision was possible. Partial results for major ion and minor ion content of the standard lab organisms and the identified marine strains are presented in Tables 5 and 6. While the ammonium formate wash had a profound effect on the major ion content of washed cells, trace element content was largely uneffected. The major ion data then reflect basic differences not only in ion content, but also in cell wall/cell membrane organization which, in reacting with the ammonium formate wash solution, allowed the escape of different quantities of the ions. The trace element data, on the other hand, probably reflected real differences in
ion content among the organisms analyzed.

The investigations reviewed indicate the need for standard growth and analytical procedures. Continuous cultivation is to be preferred for growth of organisms to be analyzed for ion content because of the variability in content of certain ions with growth rate, \( u \). Maintenance of a constant environment during growth can be achieved using this cultivation technique.
III. MATERIALS AND METHODS

A. **Organism.**

The organism used in this investigation was a marine variant of *Pseudomonas putida* (ATCC 29735) isolated by Roche (1966) and named *Pseudomonas cuprodurans* by her because it demonstrated a resistance to $2.5 \times 10^{-3}$ M Cu at the time of isolation. The organism was characterized further by McCarthy (1971) and classified to its present taxonomic location by Passman (1977).

B. **Labware preparation.**

All labware used in this investigation was subjected to a rigorous cleaning regimen in order to minimize metal contamination. This included, after dishwasher washing and single distilled water rinse, an overnight soak in concentrated HNO (Analytical grade J. T. Baker Co.) followed by several rinses with double distilled, deionized water (Q2W) (Corning Model AG-11 glass still, Corning Glass Co., Corning, N.Y.), and Millipore Milli-Q deionizer system, (Millipore Corp., Bedford, Mass.). The labware was soaked overnight in Q2W, rerinsed in Q2W, drained, wrapped in Saran wrap and stored under dust-free conditions until use. Appropriate precautions were taken to eliminate the possibility of contamination during handling.
C. M9/GCA medium preparation.

M9/GCA medium was a modification of a glucose/mineral salts medium known as M9 (per liter 75% artificial seawater adjusted to a salinity of 26.5 ppt, glucose, 5.0 g, NH₄Cl, 1.0 g, K₂HPO₄, 0.25 g). The two media were identical with the exception that the molar equivalent of sodium gluconate (ICN Pharmaceuticals, Inc., Cleveland, Ohio), 6.06 g/liter, was substituted in place of the glucose. M9/GCA medium was prepared in 15 liter quantities for each chemostat run. All salts were dissolved separately in Q2W and added to air-saturated 100% Kester seawater (KSW, Kester, et al., 1967) in the order, gluconate, NH₄Cl, K₂HPO₄. pH was adjusted to above 7.4 with 2 N NaOH prior to addition of K₂HPO₄, then adjusted to 7.40 with 1 N HCl. Q2W was added to give the 15 liter total volume. Final salinity of the medium was 26.5 ppt. In experiments in which Cu was included in the growth medium, appropriate amounts of 1 x 10⁻³ M Cu as CuCl₂ (J. T. Baker Co.) were added after the addition of K₂HPO₄, prior to the final pH adjustment. The medium was sterilized using a pressurized filtration system. An 18.9 liter stainless steel pressure vessel (Gelman Inst. Corp., Ann Arbor, Mich.) was pressurized with nitrogen gas to 3.16 kg/cm² forcing the M9/GCA medium from the vessel and through two 142 mm teflon-coated process filter holders (Millipore Corp.) stacked in series. The upper filter holder contained an AP15 prefilter and a 0.45 μm pore size Millipore filter. The lower filter holder contained a 0.2 μm
pore size Nucleopore filter (Nucleopore Corp., Pleasanton, Calif.). The entire filtration apparatus was assembled and autoclaved as a single unit. The filter-sterilized medium flowed directly into a sterile, vented 20-liter pyrex carboy which acted as the medium reservoir. After filtration was complete, the filter was bubble point tested at 4.57 kg/cm$^2$ to check the integrity of the 0.2 μm membrane filter.

D. Continuous cultivation apparatus.

Bacterial cells to be analyzed were grown in a chemostat apparatus. An 11.5-cm diameter 2.5-liter Brewer jar served as the reaction vessel (RV). The RV head was cut from a 12-mm thick ethyl propylene gasket. Ports were cut in the RV head to accept the various inlet and outlet fittings. The head was held in place by means of a specially constructed aluminum clamp. Fittings passing through the head included 1) glass gas dispersion tube, 2) combination pH electrode, 3) titrant inlet tube, 4) glass thermometer, 5) medium inlet tube, 6) culture outlet tube, 7) sampling port and 8) vent port. All glass fittings in the chemostat were acid-washed according to the procedure described for labware preparation. The pH electrode, after an initial cleansing in 1 N HCl, was stored in 0.05 N HCl and used only for the purpose of monitoring pH in the chemostat. Prior to use, the electrode was sterilized by soaking for 30 min in 5 % H$_2$O$_2$. Air introduced into the RV was supplied by a Silent Giant aquarium pump. The air was
regulated with a gas throttle valve, then filtered through a 42-mm diameter, 0.22 μm pore size Millipore filter before entering the RV. Air was supplied at a rate sufficient to maintain approximately 50% saturation of oxygen in the growth medium. Oxygen saturation was determined periodically with a YSI Model 51 Oxygen Meter (Yellow Springs Inst. Co., Yellow Springs, Ohio) on whole culture subsamples removed from the RV and handled so as to alter the oxygen content as little as possible prior to measurement. pH of the culture was maintained at 7.4 by means of a Chemtrix Type 45AR pH Controller Unit (Chemtrix, Inc., Hillsboro, Oregon). The controller unit activated a Masterflex constant-speed 10 RPM pump (Cole Parmer Inst. Co., Chicago, Ill.) fitted with a No. 7013 head (0.0315 in ID tubing), capable of delivering titrant at a constant rate of 0.6 ml/min. The titrant pump delivered autoclaved 0.1 N NaOH from a modified 500 ml Nalgene graduated cylinder to the RV upon call from the controller unit. The controller unit was able to maintain pH to ±0.02 pH units. M9/GCA medium was delivered to the RV by a variable speed Masterflex pump fitted with a No. 7013 head. All tubing used in the chemostat was the medical grade silicone type. A glass J-shaped outlet tube maintained the culture at a constant volume of 1-liter in the RV. The culture overflow was pumped from the J-tube by means of the same Masterflex pump used to supply fresh medium to the RV. However, the outlet line utilized a No. 7016 head and 0.125
in ID tubing. Thus, per revolution of the pump, the inlet line was capable of supplying 0.06 ml fresh medium while the outlet line was capable of removing 0.80 ml of the culture, a pump rate differential of 13.3x in favor of the outlet line. Thus by adjusting the height of the open end of the J-tube a constant level (volume) was maintained in the RV. Subsamples were removed from the RV via a sample port consisting of a canula passing through a serum stopper in the RV head and extending into the culture by means of a length of 0.3 mm ID tubing. A 5 ml disposable plastic syringe fitted onto the cannula was used to withdraw the subsamples. The RV vent consisted of a length of tubing passing from the top of the head space of the RV to a separate 250-ml Erlenmeyer flask fitted with a Balston Type 90 Grade AA filter (Balston, Inc., Lexington, Mass.). The separate flask eliminated the problem of wetting and clogging encountered when the filter was fitted directly to the RV head. Constant mixing of the culture was accomplished by means of a circular teflon stirring disc within the culture rotated by a magnetic stirrer beneath the RV. Temperature of the RV was maintained at 25°C by placing the entire RV in a small plastic tub filled with water, surrounding the RV with coiled copper tubing, and circulating 25°C water from a Tamson water bath (Neslab Instruments, Portsmouth, N.H.) through the copper coils. The temperature was maintained to within ±0.5°C at all times.
E. Chemostat operation.

A strict step-up inoculation sequence was followed prior to inoculation of the chemostat. An M9/GCA agar slant was inoculated from a modified 2216E (Passman, 1977) work slant and incubated overnight at 25°C. The small amount of growth present on the M9/GCA slant was rinsed from the surface with 5 ml M9/GCA medium and inoculated into 100 ml M9/GCA medium in a 250 ml Erlenmeyer flask. This 100-ml culture was incubated overnight with shaking at 25°C. The following day the culture was centrifuged at 10,000 x g at 25°C, washed once in fresh M9/GCA medium, then resuspended in fresh M9/GCA medium to an approximate density of 1.0 at 420 nm. One liter of M9/GCA medium in the chemostat RV was inoculated with the equivalent of 5 ml of an A$_{420}^\text{=1.0}$ culture, and grown as a "batch" culture with controlled pH until an A$_{420}$ of approximately 2.0 was reached. At this time the medium flow was begun. Once the population reached a steady state, usually after 36-48 h, harvest of the cells was initiated.
F. Cultural characteristics determined during growth.

Throughout the course of each chemostat run a variety of physical and biochemical parameters were determined in order to characterize the physiological state of *P. putida* in continuous culture. All parameters were determined upon a single subsample withdrawn from the RV at the specified sampling time. Subsamples were withdrawn for analysis 5 times during each chemostat run at approximately 12 h intervals. Absorbance at 420 nm was used to give an indication of bacterial population density and was determined on appropriately diluted samples using a Zeiss PM-QII Spectrophotometer (Carl Zeiss, Oberkochen, Weltenberg, Germany). Cell dry weight was determined by filtering 2 ml of culture subsample onto a tared 42-mm 0.4 μm Nucleopore filter and washing with three volumes of Q2W. Filters were dried at 60°C to a constant weight and weighed on a Mettler H10T Analytical Balance (Mettler, Inc. Princeton, N.J.). Triplicate analyses were performed at each sampling. Viable counts were determined by dilution of subsamples in 75% KSW and plating in triplicate on 2216E agar plates. Colonies were counted after 48 h incubation at 25°C.

Biochemical parameters examined included cellular DNA, RNA and protein. Cellular DNA was determined by the ethidium bromide fluorometric technique of Doonkersloot, et al, (1972), with the following modification. Cells were
harvested by centrifugation at 10,000 x g at 4°C instead of by filtration as described. Cellular RNA was determined by the orcinol colorimetric technique on cells harvested in a similar manner. Cellular protein was determined by a modification of the Lowry technique (Passman, 1977). All biochemical measurements were made on a 1.0 ml subsample. Triplicate analyses were performed in each instance.

G. Cell harvest.

Cells were harvested by continuous centrifugation in a Sorvall RC-2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) fitted with a Szent-Gyorgi Blum continuous flow head. Effluent of the RV passed directly into the centrifuge. The entire apparatus including the collection tubes was of stainless steel construction and not teflon coated. Cells were harvested at 10,000 x g at 2°C for approximately 60 h.

H. Wash procedure.

At the end of the harvest, pellets from the eight continuous flow head collection tubes were transferred to a single 250-ml polycarbonate centrifuge bottle. Care was taken throughout the entire wash procedure to maintain cell pellets and all wash solutions at ice bath temperature. Q2W (100 ml) was added to the centrifuge bottle and the bottle was shaken to break up the pellet. Microscopic cell
aggregates were broken up by subjecting the cell suspension to treatment with a perforated vibrating disc made of teflon-coated stainless steel (Vibromixer, Chemapec, Inc., Hoboken, N.J.). After mixing for 10 min the cells were pelleted by centrifuging at 10,000 x g at 2°C for 15 min. The supernatant fluid was removed and replaced with 100 ml fresh Q2W. The process was repeated two more times. After removal of the final supernatant fluid, the pellet was stored at -90°C in the centrifuge bottle. Freezing the pellet facilitated quantitative transfer to a tared teflon beaker by eliminating the need to wash the cell material from the centrifuge bottle.

I. Preparation of cell pellets for analysis.

Frozen cell pellets were transferred to tared 50 ml teflon beakers for drying. Beakers were covered with teflon watch glasses and dried at 60°C to a constant weight. After determination of total dry weight, pellets were ground to a fine powder with an agate mortar and pestle. This powder was dried in a vacuum oven and stored in linear polyethylene (LPE) vials in a dessicator. C, H and N analyses were performed directly on this ground, dried cell material. P and metal analyses were performed on samples that had been digested on a hot plate in Redistilled Nitric Acid (RDNA, 15.46 N) to remove organic matter. Approximately 1.0 g dried ground cell material was weighed into a 30-ml teflon beaker and covered with 15 ml RDNA. The solution was
covered with a small teflon watch glass and gently warmed overnight until initial digestion was complete. The temperature of the hotplate was then increased and sufficient acid added to the beakers until the digestion process, as indicated by the absence of brownish-orange fumes, was complete. During the final phase of the acid-digestion process a siliceous precipitate was always observed (Passman, 1977). This precipitate was separated from the acid-digest solution by centrifugation at 15,000 x g and washed three times in 1 % RDNA. This wash procedure consisted of centrifugation, removal of the supernatant wash solution, resuspension of the precipitate in fresh 1 % RDNA, a one h leaching period, and then recentrifugation. All washings were added to the original acid digest solution. Acid digests were stored at 4°C in acid-washed LPE scintillation vials (New England Nuclear, Boston, Mass.) until analysis.

J. Identification of products of glucose oxidation.

Products of glucose oxidation were determined by paper chromatography according to the method of Norris and Campbell (1949). Standards (gluconic acid, 2-ketogluconic acid and 5-ketogluconic acid) were used as supplied, as their Na salts (ICN Pharmaceuticals, Inc., Plainview, N.Y.), and were spotted (0.02 ml) as solutions of molar strength equivalent to that of 5 g glucose per liter (28 mM) by repeated application from a finely drawn pipette tip, onto
Whatman #1 paper. The chromatograms were irrigated for 9-12 h in a solvent system containing n-butanol:glacial acetic acid:water in the ratio 2:1:1. After drying, spots were detected with one of two spray reagents. AgNO₃ (0.1 N in 5 N NH₄OH) revealed the presence of all carbohydrate spots. Aniline oxalate (saturated solution in glacial acetic acid) reacted with glucose (light brown spot) and 2-ketogluconate (rose-pink spot), but not with gluconic acid or 5-ketogluconic acid.

K. Preparation of medium components for trace metal analysis.

NH₄Cl (1.0 g) and K₂HPO₄ (0.25 g) were weighed out and dissolved in 10 ml 1 % RDNA resulting in concentration factors of 100 X over their concentrations in M9/GCA medium. These solutions were analyzed directly. Sodium gluconate (3.03 g) was digested in concentrated RDNA in the same manner as the bacterial cell material. Following completion of the digestion process the NaNO₃ crystals which remained were dissolved in 5 ml Q2W. RDNA (5 ml of 2 %) was then added to give a 1 % RDNA solution for analysis. The concentration factor of trace metals in this solution compared to their concentration in M9/GCA medium was approximately 50 X.
Trace metals in KSW were extracted by two methods. Solvent extraction was by the method of Danielsson, et al (1978), using a combination of ammonium pyrroolidine dithiocarbamate (APDC) and diethylammonium diethyldithiocarbamate (DDTC) (J. T. Baker, Co.) as the complexing reagents and FREON-TF (Cleanway Industries, Keene, N.H.) as the extraction solvent. Diammonium hydrogencitrate (J. T. Baker Co.) was used to buffer the samples at pH 5.0. Ion exchange resin extraction was by the method of Kingston, et al (1978). The Chelex 100 resin used was 50-100 mesh (BioRad Laboratories, New York, N.Y.). NH$_4$OH was generated by bubbling ammonia gas through Q2W. The concentration of the NH$_4$OH obtained was 6.115 N. The glacial acetic acid used was ULTREX grade (J. T. Baker Co.). Ammonium acetate (4 ml of 3 M) was used to buffer each 500 ml sample to pH 5.0. The column apparatus was identical to that described by Kingston, et al (1978) with the exception that addition of sample to the reservoir was through the open bottom of the inverted bottle. This opening was covered with Saran wrap during the extraction process to prevent airborne contamination.

L. Analytical procedures.

C, H and N analyses were performed in triplicate on ground cell material using a Hewlett Packard F&M Model 180 CHN Analyzer. Sample size was 500-700 ug. Peak heights were compared to the
cyclohexanone-2,4-dinitrophenylhydrazone standard (C 51.79 %, H 5.07 %, N 20.14 %).

P analyses were performed on appropriate dilutions of acid digest solutions using the ammonium molybdate colorimetric method of Fiske and Subbarrow (1925). Cations were analyzed by either atomic absorption or atomic emission spectrometry using an IL-351 Double Beam AA/AE Spectrophotometer (Instrumentation Laboratories, Wilmington, Mass.). Flame atomic absorption/atomic emission conditions were those suggested by the manufacturer. Flameless atomic absorption analyses were performed using the IL-555 Graphite Furnace Temperature Programmer Unit. Instrument parameters for flameless AA analysis were generally those recommended by the manufacturer but with slightly longer sample drying cycles. Background correction, when required, was supplied by a Deuterium Hollow Cathode Lamp. Graphite furnaces were the cylindrical, pyrolytically-coated variety. Samples (10 ul) were injected into the furnace using an Eppendorf 10/20/25 ul pipette (Brinkman Instruments, Westbury, New York). Pipette tips (Eppendorf) were rinsed a minimum of 5 X with 1 % RDNA and 3 X with the sample before injection of sample into the furnace to minimize contamination and adsorption problems often associated with polypropylene tips (Benjamene and Jenne, 1978; Samela and Vuori, 1979). Dilutions for flame AA analyses were made in 1 % RDNA or 1 % RDHA as required. Dilutions for flameless AA were made in 1 % RDNA. Ca dilutions also contained 1000 ppm LaCl\textsubscript{3} as a
releasing agent. Sr standards and sample dilutions were prepared in 1% RDHA containing 1000 ppm K as KCl.

Qualitative estimation of trace metals associated with the siliceous precipitate after the leaching procedure was accomplished by dc-arc emission spectroscopy.

M. Statistical analysis

Statistical analyses were performed using the packaged statistics program Minitab II, Version 77.2, UNH implementation 0.1, available of the Dec-10 academic computing system of the University of New Hampshire. Statistics most frequently calculated were mean ($\bar{X}$), standard deviation (s), standard error of the mean (S.E.), least significant difference (L.S.D.) and analysis of variance, one-way (ANOVA). Documentation of calculations used to obtain mean, standard deviation, and ANOVA statistics are available from the University of New Hampshire Dec-10 Applications Library. Standard error of the mean was calculated as

$$S.E. = \frac{s}{\sqrt{n}}$$

where $n$ was the number of items in the population on which $s$
and \( \bar{x} \) were calculated. Least significant difference was calculated as

\[
\text{L.S.D.} = \frac{S_y \cdot q_{kr.05}}{\sqrt{n}}
\]

where \( S_y \) was the pooled standard deviation, \( q \) was the value from the table of percentage points of studentized range (\( k=\)number of means compared, \( r=\)degrees of freedom in the pooled standard deviation). Acceptable level of significance was taken to be 95% in all cases unless stated otherwise. Explanation of the exact application of each statistic is provided with presentation of the different data.
IV. RESULTS

A. Growth of *P. putida* in batch and continuous culture.

When preparing cells for trace metal analyses, cultivation in a defined medium was desirable. Growth of *P. putida* in the glucose/mineral salts medium, M9, was possible, but, as shown in Fig. 1, curve (a), and in Table 7, the products of glucose oxidation were highly acidic and their production rapidly reduced the pH of the medium to a point which inhibited further growth. Maintenance of a neutral pH results in continued oxidation of the substrate and greater final culture densities.

Metabolic byproducts of glucose oxidation produced by *P. putida* were examined by paper chromatography to determine if an intermediate of the glucose oxidation process existed which could be utilized as the carbon/energy source in the medium, and which could be supplied as the neutralized salt, eliminating the problem of low pH inhibition of growth. A mid-log ($A_{420} = 0.3$) batch culture of *P. putida* in M9 medium was harvested by centrifugation at 25 C at 10,000 x g. The cells were resuspended in fresh medium and incubated one h with shaking to allow time for acclimation to the new external environment, then centrifuged again and resuspended to a density of $3.5 \times 10^7$ CFU/ml in 100 ml fresh M9 medium in a 250 ml culture vessel. Agitation of the culture was
accomplished using a magnetic stirrer. pH was maintained at neutrality by manual addition of 0.1 N NaOH. At hourly intervals for 5 h, 5 ml aliquants of the culture were withdrawn. \( A_{420} \) and pH were determined on a 3 ml portion of the sample. The other 2 ml were filtered gently through a 0.45 \( \mu \) Millipore filter and the filtrate placed in a boiling water bath for 2 min to halt any extracellular enzymatic reactions. After chilling on an ice bath, 0.02 ml of the sample were spotted on Whatman #1 paper by repeated application from a finely drawn pipette tip. Samples were spotted in duplicate on opposite halves of the paper. After irrigation for 9-12 h, the chromatogram was dried quickly by exposure to mild heat to prevent diffusion and spreading of the spots. The chromatogram was cut in half so that duplicate copies were obtained. One half of the chromatogram was sprayed with the AgNO\(_3\) reagent to reveal the presence of all carbohydrate spots. The other half was sprayed with the aniline oxalate reagent which reacts with 2-ketogluconate (2KG) to give a rose-colored spot, and glucose to give a pale brown spot, but not with 5-ketogluconate (5KG), or with gluconic acid (GCA). The glucose spot was detected easily throughout the course of the experiment at an \( R(f)=0.435 \) in both the AgNO\(_3\)-sprayed and aniline oxalate-sprayed chromatograms (Table 8). Tailing of the glucose spot in the seawater medium, which increased noticeably by the fourth and fifth h made identification of separate spots in the AgNO\(_3\)-sprayed
chromatogram difficult. By the fourth and fifth h, however, the aniline oxalate-sprayed chromatogram revealed that part of the tailing noted in the AgNO$_3$-sprayed chromatogram was due to the presence of 2KG. The presence of gluconic acid as an intermediate was not demonstrated conclusively because of the close proximity of the 2KG, GCA and glucose spots. The presence of a pink tail on the glucose spot, evident in the aniline oxalate-developed chromatogram, suggested the presence of 2KG. The experiment was repeated using M9/GCA medium. In this medium, pH dropped only to $\geq 6.5$ before beginning to rise again, thus eliminating the problem of low pH inhibition (see Fig. 1, curve (b), and Table 7). Gluconate was detected in the AgNO$_3$-sprayed chromatogram throughout the course of the experiment at an $R(f)=0.38$ (Table 9). By the second h of the experiment, a faint 2KG spot was detected in the aniline oxalate-sprayed chromatogram which increased in intensity until the experiment was terminated at 23 h. Thus, the production of 2KG from gluconate in M9/GCA culture by P. putida was established. Considering the data from the two experiments, the presence of gluconate as an intermediate in glucose oxidation was indicated.

Growth rates (u) and doubling times (T-d) for P. putida in M9 and M9/GCA media were determined. During logarithmic growth, growth rates in M9 and M9/GCA batch culture were calculated to be 0.054 and 0.096, respectively, corresponding to doubling times of 12.90 and 7.22 h. The
rapid drop in pH observed in M9 was probably the cause of the slower growth rate in this medium. Buffering M9 with 25 g/liter Tris-hydroxyamino methane (Tris, Calbiochem, East Rutherford, New Jersey) allowed for an increase in u (0.087) and a reduction in T-d (7.98 h). Further increases in u were obtained using PIPES buffer (piperazine-n,n'-bis(2-ethanesulfonic acid, Calbiochem), at concentrations of 2 g/liter (u=0.111, T-d=6.27 h) and 10 g/liter (u=0.188, T-d=3.68 h). In separate experiments (data not shown) the presence of Tris in M9 at a concentration of 25 g/liter protected the inoculum from the toxic effects of 1 x 10^{-5} M Cu as evidenced by the lack of an extended lag time. While affording excellent buffering capacity, the PIPES buffer (10 g/liter) reduced only slightly the extended lag time observed in unbuffered cultures amended with 1 x 10^{-5} M Cu.

In pH-controlled continuous culture (pH=7.4), the maximum growth rate attained in M9/GCA medium was 0.156 (=u-max), corresponding to a doubling time of 4.44 h. This was equivalent to steady state growth at a medium delivery rate of 2.6 ml/min. At higher dilution rates, D-c, the critical dilution rate, was exceeded and "washout" of the culture occurred. The growth rate in continuous culture was significantly higher than that observed in batch culture. This phenomenon has been described previously by other investigators (Ellsworth and Telling, 1956). Under conditions of Cu stress in continuous culture using M9/GCA
medium, the maximum growth rate attainable was 0.096 corresponding to a $T_d$ of 7.22 h. Medium flow rate in this instance was 1.6 ml/min.

B. Protein, RNA and DNA content of *P. putida* grown under Standard conditions and under conditions of Cu stress.

Analyses for protein, RNA, and DNA were performed in order to provide insight into the relative proportions of the major biomolecular components of the cell which are, to some extent, a reflection of the metabolic state of the organism. Under the described growth conditions, *P. putida* was grown in continuous culture, harvested by continuous centrifugation at 2°C and prepared for analyses utilizing a chilled Q2W wash and RDNA wet ash technique (where applicable). Five times during each chemostat run (approximately every 12 h) samples were withdrawn from the RV and analyzed to determine protein, RNA and DNA content of the cells. Dry weight and viable counts also were determined. Triplicate analyses were performed in each instance. The parameters are reported as per cent of cell dry weight. The value recorded for each chemostat run represents an overall mean of 15 individual measurements; i.e., 5 samplings x 3 replicate analyses of each sample in each chemostat run.
1. **Protein analyses.**

Results of the protein analyses of cells grown under standard conditions (continuous culture at 25°C in M9/GCA medium at u=0.156) appear in Table 10. Sampling means ranged from 36.1 to 71.1 % protein of cell dry weight with an overall mean of 50.1 % (S.E.=1.60 %). Mean values of % protein for the individual chemostat runs ranged from 43.5 to 57.2 %. The ANOVA comparing sampling to sampling variation (within) versus variation among chemostat runs (among) indicated no significant variation among chemostat runs which was in excess of the variation observed while sampling within a single chemostat run.

Protein content of *P. putida* cells grown in M9/GCA medium amended with $5 \times 10^{-5}$ M Cu is shown in Table 11. The absolute range of sampling means was 34.3 to 50.7 % with an overall mean of 42.5 % (S.E.=1.48 %) protein of cell dry weight. Mean values of the different chemostat runs ranged from 40.9 to 43.9 %. The ANOVA comparing variation between samplings within the chemostat runs versus variation among the different chemostat runs indicated no significant variation between different runs in excess of variation observed within a single chemostat run. An overall Pooled-t analysis comparing protein content of *P. putida* grown under standard conditions and conditions of Cu stress yielded a highly significant difference ($P=0.002$; df=38) between the two populations.
2. **RNA analyses**

Results of RNA analyses of cells grown under standard conditions in M9/GCA medium appear in Table 10. Sampling means for RNA content ranged from 7.4 to 18.6 % with an overall mean of 12.4 % (S.E.=0.69 %) RNA of cell dry weight. The mean values obtained from separate chemostat runs ranged from 10.6 to 14.2 %. The ANOVA indicted significant variations among different chemostat runs. Calculation of the L.S.D. yielded a value of 4.3. None of the means could be detected as significantly different by this method.

RNA content of *P. putida* grown under conditions of Cu stress are presented in Table 11. The absolute range of sampling means ranged from 5.6 to 15.2 % with an overall mean of 11.7 % (S.E.=0.91 %) RNA. It was suspected that a value of 5.6 % obtained in the second sampling of Chemostat Run #2 (Cu stress conditions) was disparate, but could not be rejected at the 95 % confidence level as an "outlier" because of a second slightly low observation. The range observed for RNA content among the three Cu-stressed chemostat run means ranged from 9.2 to 13.6 %. The precision of the RNA analyses and the presence of several suspected disparate values in Chemostat Run #2 combine to present significant differences in RNA content among the different chemostat runs (ANOVA). However, none of the means could be detected as significantly different using the calculated L.S.D. value of 4.6 %. An overall Pooled-t
analysis of the unstressed and Cu stressed population indicates no significant differences in RNA content under the two cultural conditions (P=0.388; df=38). Because significant differences were detected by the ANOVA among chemostat runs under both standard and stressed conditions, this conclusion must be considered with caution.

3. DNA analyses.

DNA content of *P. putida* grown under standard conditions is listed in Table 10. Sampling means showed an absolute range of 1.7 to 5.7 % with an overall mean of 3.5 % (S.E.=0.27 %) DNA of cell dry weight. Mean values from separate chemostat runs ranged from 2.3 to 4.6 %. Individual DNA determinations, i.e., replicates of a single sampling were highly reproducible. This resulted in significant differences not only among the different chemostat runs, but also among samplings within a single chemostat run. Using the calculated L.S.D. value of 1.3, it was noted that Chemostat Runs #1 and #2 were significantly different from #4 and #5.

DNA content of Cu-stressed *P. putida* cells is reported in Table 11. The absolute range of sampling values was considerably less than under unstressed conditions: 2.30 to 3.69 % DNA with an overall mean of 2.95 % DNA (S.E.=0.15 %). Differences among these values were not significant (P=0.895 to P=0.952). Despite the precision obtained in analysis of
Cu-stressed cells, the variability observed in unstressed populations reduced the significance of the Pooled-t analysis to P=0.080 (df=36): the two populations cannot be considered significantly different at a confidence level of 95 %.

C. **Carbon, Nitrogen and Hydrogen Analyses.**

*P. putida* cells harvested by continuous centrifugation were washed, dried and ground using an agate mortar and pestle as previously described. C, N and H analyses were performed on the ground cell material to determine relative concentrations of these major cellular components and to assure that the wash procedure was consistently removing loosely associated cell debris. The data for *P. putida* grown in a chemostat culture at D=0.156 h⁻¹ in M9/GCA medium are presented in Table 12. Values indicated for each chemostat run represent the mean of triplicate analyses.
1. **Carbon analyses.**

The absolute range of individual observations of % C was 43.2 to 46.9 % giving an overall mean of 45.6 % (S.E.=0.31 %). The range of means among the different chemostat runs was 44.3 to 46.2 %. The ANOVA comparing variation due to Error (replicate analysis) and variation due to Factor (separate chemostat runs) indicated that no significant difference was detected in % C content of cells from the five chemostat runs (F .05[4,10] = 3.48; Fs = 1.46) that cannot be attributed to random error inherent in the analyses.

Data describing C content of *P. putida* grown in chemostat culture (D=0.096 h⁻¹) in M9/GCA medium amended with 5 x 10⁻⁵ M Cu are shown in Table 13. Absolute range of individual observations ranged from 41.3 to 47.1 % with an overall mean of 46.2 % (S.E.=0.23 %). Mean values for the three chemostat runs ranged from 44.7 to 46.8 %. Rejection of a single "outlier" value of 41.3 % in Chemostat Run #1 analysis yielded a range of means from 45.5 to 46.8 %. As in the unstressed condition, the ANOVA indicated no significant variation in % C among the three chemostat runs that was not attributable to random error inherent in the analysis (F .05[2,6] = 5.14; Fs = 1.11). A Pooled-t analysis comparing % C in *P. putida* grown under standard conditions and under Cu stress conditions indicated a very low degree of significance (P=0.778; df=22) of the test;
there was no significant difference in % C content of cells from the two populations at the 95 % confidence level.

In early chemostat investigations, P. putida was grown in M9/GCA medium containing 1 g NH₄NO₃ instead of 1 g NH₄Cl. For comparison, the C, N, H analyses using a Perkin-Elmer Model 185 CHN Analyzer are presented in Table 14. Each value is the average of duplicate analyses. Pooled-t analyses comparing % C in cells grown under standard conditions and in M9/GCA medium containing NH₄NO₃ indicated a very low level of significance of the test (P=0.329; df=8). It was concluded that no significant difference in % C content exists between cells grown in the presence of the two different inorganic N sources.

2. Nitrogen analyses

Data indicating % N content of P. putida under standard conditions are presented in Table 12. The absolute range of observations for % N was from 11.8 to 12.8 % with an overall mean of 12.3 % (S.E.=0.07 %). Mean values for % N from separate chemostat runs ranged from 12.1 to 12.5 %. The ANOVA comparing variation among replicate analyses (variation due to Error) and variation among separate chemostat runs (variation due to treatment or Factor) indicated that, despite the high degree of precision noted in these analyses, variation due to Factor was not significant (F .05[4,10] = 3.48; Fs = 0.69) when compared
to variation among replicates.

N content of *P. putida* grown in chemostat culture (D=0.096 h\(^{-1}\)) in M9/GCA medium amended with 5 \(\times\) 10\(^{-5}\) M Cu is presented in Table 13. The absolute range of observations for % N was 10.8 to 12.6 % with an overall mean of 12.3 % (S.E.=0.06 %). Mean values for % N from separate chemostat runs ranged from 12.1 to 12.4 %. The ANOVA comparing variation due to Error (among replicates) and due to Factor (among separate chemostat runs) once again indicated no significant difference (\(F_{0.05}(2,6) = 5.14;\) Fs = 0.77) in N content of cells from the different chemostat runs. A Pooled-t analysis comparing % N content of *P. putida* grown under standard and Cu stress conditions indicated no significant difference (P=0.179; df=22) between the two populations.

N content of cells grown in M9/GCA medium containing \(\text{NH}_4\text{NO}_3\) is presented in Table 14. Pooled-t analyses comparing mean % N for the separate chemostat runs under standard conditions and in M9/GCA medium containing \(\text{NH}_4\text{NO}_3\) indicated no significant difference in the two populations at the 95 % confidence level (P=0.662; df = 8). Substitution of \(\text{NH}_4\text{NO}_3\) as the N source in M9/GCA medium had no effect on the N content of *P. putida* grown in chemostat culture at D=0.156 h\(^{-1}\), even though the substitution resulted in a 34 % increase in N content of the medium.
3. **Hydrogen analyses.**

Data indicating H content of *P. putida* grown in chemostat culture (D=0.156 h\(^{-1}\)) in M9/GCA medium are presented in Table 12. Values of individual observations ranged from 5.5 to 9.6 % H giving an overall mean of 6.6 % (S.E.=0.30 %). Means for % H of the different chemostat runs ranged from 5.7 to 7.8 %. The ANOVA comparing variation in % H content due to Error (replication) and Factor (separate runs) suggested significant differences (F .05[4,10] = 3.18; Fs = 3.48) among the different chemostat runs which were in excess of that expected from random analytical error alone. Using a calculated L.S.D. value of 1.9 %, it was determined that Chemostat Run #1 was significantly different from Runs #4 and #5.

Per cent H content of *P. putida* grown in M9/GCA medium amended with 5 x 10\(^{-5}\) M Cu in chemostat culture (D=0.096 h\(^{-1}\)) is presented in Table 13. Values for individual observations ranged from 5.3 to 8.2 % with an overall mean of 7.5 % (S.E.=0.33 %) indicated. Mean % H content of cells from the three Cu-stressed chemostat runs ranged from 6.2 to 7.8 %. The ANOVA indicated no significant variation among chemostat runs due to Factor (F .05[2,6] = 5.14; Fs = 5.07). The variation observed among the different chemostat runs was attributable to random variation inherent in the analyses. Again, caution must be exercised in consideration of this conclusion since significant
variations were observed among chemostat runs under standard conditions.

Data relating % H content of _P. putida_ grown in M9/GCA medium containing NH$_4$NO$_3$ are presented in Table 14. Pooled-t analysis comparing % H in cells grown in M9/GCA medium containing NH$_4$NO$_3$ to those grown under standard conditions indicated no significant difference in % H between the two populations (P=0.515; df=6).

D. **Phosphorus Analyses.**

P analyses were performed on acid-digested cells to determine the contribution of this major protoplasmic (and cell envelope associated) element to the composition of the bacterial cell. Care was taken to maintain equivalent concentrations of RDNA in both samples and standards. Results of P analyses of cells grown under standard conditions, under conditions of Cu stress and in M9/GCA medium in which NH$_4$NO$_3$ had been substituted for NH$_4$Cl, appear in Table 15. Values reported represent the mean of triplicate analyses ± one standard deviation. Under standard conditions, P content of _P. putida_ ranged from 2.55 to 2.87 % with an overall mean of 2.65 % (S.E.=0.032 %) for the different chemostat runs. The absolute range of observations was 2.53 to 2.89 %. Under conditions of Cu stress, P content of _P. putida_ was higher: individual
observations ranged from 2.87 to 3.47 % with an overall mean of 3.13 % (S.E.=0.052 %). Mean values for the different Cu-stressed chemostat runs ranged from 3.08 to 3.26 %. A Pooled-t analysis based on mean values for the separate chemostat runs indicated a high degree of significance for the difference in P content in \textit{P. putida} when grown under standard conditions and under conditions of Cu stress (P=0.002; df=6).

P data for cells grown in M9/GCA medium containing \textit{NH}_4\textit{NO}_3 are presented in Table 15. Individual observations ranged from 1.98 to 2.80 % with an overall mean of 2.53 %. Mean values for the separate chemostat runs ranged from 2.01 to 2.78 %. The observation of 2.01 % for Run #5 was significantly different from the values for the other four chemostat runs (P<0.05) and was rejected as an "outlier." Recalculation of the overall mean yields a value of 2.65 %, precisely the value observed in the standard M9/GCA medium. It was concluded that P content of \textit{P. putida} is unaffected by replacement of \textit{NH}_4\textit{Cl} with \textit{NH}_4\textit{NO}_3 in M9/GCA medium.

The high degree of precision in the P analyses, as denoted by low standard deviations relative to the magnitude of the means (average coefficient of variation 1.2 %) resulted in all variations among chemostat means obtained under standard conditions and in M9/GCA medium containing \textit{NH}_4\textit{NO}_3, determined as significant (P< 0.05). The ANOVA comparing variations among replicates to variation among
chemostat runs (standard conditions) yielded an F-ratio of 162.37 (F .05[4,10] = 3.48) indicating a highly significant variation among these five chemostat runs. The relatively wide variation among replicates in Chemostat Run #3 under Cu stress conditions prevented the variations among these three chemostat runs from being significant (F .05 [2,5] = 5.79; Fs = 1.67). Variations among chemostat runs using M9/GCA medium containing NH₄NO₃ were highly significant (F .05[4,10] = 3.48; Fs = 1211.14).

E. Major ion analyses.

Na, K, Mg and Ca comprise the major portion of the inorganic ion content of *P. putida*. Atomic absorption and atomic emission analyses for these ions were performed on RDNA-ashed samples. In performing flame analyses (AA or AE) output signal of the instrument was integrated over a preset time period. Throughout this investigation a 1/4 sec integration period was used when performing flame analyses. Values reported for flame analyses, therefore, represent an instrument-derived integrated mean. Replicate aspirations of a single sample yielded precisely the same integrated signal. Hence, standard deviations based on random noise were without meaning and were not reported. Because of the many operational difficulties encountered in cultivation of *P. putida* in M9/GCA medium containing NH₄NO₃, results of analyses of cells grown under these conditions were
considered less accurate and so are presented in tabular form only for the purposes of comparison. Statistical analyses of these data are presented at the time of discussion of these data.

Major ion content of *P. putida* grown in M9/GCA medium in chemostat culture (D=0.156 h\(^{-1}\)) is presented in Table 16. Na values ranged from 4250 to 6080 ppm with a mean of 5356 ppm (S.E.=394 ppm). K values were more variable and ranged from 3360 to 6440 ppm with a mean of 4470 ppm (S.E.=543 ppm). Mg values showed a greater consistency and ranged from 6320 to 7280 ppm indicating a mean content of 6905 ppm (S.E.=167 ppm). Ca values, like Mg, were reproducible among chemostat runs with values ranging from 1640 to 1840 ppm, yielding a mean Ca content of 1766 ppm (S.E.=37 ppm). Under conditions of Cu stress, all major ion values were shifted to some degree (Table 17). Na values ranged from 2460 to 4450 ppm with a mean of 3327 ppm (S.E.=588 ppm) and K values ranged from 2550 to 4600 ppm with a mean of 3263 ppm (S.E.=666 ppm). Pooled-t analysis of the Na data indicates a significant difference (P=0.024; df=6) in Na content of *P. putida* when grown under conditions of Cu stress. Because of the variation observed in K values, the reduction in K content under conditions of Cu stress, while noted, cannot be considered significant (P=0.216; df=6). Major divalent cation content was significantly higher under conditions of Cu stress. Mg content ranged from 7630 to 8560 ppm with a mean of 8133 ppm (S.E.=273 ppm). Ca content ranged from
2270 to 2600 ppm yielding a mean of 2489 ppm (S.E.=110 ppm). Pooled-t analyses comparing major divalent cation content of *P. putida* under standard conditions and under conditions of Cu stress indicated highly significant differences for Mg (P=0.006; df=6) and for Ca (P=0.0003; df=6). Cellular content of the monovalent ions, Na and K was reduced; Mg and Ca were elevated under conditions of Cu stress compared to results obtained under standard conditions. Major ion content of *P. putida* grown in M9/GCA medium containing NH$_4$NO$_3$ is presented in Table 18.

**F. Minor ion analyses.**

Multivalent cations, often of biological interest, present in *P. putida* at concentrations less that the major ions, yet greater than the trace elements include Fe, Zn, Sr and Cu. Concentrations of these elements detected in *P. putida* grown under standard conditions in M9/GCA medium in chemostat culture (D=0.156 h$^{-1}$) are reported in Table 19. Values obtained under conditions of Cu stress are presented in Table 20. Minor ion content data for *P. putida* cultivated in M9/GCA medium containing NH$_4$NO$_3$ are presented in Table 21. Analyses for minor ions were performed on RDNA-ashed samples by AA and AE spectrometry. As with the major ions, values reported represent instrument-integrated means.
Fe concentrations observed under standard conditions ranged from 110 to 142 ppm with a mean of 125 ppm (S.E.=5.8 ppm). Under conditions of Cu stress the range of values observed was 118 to 130 ppm with a mean of 124 ppm (S.E.=3.5 ppm). Pooled t analyses comparing the two overall means indicated no significant difference (P=0.881; df=6) in Fe content of P. putida when grown under the two different cultural conditions.

Zn content of P. putida under standard conditions varied from 27.0 to 61.9 ppm with an overall mean of 49.4 ppm (S.E.=6.29 ppm). Under conditions of Cu stress Zn content ranged from 44.1 to 56.6 ppm yielding an overall mean of 49.4 ppm (S.E.=3.71 ppm). Pooled-t analysis of the two populations indicates no significant difference (P=0.995; df=6) in Zn content of cells grown under the two different cultural conditions.

Sr content of P. putida ranged from 24.0 to 28.7 ppm with a mean of 25.9 ppm (S.E.=0.91 ppm) under standard conditions. Under conditions of Cu stress, Sr content ranged from 29.1 to 47.1 ppm with a mean of 37.6 ppm (S.E.=5.22 ppm). Pooled-t analysis comparing means of the two populations indicates a level of significance of P=0.026 (df=6). It was concluded that Sr content of P. putida was significantly different under conditions of Cu stress.
Cu content of P. putida grown under standard conditions ranged from 6.8 to 15.5 ppm indicating a mean Cu content of 11.6 ppm (S.E. = 1.47 ppm). Under conditions of Cu stress Cu content of P. putida was significantly elevated. Cu concentrations under these conditions ranged from 80.4 to 368 ppm. Though the Cu content of M9/GCA medium was maintained at $5 \times 10^{-5}$ M in investigations of the responses of P. putida to conditions of Cu stress, the Cu content of the cells grown under these conditions varied considerably. This point will be discussed subsequently. Under Cu stress conditions a mean Cu concentration of 219 ppm was calculated. This value was significantly different ($P = 0.014; \; df = 6$) from the concentration observed under standard growth conditions.

G. Trace element analyses.

Also detected in P. putida were a number of inorganic cations present at concentrations below those of the minor ions and considerably below those observed for the major ions. Analyses for Mn, Ni, Cd, Pb and Cr were performed during this investigation by graphite furnace AA spectrometry. Because of the transient nature of the signal observed in flameless AA analyses, an integration time of 1/16 sec was used. Samples were analyzed by injection in triplicate into the furnace. Matrix effects were investigated by spiking a known amount of the element to be
analyzed into the furnace concurrently with the sample. Peak height differences between sample and sample-plus-spike were compared to equivalent peak heights in the standard curve of the element under consideration. Reduced (or enhanced) peak heights would indicate the presence of matrix effects. The high dilution factors required for analysis of these samples appeared to eliminate effectively any matrix effects which existed, as no corrections were necessary. The results of these analyses for *P. putida* grown under standard conditions and under conditions of Cu stress are presented in Tables 22 and 23, respectively. Results of trace element analyses of *P. putida* grown in M9/GCA medium containing NH$_4$NO$_3$ are reported in Table 24.

Mn content of *P. putida* grown in M9/GCA medium in chemostat culture (D= 0.156 h$^{-1}$) ranged from 2.4 to 5.7 ppm yielding a mean Mn content of 3.5 ppm (S.E.=0.61 ppm). Under conditions of Cu stress Mn content varied from 4.6 to 5.3 ppm yielding a mean of 4.9 ppm (S.E.=0.22 ppm). The Pooled-t analysis comparing means of the two populations indicated that no significant difference in Mn content existed when *P. putida* was grown under the two different cultural conditions.

Ni content of *P. putida* grown in M9/GCA medium in chemostat culture (D=0.156 h$^{-1}$) ranged from 3.1 to 4.4 ppm with a mean of 3.8 ppm (S.E.=0.28 ppm). The value of 10.8 ppm was rejected as an "outlier", (P<0.05). Under
conditions of Cu stress, values of Ni concentrations ranged from 4.3 to 4.8 ppm, yielding a mean Ni content of 4.6 ppm (S.E.=0.15 ppm). Based on comparison by Pooled-t analysis, it was concluded that Ni content did not vary significantly (P=0.094; df=5) when grown under standard conditions and under conditions of Cu stress.

Concentrations of Cd observed in P. putida grown in M9/GCA medium in chemostat culture ranged from 1.4 to 2.6 ppm with a mean of 2.0 ppm (S.E.=0.25 ppm). Under conditions of Cu stress, Cd content ranged from 1.6 to 2.5 ppm yielding a mean Cd content of 1.9 ppm (S.E.=0.30 ppm). The value of 2.5 ppm can be rejected as an outlier (P<0.05) yielding a mean of 1.6 ± 0.05 ppm, but because of the low number of replicates all values were considered.

Pb content of P. putida grown in M9/GCA medium in chemostat culture (D=0.156 h⁻¹) ranged from 2.2 to 4.0 ppm indicating a mean Pb content under these conditions of 3.2 ppm (S.E.=0.36 ppm). Under conditions of Cu stress, Pb content of P. putida was observed to be significantly different; (P=0.002; df=6). Values ranged from 0.10 to 0.87 ppm indicating a mean Pb content of 0.37 ppm (S.E.=0.25 ppm). As with Cd, the value reported for Chemostat Run # 1 could be rejected as an outlier (P<0.05), but was considered because of the low number of replicates.
Cr analyses of P. putida grown in M9/GCA medium in chemostat culture (D=0.156 h\(^{-1}\)) exhibited wide variation, with values ranging from 2.0 to 14.2 ppm. None of the observations can be rejected statistically as an "outlier." Mean Cr content detected was 5.8 ppm (S.E.=2.40 ppm). Growth under conditions of Cu stress resulted in no significant difference in Cr content detected (P=0.903; df=6). Observed values ranged from 3.31 to 9.21 ppm with an average content of 5.4 ppm (S.E.=1.9 ppm).

H. **Medium trace metal analysis.**

In reporting data on elemental composition or on accumulation of metals by microorganisms it is essential to provide information on the content of the growth medium used. To provide a basis for comparison of the results of this investigation with those obtained by other investigators all components of the medium were analyzed for trace metal content.

1. **KSW Extraction Analysis.**

Because extremely low amounts of the elements are often encountered, determination of trace metal content in artificial seawater, as in natural saline waters, requires techniques which allow for concentration of the metals from a large sample volume into a smaller volume to a
concentration above the detection limit of the analytical technique to be used. Also required of an extraction technique is the removal of the trace elements from the alkali and alkaline earth salt matrix which may interfere with the analyses. Data presented in Table 25 report the results of the analyses of 100 % KSW by two extraction methods. The Chelex ion-exchange extraction technique of Kingston, et al (1978) has been reported to provide recoveries in the range of 90 to 100 % for the following metals from saline waters: Cd, Co, Cu, Fe, Mn, Ni, Pb, and Zn. In this technique the alkali and alkaline earth metals were eluted selectively from the column with 1.0 M ammonium acetate prior to elution of the trace metals with 2.5 M RDNA. A modification of the APDC-DDTC extraction technique of Kinrade and VanLoon (1974), as described by Danielsson, et al (1978), was reported to provide extraction efficiencies of between 90 and 100 % for the following ions in saline waters: Cd, Co, Cu, Fe, Ni, Pb and Zn. Trace metals were removed from the major ion salt matrix by extraction as the carbamate complex into the organic solvent FREON-TF. Following this extraction, trace metals were released back into acidic aqueous solution prior to analysis. Both techniques provided for a 50 X concentration factor when extracting a 500 ml sample and eluting or back-extracting into a final volume of 10 ml. Data are reported for the two techniques in Table 25 as concentration detected in 100 % KSW, concentration detected in sample
spiked with a known amount of a given metal and calculated extraction efficiency, determined as follows:

\[ \% \text{Ex} = \frac{(M_2 - M_1)}{S} \]

derived from the equations

\[ (1) \% \text{Ex(KSW)} = M_1 \]

and \[ (2) \% \text{Ex(KSW+S)} = M_2 \]

where \% Ex = extraction efficiency, KSW = true value of the concentration of a particular metal in 100 \% KSW, M_1 = amount detected of a particular metal in unspiked 100 \% KSW, S = value of the spike added, and M_2 = concentration detected in 100 \% KSW to which the spike has been added.

Extractions were performed three times. Extraction efficiencies for the Chelex extraction technique were below those reported by Kingston, et al (1978). Mn extraction was particularly poor; Fe extraction was low as well. Cr, which was not considered by the authors, gave the lowest recovery efficiency. The APDC-DDTC/FREON-TF technique gave excellent recoveries for Fe, Zn and Cu, a curiously high recovery (119 \%) for Cd, and less efficient recovery of Ni and Pb. Mn is not extracted by this technique, and Cr, which is not usually extracted using carbamate techniques, and was not considered by the authors, gave a recovery of 31.7 \%.
2. **Trace metal content of M9/GCA medium.**

The remaining components of M9/GCA medium, NH₄Cl, K₂HPO₄ and Na-Gluconate were prepared for metal analysis as previously described. The medium was analyzed as separate components for two reasons. The first was to learn the contribution of each of the different components to the total trace metal content of the medium, especially of the KSW used routinely in this laboratory. The second was to assure that gluconic acid, which forms stable complexes with many transition elements, did not cause unrealistically low values to be observed by competing with the carbamate extraction reagents, thereby reducing the efficiency of the overall extraction process. The results of the analyses of M9/GCA medium components, and a total medium trace metal content based on the sum of the separate components are presented in Table 26. KSW (75 %) values are calculated from observed concentration in the extraction of 100 % KSW, corrected for extraction efficiency and reduction in salinity to 26.5 ppt. GCA was the major contributor of Fe, Zn, Cu and Cr to the trace metal content of the medium. The salts of KSW were the major contributors of Ni, Cd and Pb to the medium. Mn content of the medium was inflated by the low extraction efficiency observed. The accuracy of this value was, therefore, highly suspect.

3. **Major ion content of M9/GCA medium.**
Major ion content of M9/GCA medium was calculated from weights of the salts used in preparation of the medium. This was felt to give a more accurate indication of the concentration of these ions than analysis by AA or AE. Analysis by these techniques would require such high dilution factors to obtain samples within the analytical range of the instrument that much accuracy would be lost. Na, K, Mg, Ca and Sr content of M9/GCA medium were calculated to be 8234, 332.5, 971.3, 310.5 and 5.9 ppm, respectively.
A. Growth of *P. putida* in M9/GCA medium.

Although it has been accepted generally that the elemental composition of microorganisms reflects to some extent the composition of the environment in which they have been cultivated, the analytical and cultural problems encountered establishing this theory are manifold, and little data exists on the subject. The need for establishing a defined medium whose content, both organic and inorganic, may be reproduced accurately and consistently was essential in an investigation whose aim was to examine the phenomenon of uptake of inorganic ions by a marine bacterium. The seawater-based glucose/mineral salts medium, M9, which has been used successfully in the past (Rake, 1978) was inadequate for these purposes. As shown in Table 7, pH limitation of growth posed a serious problem. The addition of pH buffers was considered undesirable because of the ability of these compounds to form stable complexes with many metals. Toxic effects on microorganisms have been reported, the action being similar to that of EDTA in extraction of divalent cations from the cell walls of gram-negative bacteria (Cox and Eagon, 1968). An examination of the products of glucose oxidation was undertaken in an attempt to determine if any intermediate of the oxidative process yielded growth comparable to that
observed with glucose, yet whose oxidation products were less acidic than those of glucose. The data in (Tables 8 and 9) indicated that *P. putida* (ATCC 29735) was similar in its glucolytic capabilities to other aerobic fluorescent pseudomonads. These bacteria utilize a combination of the hexose monophosphate shunt and the Entner-Doudoroff pathway to produce gluconate and 2-ketogluconate via particulate glucose and gluconate oxidases (MacKechnie and Dawes, 1969; Orsten, 1971; Vincente and Canovas, 1973; Eisenberg, et al., 1974). The oxidation of glucose to 2KG via gluconate is mediated by enzymes located at the cell periphery (Midgely and Dawes, 1973). Carbon flow through these extracellular oxidative pathways was some 10-fold that transported into the cell by glucose transport systems. Depending on cultural conditions, significant proportions of glucose and gluconate were converted to gluconate and 2KG, respectively, in the medium (Whiting, et al., 1976; Lynch, et al., 1975; Lynch and Franklin, 1978).

In this investigation, replacement of glucose with the first major byproduct of glucose oxidation, gluconic acid, as the neutralized Na salt eliminated the problem of pH inhibition and thus the need for addition of buffering compounds, or in the case of continuous culture, large quantities of concentrated base, to the culture (Table 7). No organic compounds were introduced into the medium to maintain a neutral pH which were not present during glucose oxidation. Growth in gluconate-based medium has been shown
to give molar growth yields (MacKechnie and Dawes, 1969 and
doubling times (Lynch, et al, 1975) equal to those obtained
in the glucose-based medium.

Doubling times observed for *P. putida* (ATCC 29735) were
slower in all media than those reported for related strains.
Vincente and Canovas (1973) reported a T-d for *P. putida*
A.3.12 (designation Stanier, 1966) of 60 min in both glucose
reported minimal doubling times of 150 min at 20°C and 90
min at 30°C in gluconate-based media. MacKechnie and Dawes
(1969) reported doubling times of 77.6 ± 6, 84.0 ± 4 and
90.8 ± 4 min at 30°C for growth in media containing glucose,
gluconate and 2KG, respectively. The minimum doubling time
observed for *P. putida* (ATCC 29735) in modified 2216E medium
at 25°C was 70 min (McCarthy, 1971), and in defined, single
carbon source media at 25°C the T-d's were 221 min
(M9/PIPES), 266 min (M9/GCA), 498 min (M9 amended with Tris
at 25 g/liter) and 774 min (M9) by this investigator.

Under conditions of Cu stress in chemostat culture
using M9/GCA medium, growth rate was reduced (*u* = 0.096, T-d
= 433 min) compared to the unstressed condition. In an
earlier investigation in this laboratory, McCarthy (1971)
oberved reduced oxygen utilization, increased respiratory
deficiency as detected using triphenyltetrazolium chloride
and alterations in relative amounts of cytochromes b-1 and c
in *P. putida* under conditions of Cu stress. Sadler and
Trudinger (1967) observed decreases in respiration for two soil pseudomonads, A-50 and C-1 in a medium amended with $10^{-4}$ to $10^{-2}$ M Cu. Increases in glucose turnover times were observed for natural populations under conditions of Cu stress by Albright and Wilson (1974). Based on information available in the literature and on past work on P. putida in this laboratory, it was concluded that the reduced growth rate observed under conditions of Cu stress in chemostat culture was indicative of a "steady-state stress" condition in which growth of the organism was maintained at a submaximal rate by continuous introduction of Cu with fresh medium into the reaction vessel. The reduction in $D_c$, the critical dilution rate, was thus a direct reflection of the degree of stress experienced by the microorganism in culture. A similar phenomenon has been reported recently by Zevenhuizen, et al (1979) in examining the response of Klebsiella aerogenes to Cu stress in chemostat culture. At a constant dilution rate ($D = 0.033 \text{ h}^{-1}$), increasing the Cu concentration in the incoming medium eventually resulted in washout of the culture, indicating that dilution rate (a constant) was no longer balanced by growth rate of the organism.

In summary, a gluconate-based seawater medium has been described and used successfully which obviated the need for addition of pH buffers in the case of batch culture, or the addition of large amounts of concentrated base, in the case
of continuous culture. Acceptable growth rates were obtained under the prescribed conditions. The highest stability constant reported in Sillen and Martel (1964) for gluconic acid was for the gluconic acid-Cu complex, $K_s = 19.3$. Hence, in studies involving Cu toxicity, speciation calculations were simplified greatly in this medium, as the system was reduced, in essence to a one-ion, one ligand milieu.

B. Protein, RNA and DNA analyses.

The cellular content of the major biomolecules, protein, RNA and DNA was monitored to gain insight into the distribution of the major organic components, C and N, and to determine if under conditions of Cu stress any major shifts in organic content of the cells occurred. Data presented in Table 10 indicated that under standard growth conditions protein accounts for 51% of the cell dry weight. This value was somewhat lower than that reported by Warren, et al. (1966) for determination of protein content of P. aeruginosa grown in a glucose/mineral salts medium (57%). However, their results were obtained with cells harvested in stationary phase in batch culture. The nucleic acids accounted for another 15% and together these three major classes of biomolecules accounted for a total of 65% of the dry weight of the cell (Table 10). Calculation of a DNA:RNA:Protein ratio indicated results noticeably different
from that obtained for analysis of the same organism by McCarthy (1971). For cells of *P. putida* grown to early-mid log phase in modified 2216E medium in batch culture the ratio obtained was 1:1.9:9.1. The ratio obtained in this investigation was 1:3.64:15 indicating a higher degree of metabolic activity within the cell in continuous culture, even though the M9/GCA medium was less complex than modified 2216E. RNA:protein ratios were not as greatly at variance: 1:4.11 in chemostat M9/GCA culture vs. 1:4.8 in modified 2216E batch culture.

Under conditions of Cu stress, average protein content of *P. putida* was reduced significantly (from 51.0 to 42.5 % cell dry weight) by approximately 17 % (Table 11). Although the reductions in DNA and RNA content were not statistically significant due to variability of some of the observations, examination of the data for the three classes of biomolecules suggested a reduction in each class as % dry weight of the cells. While reduction in protein content was greatest compared to results obtained under standard conditions (17 %), reductions in average RNA and DNA contents of 7.3 and 13.3 %, respectively, were calculated. The overall % of cell dry weight accounted for by protein, RNA and DNA under conditions of Cu stress was 57 % (Table 11). Two possible situations may account for the observed reduction in each of the components as a % of cell dry weight. Either a lesser amount of each of these components was produced in the cell under conditions of Cu stress,
relative to growth under standard conditions, or a greater amount of another cell component was produced under stress conditions so that the relative amounts of protein, RNA and DNA appeared reduced. The actual situation was probably a combination of these two suggested possibilities. Under condition of Cu stress, a reduced growth rate of *P. putida* suggested a reduced rate of metabolic activity. As will be discussed in the following sections, relative increases in other cell components were by percentages roughly equivalent to decreases in protein, RNA and DNA. Other components remained unchanged in relation to cell dry weight.

Calculation of the DNA:RNA:PROTEIN ratio observed under condition of Cu stress yields values of 1:3.9:14.4. The RNA:protein ratio indicated was 1:3.7 reflecting the observed reduction in protein content. The data suggested the occurrence of a phenomenon reported by other investigators (Blundell and Wilds, 1968) involving selective inhibition of protein synthesis relative to RNA synthesis in the presence of heavy metal salts. Comparing absolute amounts of protein and nucleic acids in *E. coli* culture containing 30 uM Co relative to the amounts in culture without Co, a 12 % reduction in protein was detected relative to the amount of RNA detected. In this investigation a similar reduction in protein content relative to RNA content under conditions of Cu stress (50 uM Cu) was observed (Tables 10 and 11). This observation was at variance with that of McCarthy (1971) who detected no
alteration in DNA:RNA:PROTEIN ratios in *P. putida* at increasing concentrations of Cu in the growth medium. It is likely that the content of complex organic matter in modified 2216E medium used by McCarthy (1971) reduced the toxicity of Cu to a concentration below which effects on nucleic acid and protein synthesis were observed.

Finally, in regard to the effect of Cu on RNA content of *P. putida*, while no significant difference in average RNA content was observed when comparing cells grown under standard and under Cu stress conditions, the RNA content under conditions of Cu stress co-varied inversely with the elevated Cu content of the cells (Fig. 2). Protein content did not co-vary with the Cu content of the cell, but in all instances, was present at a reduced level (Table 11). This situation might have occurred under conditions where protein synthesis was reduced by a concentration of Cu lower than that which adversely affected RNA synthesis; only when Cu concentrations approached 100 ppm in the cell was an effect on RNA synthesis noted. At this threshold of toxicity, RNA content varied inversely with the concentration of the toxic ion. Protein synthesis, already at a reduced rate, was not affected by the fluctuations in RNA content which still approximated RNA content of the unstress condition.

Summarizing these observations, under standard growth conditions in M9/GCA medium protein, RNA and DNA together comprise 67% of cell dry weight. Under conditions of Cu
stress this total was reduced to 57%, primarily due to a reduction in protein content. RNA content also was affected by elevated Cu concentrations, though less drastically than protein content, and varied inversely with cell Cu content within the range of 80 to 368 ppm.

C. **Carbon, nitrogen and hydrogen analyses.**

C, N and H content of *P. putida* was monitored for several reasons. Together these three elements generally comprise approximately 70% of the bacterial cell dry weight. In an investigation purporting to examine elemental composition in a marine bacterium, analyses for C, N and H content was **de rigueur**. Initially, C, N and H analyses were included in the experimental protocol as a check to insure that the wash procedure was performed completely, effectively and consistently. If any major shifts in metabolism occurred under conditions of Cu stress, for example, these metabolic aberrations might be detected as variations in the C:N ratios of the cells.

Reproducibility of the C and N data revealed that the wash procedure was, indeed, complete and consistent (Tables 12-14). Values for C and N for the different chemostat runs fell generally within the range of precision of the analytical technique. H content was inherently more variable, as daily humidity fluctuations during analysis
directly affected moisture content of the samples and thus, H content. C and N values were somewhat lower than expected as C, N and H analyses of *P. putida* grown in modified 2216E medium using a similar protocol previously had yielded % C values of 54.1 ± 1.02 % and % N values of 14.5 ± 0.21 % (Passman, 1977). In cells of *P. putida* grown in chemostat culture in 2216E medium, C, N and H together accounted for 75.8 % of the cell dry weight (Passman, 1977). In the current investigation C, N and H accounted for only 64.4 % of cell dry weight under standard conditions (Table 12). Calculation of the C:N ratios indicated that reduction in C and N content as determined in M9/GCA medium were proportional, and that an average C:N ratio of 3.75 was maintained. A C:N ratio of 3.78:1 in *P. putida* grown in modified 2216E was calculated from the data of Passman (1977). While the absolute dry weight C and N content of the cells varied under the two cultural conditions (2216E vs M9/GCA media, both in chemostat culture), their relative concentrations were unchanged. Examination of C, N and H content of cells grown under conditions of Cu stress indicated only a slight increase in the C:N ratio which was ascribed to the reduction in protein content discussed earlier. This increase in C:N ratio was significant at the 98 % confidence level (P = 0.021; df = 6), indicating that a slight rearrangement in major cellular components did occur under conditions of Cu stress.
Under conditions of growth in M9/GCA medium containing NH$_4$NO$_3$ in place of NH$_4$Cl, a C:N ratio of 3.74 was observed. In general, although absolute concentrations of C, N and H varied under different cultural conditions, the relative concentrations of C and N were maintained unless the cells were placed under stress conditions.

D. Major Ion Analyses: Na and K

Major ion data are discussed in two sections. Na and K are primarily intracellular ions and are considered together. The factors influencing the concentration of one of these ions equally influence the other. The factors which affected Mg and Ca values in this investigation were attributed primarily to their effect of the cell wall/cell envelope. Thus, Mg and Ca, and also Sr and P, other cell wall-associated elements, are discussed together in the following section.

In the literature review, data have been presented indicating that Na and K are present within the cell in osmotically active forms. Intracellular Na content of Alteromonas haloplanktis, B-16 was approximately equal to extracellular Na content over a range of 0-1 M extracellular Na (Takacs, et al., 1964). Intracellular K in Alteromonas haloplanktis, B-16 was 2 X the extracellular K concentration at 0.01 and 0.15 M extracellular K and was independent of Na.
concentration. Na content of E. coli was determined by Na content of the medium and was independent of osmolality of the medium (Epstein and Shultz, 1965). A sudden drop in osmolality resulted in a rapid efflux of K from the cell. Jones, et al (1979) corroborated an earlier report by Kung, et al (1976) indicating that the amount of a particular metal detected was highly dependent upon the wash procedure used in cell preparation. Jones, et al, (1979) also observed varying sensitivities of different organisms to different washing techniques. P. putida was particularly sensitive to the chilled 0.5 M ammonium formate wash employed in that investigation. Additional factors were the relative amounts of Na and K which were cell envelope-associated compared to the cytoplasmic concentrations and method of presentation of the data, whether as % cell dry weight or as cell wet weight (i.e., amount of a metal detected relative to the amount present in a volume of medium equivalent in weight to that occupied by a viable cell). For an element not concentrated at all by a cell (i.e., [M+]in = [M+]out), that is 80 % water by weight, the concentration factor reported for that element on a cell wet weight basis would be 1, indicating no concentration of that element by the organism, but reported on a dry weight basis, the organism would appear to concentrate the element from the environment by a factor of 5 X. This calculation presumes that the element was not washed out of the cell during preparation for analysis.
Without consideration of these factors, accurate estimates of cellular Na and K in situ would not be possible. The data are useful only for comparison to other data obtained in the same manner from the same organism. Extrapolations, however, are useful for the purposes of discussion, and so if one assumes an 85 % water content for P. putida and, as per Takacs, et al (1964) a Na(in)/Na(out) ratio of 1 and a K(in)/K(out) ratio of 2, the expected Na content of P. putida would be 54,900 ppm and the expected K content, 4433 ppm if no losses occurred during the wash process. The mean Na value of 5356 ppm (Table 16) would then indicate that approximately 90 % of the Na had been removed in the wash procedure. The mean K value of 4470 ppm, however, would indicate that use of the chilled Q2W wash, as described, resulted in virtually no K loss from the cell. Based on past assumptions and on data for E. coli (Epstein and Shultz, 1965; Kung et al, 1976) indicating rapid efflux of K under conditions of osmotic downshock, one is forced to reconsider the assumptions under which the calculations were made. The assumption of an 85 % water content of the cell is not unreasonable. A range of 80-90 % water is reported in Stanier, et al (1976). The assumption that approximately 100 % of the monovalent cations was associated with the cell cytoplasm in P. putida is surely an over-estimate, since Na and K are associated transiently with symport and antiport systems in the cell membrane. Based on the relative stability constants of divalent and
monovalent cations in organic complexes (Sillen and Martell, 1964), the relative affinities of divalent vs monovalent cations for cell wall materials (Beveridge and Murray, 1976) and abundance of divalent cations in natural and KSW, one can assume that a majority of the available binding sites on the cell wall are occupied by divalent cations. If, then, the data reported by Takacs, et al are valid for other cells, it would appear that in the case of the marine pseudomonad P. putida (ATCC 29735) careful maintenance of ice cold temperatures from the time of harvest through the wash procedure resulted in retention of intracellular K, but not of intracellular Na. The association of K, but not Na with many macromolecular structures in the cell may have accounted for this phenomenon.

Under conditions of Cu stress reduced amounts of both alkali metals were detected. Increased osmotic sensitivity in Pseudomonas sp. when grown in the presence of $4 \times 10^{-4}$ M Cu was reported by Sadler and Trudinger (1967). Similar observations have been reported by Zevenhuizen, et al (1979) for Klebsiella aerogenes grown in chemostat culture under conditions of Cu stress. The same phenomenon appeared to occur in Cu-stressed P. putida. Na and K were both more easily removed from the cell by the wash procedure, with Na, again, more drastically affected (Table 17).
Na and K data for \textit{P. putida} grown in chemostat culture in M9/GCA medium with NH$_4$NO$_3$ as the N-source (Table 18) demonstrated reduced values compared to those obtained under standard conditions. The reduction in Na content was not significant ($P = 0.662$; df = 8); the reduction in K content was significant ($P = 0.014$; df = 8). During these experiments equipment failures prevented continual maintenance of chilled temperatures. Otherwise the cells were prepared in an identical manner to those under standard conditions. The data indicated greater efflux of alkali metals when low temperatures were not maintained. Under these conditions of growth and temperature maintenance, results approximating those of Passman (1977) were obtained.

Summarizing observation of alkali metal content of \textit{P. putida} in this investigation, Na and K were detected at higher concentrations than previously reported using a Q2W wash with special attention paid to maintenance of ice cold conditions during harvest and preparation of the cell material. Based on available information from the literature, it would appear that 90\% of the intracellular Na was removed by the wash procedure while intracellular K was retained. Under conditions of Cu stress Na and K were more sensitive to the osmotic effects of the wash procedure, with Na again more depleted.
E. **Major ions: Mg and Ca, and Sr and P.**

Mg and Ca values reported in Table 16 were different from those reported previously for *P. putida* (Passman, 1977; Jones, ET AL, 1979). Mg content reported by Passman (1977) was $4000 \pm 1320$ ppm, and the Ca content was $2600 \pm 2550$ ppm; percent relative standard deviations for the analyses were 33.3 and 99.6 % for Mg and Ca, respectively. In the current investigation coefficients of variation for Mg and Ca were 5.4 and 4.7 %, respectively. Differences in results between the two investigations were due presumably to a variety of factors including medium type and growth rate in chemostat culture. Differences in handling and analytical procedures are to be considered, although identical protocols for harvest, wash and analysis were used. Variability observed in the results of Passman (1977) suggested that caution be exercised when considering accuracy of his mean values.

Consideration of the Mg value obtained in the current investigation on a wet weight basis, again, provides some insight into the possible relative concentration factors for the different elements. Based of a water content of 85 % (w/v), a dry weight Mg content of 6483 ppm would be expected if Mg was not concentrated by *P. putida*. Previous investigations have indicated that Mg was bound tightly by cells and not removed by distilled water washes (Beveridge and Murray, 1976; Kung, ET AL, 1976). The concentration factor in wet cells based on the dry weight Mg content
(Table 16) would be 1.06 X, suggesting that little energy need be expended by the organism to satisfy its Mg requirement. Ca was exported from cells under normal conditions by most organisms (Silver, 1977). However, under conditions where active transport (export) was inhibited, either by cold or by metabolic inhibitors, Ca re-entered the cell by facilitated transport and accumulated to concentrations approaching those outside the cell (Silver, et al, 1975). Assuming an 85 % water content of P. putida, the concentration of Ca expected in the cell if not concentrated (or exported) would be 1552 ppm. On this basis a wet weight concentration factor of 1.14 X was calculated. However, one must be cautious when considering calculations such as these for ions which are partially or primarily cell wall-associated. A cell which has a relatively low cytoplasmic Ca content, but a high cell wall Ca content may appear to neither actively export or concentrate Ca when considered on a whole cell basis.

The mean Sr value of 25.9 ppm obtained in this investigation (Table 19) was the first reported for P. putida and so no basis for comparison was available. Sr has been reported to be taken up by the marine bacterium, Serratia marinorubra (Lear and Oppenheimer, 1962) and concentrated by a factor of 1300X (based on cell wet weight).
P values observed in Table 15 were greater than those observed by Passman (1977; 1.75 ± 0.20 %). Failure to leach P, as well as other trace elements from the siliceous precipitate recovered during the digestion process may have been responsible for the low values for P, and other trace elements, as well, observed by Passman (1977).

Under conditions of Cu stress Mg, Ca, P and Sr content increased (Tables 15, 17, 20). These changes presumably were related to subtle changes in the cell wall of P. putida under these conditions. P. putida is a gram-negative bacterium whose cell wall contains a dense inner layer composed of peptidoglycan and lipoprotein, and an outer membrane different in structure and function from the cytolasmic membrane, consisting of proteins, lipopolysaccharide and lipoproteins. Mg, Ca and Sr in other bacteria have been implicated in crosslinking of peptide subunits of peptidoglycan in maintenance of integrity of superficial cell wall layers and in crosslinking between lipopolysaccharide subunits and other cell wall layers (Asbell and Eagon, 1966; Rayman and MacLeod, 1975; Beveridge and Murray, 1976). P is associated primarily with two classes of compounds in the cell, the nucleic acids and the phospholipids of the membrane. The cell wall of P. aeruginosa contained 24.3 % lipid, 29-33 % of which was phospholipid; P content of the cell wall was 2.6 % (Cox and Eagon, 1968). Bobo and Eagon (1968) observed Mg and Ca associated with the phospholipids of P. aeruginosa,
especially with phosphatidylethanolamine and phosphatidylglycerol. P (1.5 %), Mg (0.15 %), Ca (0.7 %) and Zn (0.09 %) was determined in the ash of \textit{P. aeruginosa} cell wall (Eagon, et al 1965).

In chemostat studies of \textit{K. aerogenes} and \textit{P. putida} Tempest (1969) observed an RNA:Mg ratio of 5. This ratio was constant at all growth temperature and dilution rates (growth rates). Absolute quantities of RNA and Mg, however, varied directly with growth rate, and hence with ribosome content of the cells. The large requirement of bacteria for Mg results principally from a specific stoichiometric requirement of their ribosomes for this cation. In \textit{P. putida} an RNA:Mg ratio of 1.28 was observed (M.W. of RNA taken as 340) under standard growth conditions. Under Cu stress conditions this ratio was reduced to 1.01. Since RNA content was not significantly different (slightly reduced), the decrease in RNA:Mg ratio was due primarily to the increase in Mg and was independent of the role of Mg in the RNA/ribosome system. It was suspected that, had growth rate of the unstressed \textit{P. putida} culture been reduced to that of the stressed culture by reducing dilution rate of the chemostat, the effect would be to decrease the amounts of Mg, RNA and P detected in unstressed cells. Hence, if those two cultural systems were compared: unstressed \textit{P. putida} growing at less than maximal growth rate due to energy limitation, and Cu-stressed \textit{P. putida} growing at the same growth rate due to the action of the heavy metal, the
relative increases calculated for Mg, P, Sr and Ca would be even greater.

Gordon and MacLeod (1966) observed a P:Mg molar ratio of 7.3:1 in phospholipid-containing extract of envelopes of P. aeurginosa and of Alteromonas haloplanktis (NCMB 19). In P. putida, a whole cell P:Mg molar ratio of 2.91 was observed. Under conditions of Cu stress the ratio was unchanged (P:Mg = 2.93) indicating a correlated increase in the two elements under Cu stress. Likewise, the Ca:Sr molar ratios were unchanged under standard and Cu stress conditions, indicating proportional increases in the two elements under Cu stress. Under conditions of Cu stress increases in Mg and P were related to increases in production of outer cell wall components which may have compensated for increased leakiness of the cell's permeability barrier. This concurrently would increase available binding sites for Ca and Sr associated with the outer cell wall structure in gram negative bacteria.

Mg and Ca data for P. putida grown in chemostat culture with NH$_4$NO$_3$ as the N-source approximated more closely the values obtained under Cu stress conditions than those obtained under standard conditions (Table 18). It is difficult to perceive that substitution of N source could have so profound an effect on Mg and Ca content of the cell, but other than exposure to temperatures above 2 C, growth and preparative conditions were identical. P content was
the same as under standard growth conditions and Sr content, while more variable, was not significantly different (Tables 15 and 20). Additional investigation would be required to explain the observed results.

Summarizing the observations of Mg, Ca, Sr and P content of *P. putida* grown in chemostat culture in M9/GCA medium, consistent, more realistic Mg values have been obtained; however, calculations of Mg content on a wet weight basis indicated that Mg was not concentrated significantly by *P. putida* to satisfy its requirement for Mg. The increase in Mg content under Cu stress was correlated with an increase of equal magnitude in P content. It is suspected that increases in both ions were related to an increased production of outer cell wall material, including the phospholipids. Ca values were reported with greater precision than in previous investigations. Whether the Ca values obtained reflected the *in situ* Ca content of *P. putida* cannot be ascertained; investigation of facilitated transport of Ca into the cell under reduced temperatures must be investigated first. The increase in Ca and Sr content of *P. putida* under Cu stress resulted from additional availability of binding sites in the added cell wall material produced under these conditions.

F. Minor ion analysis: Fe and Zn.
Fe and Zn values for *P. putida* grown in M9/GCA medium (Table 19) were greater than observed in M9 medium (37 ppm Fe, 20 ppm Zn) by Murray (1974), but less than those observed in 2216E medium by Passman (1977; 170 ppm Fe, 97 ppm Zn). Data presented by Jones, et al (1979) indicated that trace metals were bound tightly to the cell and not removed by any of the wash solutions investigated. The reported differences, then, likely reflected differences in medium composition. Murray (1974) reported Fe and Zn concentrations for M9 medium of 356 and 29 ppb, respectively.

Fe and Zn content of *P. putida* grown under Cu stress conditions were not significantly different from content observed under standard conditions (Table 20). Content of these metals observed in M9/GCA medium with NH₄NO₃ as the N-source, while appearing to be slightly increased, were not significantly different (Fe: \( P = 0.026, \text{df} = 8 \); Zn: \( P = 0.101, \text{df} = 8 \); Table 21). The data indicated that Fe and Zn content were determined primarily by concentrations of these trace metals in the growth medium. Even in the presence of elevated concentrations of a highly reactive element such as Cu, the uptake of these physiologically important trace metals by the cell was unaltered.

G. Minor ion analysis: Cu.
Under standard conditions Cu content of *P. putida* was 11.6 ppm (Table 15). This value was below the values reported by Passman (1977), 27.6 ± 7.01 ppm, and Jones, et al (1979), 19 ppm, for Cu content of *P. putida* grown in modified 2216E and 0.1 % tryptone/yeast extract seawater media, respectively. While certain activities as enzyme cofactors have been described for Cu in microbial systems, obligate requirements for Cu have not been demonstrated. Variation in Cu content in the two cited investigations and this investigation were probably the result of differences in medium Cu content. *P. putida* grown in M9 medium (Cu content, 13 ppb in the medium) exhibited a Cu content of 13 ppm (Murray, 1974). The value of 11.6 ppm reported in the current investigation was obtained in a medium containing 7.11 ppb Cu.

Under the same growth conditions in a medium containing NH₄NO₃ as the N source, Cu content was significantly higher (Table 21; P = 0.014, df = 8). This difference may have been a reflection of differences in medium Cu values, although analysis of NH₄NO₃ for Cu was not performed.

Under conditions of Cu stress greatly elevated concentrations of Cu were observed in *P. putida* (Table 20). The concentration of Cu in the cells was observed to vary significantly among the three Cu stressed chemostat runs, even though the Cu content of the medium was maintained at 5 x 10⁻⁵ M Cu in all instances. Variations in Cu content were
inversely related to the average cell number in the reaction vessel (Fig. 3) which varied somewhat from chemostat run to chemostat run. The phenomenon was similar to that observed by Sadler and Trudinger (1967) and by Zevenhuizen, et al (1979) in which Cu content of a cell was determined by the ratio of available Cu to biomass within a certain range of Cu concentrations. Beyond this linear range uptake of Cu increased drastically as cell injury allowed entry of the ion into the cell. This phenomenon was observed by Jones, et al (1976) in their investigation of Zn uptake by Alcaligenes marinus in a complex seawater medium containing Zn in the range of $1 \times 10^{-5}$ to $3 \times 10^{-4}$ M. Concentration factors decreased with increasing Zn up to a concentration of $1 \times 10^{-4}$ M Zn where presumably all available binding sites were occupied, but major cell injury had not occurred. With increasing Zn concentration, more binding sites became available in the injured cell and concentration factors once again increased. In the current investigation Cu/biomass ratios were maintained within the linear region of the observed relationship. However, at lower cell densities, increasing absolute amounts of Cu were cell-associated indicating increased uptake or increased binding of Cu with increasing Cu/biomass ratio.

A second complicating factor existed in the medium used in this investigation which should be discussed at this juncture. As mentioned previously, the the stability constant for the Cu-gluconate complex was given by Sillen.
and Martell (1964) as 19.3. Transport mechanisms for gluconate have been described, and the possibility exists that Cu concentrations detected were the result of uptake of Cu as the gluconate complex. With increasing population densities in the chemostat, increasing amounts of gluconate would be converted extracellularly to 2KG. Although the stability constant for the 2KG-Cu complex was not given by Sillen and Martell (1964) it was expected that it approximated that of the gluconate-Cu complex. Since 2KG transport mechanisms are not activated until gluconate is nearly exhausted from the medium, an increasing concentration of excluded complexes in the form of the 2KG-Cu complex could be observed with increasing cell densities. This situation would have caused increasing amounts of Cu to be maintained outside the cell at increasing cell densities and would account for the decrease in the absolute amount of cell-associated Cu detected. Monitoring of 2KG content of the RV effluent would provide further insight into this situation.

Summarizing the results for Cu uptake by P. putida, the results obtained in M9/GCA medium agreed with those obtained by Murray (1974) in M9 medium which had a Cu content roughly equivalent to that found in M9/GCA medium. During growth in M9/GCA containing $5 \times 10^{-5}$ M Cu, Cu content of the cells was elevated, and varied inversely with cell density in the medium. The absolute amount of Cu bound to the cells at different cell densities was not constant.
H. Trace element analyses: Mn, Ni, Cd, Pb and Cr.

Trace ions detected in *P. putida* under standard conditions (Table 22) were not greatly different than those detected by Murray (1974) after growth in M9 medium. The slightly higher Ni, 9.9 ppm, and Pb, 18 ppm, values detected by Murray (1974) were likely a reflection of the higher medium concentrations of these elements (14 ppb and 32 ppb, respectively). Of the trace elements considered in this investigation, only Mn and occasionally Ni are considered biologically important from a nutritional standpoint. The other ions are of interest primarily for the toxic properties they exhibit. All are accumulated primarily by adsorption, but specific active transport systems (Silver, 1970) and cross-reactivity with other metal transport systems (Webb, 1970) also have been reported.

Under conditions of Cu stress only mean Pb content of *P. putida* was altered significantly. All other ions were present at concentrations approximating those observed under standard conditions. Among the individual chemostat runs, Pb and Cd were present in *P. putida* at lower concentrations in cells containing higher Cu contents (Table 23). Pb and Cd are lower than Cu on the Irving and Williams (1949) series for stability of chelates. In this investigation Cu at high Cu/biomass ratio was able to displace Cd and Pb, metals which have been reported to be primarily adsorbed to, rather than transported into cells (Tornabene and Edwards,
1972; Doyle, et al., 1975). Other elements which were transported into the cell were not displaced by the elevated Cu concentrations in the growth medium.

Trace element content of *P. putida* grown in M9/GCA medium containing NH₄NO₃ as the N source was not significantly different from content observed under standard conditions (Table 24), with the exception of Pb which was significantly higher (*P* = 0.012; df = 8) possibly due to a higher Pb concentration in the medium. Substitution of NH₄NO₃ as the N source in M9/GCA medium apparently had no effect on the trace metal content of *P. putida*.

Co analyses were attempted during this investigation but were unsuccessful. Difficulty was encountered because of the proximity of an intense Ca emission line at 239.9 nm to the absorbance line for Co (240.7 nm). Attempts to use other Co absorbance lines were unsuccessful due to the relatively low intensity of the Co hollow cathode lamp. Extraction of Co from the seawater-salt matrix by various modifications of the APDC/DDTC solvent extraction technique also were not successful.

Analysis of the washed siliceous precipitates (Materials and Methods) from Chemostat Runs #5 (standard conditions) and #2 (M9/GCA medium containing NH₄NO₃) by dc-arc emission spectroscopy revealed that aside from Si, only Al and Fe were present at significant concentrations (<100 ppm). Based on an estimated sample weight of 10 mg
for precipitate obtained from 1 g dry weight cell material, the indicated concentration losses for Al and Fe due to removal with the precipitate was 1.0 ppm. Attempts to detect Al by graphite furnace AA were not successful due to interfering furnace emissivity and recurring "memory" effects. The arc emission analyses indicated that Al was present in the cell at concentrations up to 1 ppm, and possibly higher. Cu was detected in the emission spectrum at <5 ppm, Mg at <10 ppm and Ba at <5 ppm (i.e., corresponding to 50 ppb, 100 ppb and 50 ppb, respectively, in the cell). Trace amounts of Na, Ca, Sn, Zn and possibly Ti were noted in the emission spectrum. P, Cd, Mn, Ni and Pb were conspicuously absent. The dc-arc emission analysis indicated that the 1 % HNO₃ wash procedure employed had leached successfully a majority of the associated metals from the precipitate. Because the quantity of precipitate that was recovered after digestion was not insignificant, precipitates obtained in subsequent Cu stress experiments were retained and weighed to give a rough estimate of their relative presence in the cell material. Values of 8.13, 8.75 and 14.33 mg washed siliceous precipitate were obtained from 1.00 g dry cell material from Chemostat Runs #1 – #3 (Cu stress conditions). A range of 0.81 to 1.43 % cell dry weight was indicated with a calculated mean content of 1.04 ± 0.34 %. The quantity of siliceous material obtained was surprising. It can only be assumed that the silicate was provided in the form of contaminants of the salts used in
preparation of the medium. This represents a heretofore overlooked component of bacterial cells which was present in significant quantities under conditions where no Si has been supplied in the growth medium. Of the other inorganic salts, only Mg was detected in comparable quantities. The possibility of significant Si uptake by marine microorganisms in natural waters where measureable quantities of Si are available is noted.
I. **Trace metal analyses of M9/GCA medium**

Analyses of the separate medium components provided information on trace metal content of KSW and on the relative contributions of each component to the overall trace metal content of the medium. Results of extraction of trace metals from KSW by the two techniques, ion-exchange and chelate/solvent extraction, indicated relative strengths and weaknesses in both techniques (Table 25).

Using the Chelex 100 ion-exchange technique less than satisfactory recoveries were obtained in all instances. The only departure from the described protocol of Kingston, *et al* (1978) was the use of 50-100 mesh Chelex 100 in place of 200-400 mesh Chelex 100. Poor extraction efficiencies using Chelex 100 have been reported by other investigators as well. Bruland, *et al* (1979) reported poor recoveries from natural waters for Cu (~40 %) and Ni (~75%) using a Chelex technique. Zn recoveries were also suboptimal. The ineffectiveness of the resin in extracting colloidal materials was suggested as the reason for the poor recoveries. Riley and Taylor (1968) reported that recovery of Fe and Mn using Chelex 100 was incomplete below a pH of 9.0. Biechler (1965) reported incomplete retention of Fe(III) and Cr(III) on Dowex-Al (50-100 mesh) in the Na form. Palkalns, *et al* (1978) suggested that if washing of OH- from the pore structure of the resin was incomplete, readily hydrolyzed metals such as Cr, Mn and Fe were
precipitated on the column and not recovered. Mn was reported not to be completely eluted from the column by 2 M HNO₃. An additional elution with 2 M HCl was required. Lee, et al. (1977) found 4 M HNO₃ was required for complete removal of all trace metals from Chelex 100. Both incomplete extraction and incomplete recovery plague the ion-exchange technique when used to extract trace metals from seawater.

Using APDC/DDTC Freon TF extraction technique of Danielsson, et al. (1978), acceptable recoveries for Fe, Zn and Cu were obtained. Mn-thiocarbamate complexes are notoriously unstable (Wijkstra and Van der Sloot, 1978; Bone and Hibbert, 1979) and were not detected during this investigation. Modifications of the original technique described by Kinrade and Vanloon (1974) have been reported which yield acceptable extraction efficiencies for Mn. Only Cr(VI) was extracted under normal conditions by thiocarbamate chelates (Bone and Hibbert, 1979). The values reported in this investigation were representative only of Cr(VI) content of KSW. Total Cr can be obtained either by oxidation of Cr(III) to Cr(VI) or by boiling the APDC/sample mixture to allow the APDC-Cr(III) complex to form (Jan and Young, 1978). Alternatively, use of DDTC alone at a pH of 2-4 will extract the Cr(III) hexahydrate complexes (Kinrade and Vanloon, 1974). Ni and Pb extractions by thiocarbamate techniques generally have been reported to be high. The reason for reduced efficiency of extraction in this
investigation was not apparent.

Comparison of ease of operation of the two techniques indicated the APDC/DDTC technique was the technique of choice. Preparation of fresh reagents and extraction time together required approximately 1.5 h and several samples could be extracted simultaneously. In the Chelex technique, elution, regeneration, washing of the column, elution of the major ions, and elution of the transition metals required a minimum of 24 h for a 500 ml sample. Based on extraction efficiencies observed in this investigation and ease of preparation and operation, the APDC/DDTC Freon TF solvent extraction would be more highly recommended.

Analysis of the separate medium components for trace metal content revealed that only gluconic acid contributed appreciable quantities of trace metals to the medium (Table 26). It is probably fortuitous that the metals contributed in the greatest quantities, Fe and Zn, were those which are required in greatest amounts by microorganisms in their growth media. Concentrations of the trace metals detected in M9/GCA medium were below those reported by Murray (1974) for M9 medium.

Concluding remarks

Analysis of biological samples for inorganic ion content continues to present problems for investigators seeking to understand interactions between these two
systems. Specimen collection/sampling procedures, handling and preparation of samples and analytical techniques all must be carefully controlled if meaningful results are to be obtained.

Analysis of microorganisms involves special problems not encountered in analysis of larger organisms. Collection of sufficient biomass from the environment to allow analysis for trace metal content is still not possible with available techniques. The utilization of continuous cultivation techniques has been an important step in overcoming limitations of laboratory cultivation of environmental samples.

Once suitable biomass has been obtained, the crucial preparative step is indisputably the wash procedure employed to free cells from extracellular debris. The question of whether or not to use a wash procedure is not a clear-cut one. If cells in the environment are normally surrounded by an extracellular organic matrix to which is adsorbed a variety of inorganic ions, does analysis after removal of this matrix yield results reflecting the in situ composition? Trapping of particulate debris within the pellet of centrifuged cells, however, also would cause inaccurate results to be observed. An ideal wash solution would be of composition and tonicity similar to the growth medium. Inclusion of a radiolabelled excluded polymer such as carboxymethylcellulose would allow for the estimation of
salts in the extracellular pore space of the pellet. Only in this way can realistic analyses for all major ion be obtained simultaneously.

The results of this investigation indicate that alterations in basic cellular metabolism occur when *P. putida* is grown under Cu stress. It is likely that this response is not unique to *P. putida*, and in fact may be widespread in aquatic systems. The importance of such changes in metabolism is currently a matter of speculation, but it is suggested that any situation which alters the flow of carbon through the biosphere and reduces the production of biomass is of significance to the ecosystem and warrants further careful investigation.
LITERATURE CITED


Table 1. Uptake of metals by mixed sediment isolates.

<table>
<thead>
<tr>
<th></th>
<th>Cr</th>
<th>Cu</th>
<th>Mn</th>
<th>Fe</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no metals added</td>
<td>109.09&lt;sup&gt;1&lt;/sup&gt;</td>
<td>213.22</td>
<td>16.53</td>
<td>289.26</td>
<td>119.01</td>
<td>206.61</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 1 ppm each metal</td>
<td>983.00</td>
<td>765.87</td>
<td>292.83</td>
<td>225.26</td>
<td>409.56</td>
<td>1648.46</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 2 ppm each metal</td>
<td>2850.00</td>
<td>1068.00</td>
<td>558.00</td>
<td>3450.00</td>
<td>720.80</td>
<td>2010.00</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values ppm dry weight

Data from Patrick and Loutit (1976)
Table 2. Trace element content of *Escherichia coli*, *Micrococcus roseus*, *Sphaerotilus natans* and *Bacillus cereus* grown in complex organic media in batch culture.

<table>
<thead>
<tr>
<th>Cell dry weight, ppm</th>
<th>Cr</th>
<th>Mn</th>
<th>Ni</th>
<th>Cu</th>
<th>Sr</th>
<th>Ag</th>
<th>Sn</th>
<th>Pb</th>
<th>Fe</th>
<th>Zn</th>
<th>Ti</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>3</td>
<td>27</td>
<td>6</td>
<td>12</td>
<td>4</td>
<td>0.6</td>
<td>5</td>
<td>12</td>
<td>300</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>M. roseus</td>
<td>2</td>
<td>9</td>
<td>7</td>
<td>18</td>
<td>5</td>
<td>0.7</td>
<td>14</td>
<td>14</td>
<td>200</td>
<td>300</td>
<td>7</td>
</tr>
<tr>
<td>S. natans</td>
<td>1</td>
<td>53</td>
<td>9</td>
<td>31</td>
<td>8</td>
<td>1.0</td>
<td>30</td>
<td>15</td>
<td>26000</td>
<td>300</td>
<td>8</td>
</tr>
<tr>
<td>B. cereus</td>
<td>0.5</td>
<td>620</td>
<td>3</td>
<td>41</td>
<td>4</td>
<td>2.0</td>
<td>5</td>
<td>8</td>
<td>300</td>
<td>300</td>
<td>3</td>
</tr>
</tbody>
</table>

from Rouf (1964)
Table 3. Elemental composition of *Alcaligenes marinus* and *Pseudomonas putida* grown in 0.1% tryptone/yeast extract seawater medium at 25°C.

<table>
<thead>
<tr>
<th></th>
<th>A. marinus</th>
<th></th>
<th>P. putida</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>CF</td>
<td>ppm</td>
<td>CF</td>
</tr>
<tr>
<td>Na</td>
<td>5600</td>
<td>0.7</td>
<td>225</td>
<td>0.03</td>
</tr>
<tr>
<td>Mg</td>
<td>1580</td>
<td>1.6</td>
<td>292</td>
<td>0.3</td>
</tr>
<tr>
<td>Ca</td>
<td>790</td>
<td>2.6</td>
<td>272</td>
<td>0.9</td>
</tr>
<tr>
<td>K</td>
<td>700</td>
<td>2.3</td>
<td>36</td>
<td>0.1</td>
</tr>
<tr>
<td>Zn</td>
<td>122</td>
<td>622</td>
<td>112</td>
<td>571</td>
</tr>
<tr>
<td>Fe</td>
<td>257</td>
<td>1548</td>
<td>422</td>
<td>2542</td>
</tr>
<tr>
<td>Cu</td>
<td>14.4</td>
<td>720</td>
<td>19</td>
<td>950</td>
</tr>
<tr>
<td>Pb</td>
<td>9.7</td>
<td>510</td>
<td>3</td>
<td>158</td>
</tr>
<tr>
<td>Ni</td>
<td>1.7</td>
<td>106</td>
<td>4.5</td>
<td>281</td>
</tr>
<tr>
<td>Mn</td>
<td>1.7</td>
<td>131</td>
<td>0.9</td>
<td>69</td>
</tr>
<tr>
<td>Cd</td>
<td>2.8</td>
<td>1400</td>
<td>4.6</td>
<td>2300</td>
</tr>
</tbody>
</table>

from Jones, et al (1976)

1 concentration factor
Table 4. Trace element composition of *Alcaligenes marinus* grown in 0.1 % tryptone/yeast extract medium containing elevated concentrations of Zn in batch culture, T=25 C.

<table>
<thead>
<tr>
<th>Zn conc. medium</th>
<th>Zn</th>
<th>Zn CF</th>
<th>Fe</th>
<th>Cu</th>
<th>Mn</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.350</td>
<td>70.1</td>
<td>200</td>
<td>176</td>
<td>14.2</td>
<td>2.3</td>
<td>1927</td>
<td>937</td>
</tr>
<tr>
<td>1.050</td>
<td>101.5</td>
<td>97</td>
<td>784</td>
<td>7.6</td>
<td>3.0</td>
<td>1714</td>
<td>742</td>
</tr>
<tr>
<td>3.650</td>
<td>201.3</td>
<td>55</td>
<td>249</td>
<td>7.1</td>
<td>3.0</td>
<td>1641</td>
<td>536</td>
</tr>
<tr>
<td>6.960</td>
<td>333.3</td>
<td>48</td>
<td>227</td>
<td>12.1</td>
<td>1.8</td>
<td>1208</td>
<td>275</td>
</tr>
<tr>
<td>13.39</td>
<td>964.4</td>
<td>72</td>
<td>167</td>
<td>4.4</td>
<td>2.0</td>
<td>1740</td>
<td>313</td>
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<tr>
<td>18.35</td>
<td>3712.4</td>
<td>202</td>
<td>250</td>
<td>7.3</td>
<td>1.8</td>
<td>1272</td>
<td>1326</td>
</tr>
</tbody>
</table>

from Jones, *et al* (1976)
Table 5. Major ion content of microorganisms grown in 0.1% tryptone/yeast extract seawater medium in batch culture at 25°C.

<table>
<thead>
<tr>
<th>Cell dry weight, ppm</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alteromonas haloplanktis</td>
<td>299</td>
<td>36</td>
<td>420</td>
<td>267</td>
</tr>
<tr>
<td>Pseudomonas putida (ATCC 29735)</td>
<td>255</td>
<td>36</td>
<td>292</td>
<td>272</td>
</tr>
<tr>
<td>Pseudomonas sp. (unidentified)</td>
<td>327</td>
<td>55</td>
<td>156</td>
<td>289</td>
</tr>
<tr>
<td>Vibrio fisherii</td>
<td>357</td>
<td>89</td>
<td>359</td>
<td>165</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>298</td>
<td>149</td>
<td>1178</td>
<td>1655</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>3341</td>
<td>1789</td>
<td>1351</td>
<td>42</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>365</td>
<td>99</td>
<td>544</td>
<td>48</td>
</tr>
<tr>
<td>Pseudomonas ovalis</td>
<td>221</td>
<td>26</td>
<td>660</td>
<td>834</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>66</td>
<td>207</td>
<td>776</td>
<td>112</td>
</tr>
</tbody>
</table>

from Jones, et al (1979)
Table 6. Minor ion content of microorganisms grown in 0.1 %
tryptone/yeast extract seawater medium in batch culture at 25 C.

<table>
<thead>
<tr>
<th>Cell dry weight, ppm</th>
<th>Fe</th>
<th>Zn</th>
<th>Mn</th>
<th>Cu</th>
<th>Ni</th>
<th>Cd</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alteromonas haloplanktis</td>
<td>262</td>
<td>159</td>
<td>0.4</td>
<td>22</td>
<td>1.5</td>
<td>39</td>
<td>3</td>
</tr>
<tr>
<td>Pseudomonas putida (ATCC)</td>
<td>422</td>
<td>112</td>
<td>0.9</td>
<td>19</td>
<td>4.5</td>
<td>4.6</td>
<td>3</td>
</tr>
<tr>
<td>Pseudomonas sp. (unidentified)</td>
<td>365</td>
<td>180</td>
<td>0.5</td>
<td>49</td>
<td>1.5</td>
<td>8.8</td>
<td>2</td>
</tr>
<tr>
<td>Vibrio fisheri</td>
<td>351</td>
<td>101</td>
<td>1.5</td>
<td>29</td>
<td>1.5</td>
<td>10.4</td>
<td>2</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>339</td>
<td>186</td>
<td>10.5</td>
<td>36</td>
<td>2.4</td>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>111</td>
<td>76</td>
<td>2.7</td>
<td>11</td>
<td>0.6</td>
<td>3.1</td>
<td>11</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>99</td>
<td>93</td>
<td>3.1</td>
<td>19</td>
<td>2.6</td>
<td>2.9</td>
<td>39</td>
</tr>
<tr>
<td>Pseudomonas ovalis</td>
<td>256</td>
<td>108</td>
<td>0.5</td>
<td>12</td>
<td>2.8</td>
<td>0.4</td>
<td>18</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>86</td>
<td>104</td>
<td>4.4</td>
<td>14</td>
<td>0.6</td>
<td>4.3</td>
<td>30</td>
</tr>
</tbody>
</table>

from Jones, et al (1979)
Table 7. Comparison of cell density ($A_{420}$) and pH change during growth of *P. putida* in batch culture in M9 and M9/GCA media.

<table>
<thead>
<tr>
<th>Time, h</th>
<th>M9</th>
<th>M9/GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{420}$</td>
<td>pH</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>7.0</td>
</tr>
<tr>
<td>21.5</td>
<td>0.006</td>
<td>7.15</td>
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<tr>
<td>26.5</td>
<td>0.012</td>
<td>7.13</td>
</tr>
<tr>
<td>29.0</td>
<td>0.013</td>
<td>7.06</td>
</tr>
<tr>
<td>44.5</td>
<td>0.041</td>
<td>6.71</td>
</tr>
<tr>
<td>47.0</td>
<td>0.056</td>
<td>6.64</td>
</tr>
<tr>
<td>50.0</td>
<td>0.101</td>
<td>6.58</td>
</tr>
<tr>
<td>52.0</td>
<td>0.150</td>
<td>6.40</td>
</tr>
<tr>
<td>64.0</td>
<td>0.735</td>
<td>3.89</td>
</tr>
<tr>
<td>95.0</td>
<td>0.690</td>
<td>3.50</td>
</tr>
<tr>
<td>115.0</td>
<td>0.650</td>
<td>3.42</td>
</tr>
</tbody>
</table>
Table 8. Chromatographic identification of oxidation products produced by *P. putida* in M9 medium grown at 25°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>R_f(s)</th>
<th>Detected by AgNO_3</th>
<th>Detected by aniline oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.440</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>Gluconate</td>
<td>0.370</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>2KG</td>
<td>0.365</td>
<td>+++</td>
<td>rose-pink, +++</td>
</tr>
<tr>
<td>5KG</td>
<td>0.543</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>T=0 h</td>
<td>0.435</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>T=1 h</td>
<td>0.435</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>T=2 h</td>
<td>0.435</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>T=3 h</td>
<td>0.435</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>T=4 h</td>
<td>0.435, tailing</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
<tr>
<td>T=5 h</td>
<td>0.435, tailing</td>
<td>+++</td>
<td>pale brown, +++</td>
</tr>
</tbody>
</table>

ND = Not detected
+
+, ++, +++ - Increasing intensity of spot detected
Table 9. Chromatographic identification of oxidation products produced by *P. putida* in M9/GCA medium grown at 25 °C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;(s)</th>
<th>Detected by AgNO&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Detected by aniline oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconate</td>
<td>0.383</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>2KG</td>
<td>0.380</td>
<td>+++</td>
<td>rose-pink, +++</td>
</tr>
<tr>
<td>5KG</td>
<td>0.560</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>T=0 h</td>
<td>0.380</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>T=1 h</td>
<td>0.380</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>T=2 h</td>
<td>0.380</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>T=3 h</td>
<td>0.380</td>
<td>+++</td>
<td>pink, +</td>
</tr>
<tr>
<td>T=4 h</td>
<td>0.380</td>
<td>+++</td>
<td>pink, ++</td>
</tr>
<tr>
<td>T=23 h</td>
<td>0.390</td>
<td>+++</td>
<td>pink, +++</td>
</tr>
</tbody>
</table>

ND = Not detected

+, ++, +++ - Increasing intensity of spot detected
Table 10. Protein, RNA and DNA content of *P. putida* grown in M9/GCA medium in continuous culture; \( D=0.156 \text{ h}^{-1} \), \( T=25 \degree C \).
Values as \( \bar{X} + s \) for triplicate analyses at 5 samplings (n=15).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Protein Percent dry weight</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50.5 ± 6.53</td>
<td>14.2 ± 3.91</td>
<td>2.3 ± 0.35</td>
</tr>
<tr>
<td>2.*</td>
<td>51.0 ± 4.43</td>
<td>13.9 ± 2.74</td>
<td>2.6 ± 0.74</td>
</tr>
<tr>
<td>3.</td>
<td>52.9 ± 6.21</td>
<td>10.6 ± 1.85</td>
<td>3.3 ± 0.32</td>
</tr>
<tr>
<td>4.</td>
<td>43.5 ± 5.10</td>
<td>13.9 ± 1.47</td>
<td>4.1 ± 1.01</td>
</tr>
<tr>
<td>5.</td>
<td>57.2 ± 9.76</td>
<td>10.5 ± 1.36</td>
<td>4.6 ± 0.60</td>
</tr>
<tr>
<td>( \bar{X} ** )</td>
<td>51.0</td>
<td>12.4</td>
<td>3.5</td>
</tr>
<tr>
<td>S.E.</td>
<td>1.60</td>
<td>0.69</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Based on three samplings.
** Mean of sampling means (n=23).
Table 11. Protein, RNA and DNA content of *P. putida* grown in M9/GCA medium containing $5 \times 10^{-5}$ M Cu in continuous culture; $D=0.096$ h$^{-1}$, $T=25$ °C. Values as $\bar{X} \pm s$ for triplicate analyses at 5 samplings (n=15).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Percent dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>1.</td>
<td>40.9 + 2.76</td>
</tr>
<tr>
<td>2.</td>
<td>42.8 + 6.08</td>
</tr>
<tr>
<td>3.</td>
<td>43.9 + 4.35</td>
</tr>
<tr>
<td>$\bar{X}$*</td>
<td>42.5</td>
</tr>
<tr>
<td>S.E.</td>
<td>1.48</td>
</tr>
</tbody>
</table>

*Mean of sampling means (n=15).
Table 12. Carbon, nitrogen and hydrogen content of *P. putida* grown in M9/GCA medium in chemostat culture; $D=0.156 \, h^{-1}$, $T=25 \, ^\circ C$. Values as $\bar{X} \pm s$ for triplicate analyses ($n=3$).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>C</th>
<th>N</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>45.5 ± 1.43</td>
<td>12.4 ± 0.48</td>
<td>7.8 ± 1.78</td>
</tr>
<tr>
<td>2.</td>
<td>46.2 ± 0.75</td>
<td>12.5 ± 0.25</td>
<td>7.3 ± 0.39</td>
</tr>
<tr>
<td>3.</td>
<td>46.1 ± 1.00</td>
<td>12.5 ± 0.27</td>
<td>6.2 ± 0.89</td>
</tr>
<tr>
<td>4.</td>
<td>44.3 ± 0.54</td>
<td>12.1 ± 0.19</td>
<td>5.7 ± 0.26</td>
</tr>
<tr>
<td>5.</td>
<td>45.3 ± 1.37</td>
<td>12.3 ± 0.26</td>
<td>5.8 ± 0.22</td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>45.6</td>
<td>12.3</td>
<td>6.6</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.31</td>
<td>0.07</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses ($n=15$).*
Table 13. Carbon, nitrogen and hydrogen content of *P. putida* grown in M9/GCA medium containing $5 \times 10^{-5}$ M Cu in continuous culture; $D=0.096$ h$^{-1}$, $T=25\, ^\circ$C. Values as $\overline{X} \pm s$ for triplicate analyses ($n=3$).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Percent dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>1.</td>
<td>46.4 ± 0.63</td>
</tr>
<tr>
<td>2.</td>
<td>45.5 ± 0.05</td>
</tr>
<tr>
<td>3.</td>
<td>46.8 ± 0.27</td>
</tr>
<tr>
<td>$\overline{X}^*$</td>
<td>46.2</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses ($n=9$).*
Table 14. Carbon, nitrogen and hydrogen content of *P. putida* grown in M9/GCA medium containing NH$_4$NO$_3$ in chemostat culture; $D=0.156$ h$^{-1}$, $T=25$ °C. Values as $\bar{X} \pm s$ for triplicate analyses (n=3).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Percent dry weight</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>N</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>44.4 ± 0.22</td>
<td>12.2 ± 0.08</td>
<td>6.8 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>44.4 ± 0.29</td>
<td>11.9 ± 0.13</td>
<td>6.7 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>46.3 ± 2.00</td>
<td>12.3 ± 0.13</td>
<td>6.8 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>44.8 ± 0.10</td>
<td>12.2 ± 0.01</td>
<td>6.9 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>44.8 ± 0.20</td>
<td>11.5 ± 0.12</td>
<td>6.9 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>$\bar{X}$</td>
<td>45.0</td>
<td>12.0</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>S.E.</td>
<td>0.32</td>
<td>0.10</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=10).*
Table 15. Phosphorus content of *P. putida* grown in chemostat culture in M9/GCA medium, M9/GCA containing $5 \times 10^{-5}$ M Cu, and M9/GCA with NH$_4$NO$_3$ in place of NH$_4$Cl. Values as $\overline{X} \pm s$ for triplicate analyses (n=3).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Percent dry weight in media</th>
<th>M9/GCA</th>
<th>M9/GCA(Cu)</th>
<th>M9/GCA(NO$_3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td>2.67 ± 0.014</td>
<td>3.09 ± 0.082</td>
<td>2.68 ± 0.008</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>2.64 ± 0.010</td>
<td>3.08 ± 0.031</td>
<td>2.78 ± 0.017</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>2.55 ± 0.016</td>
<td>3.26 ± 0.196</td>
<td>2.62 ± 0.008</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>2.87 ± 0.024</td>
<td></td>
<td>2.54 ± 0.014</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>2.55 ± 0.222</td>
<td></td>
<td>2.01 ± 0.023(R)*</td>
</tr>
<tr>
<td>$\overline{X}$</td>
<td></td>
<td>2.65</td>
<td>3.13</td>
<td>2.66</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>0.032</td>
<td>0.052</td>
<td>0.027</td>
</tr>
</tbody>
</table>

*(R)=rejected as an outlier at 95% confidence level in calculation of overall mean.

**Overall mean of individual analyses (n=15 for M9/GCA, n=8 for M9/GCA(Cu), n=15 for M9/GCA(NO$_3$)).
Table 16. Major ion composition of *P. putida* grown in M9/GCA medium in chemostat culture; $D=0.156 \text{ h}^{-1}$, $T=25 ^\circ \text{C}$.

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Cell dry weight, ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na</td>
</tr>
<tr>
<td>1.</td>
<td>5810</td>
</tr>
<tr>
<td>2.</td>
<td>6080</td>
</tr>
<tr>
<td>3.</td>
<td>4250</td>
</tr>
<tr>
<td>4.</td>
<td>6080</td>
</tr>
<tr>
<td>5.</td>
<td>4560</td>
</tr>
<tr>
<td>$\bar{X}*$</td>
<td>5356</td>
</tr>
<tr>
<td>S.E.</td>
<td>394</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=5).*
Table 17. Major ion composition of *P. putida* grown in M9/GGA medium containing $5 \times 10^{-5}$ M Cu in chemostat culture; $D=0.096 \text{ h}^{-1}$, $T=25{\degree}\text{C}$.

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3080</td>
<td>2650</td>
<td>8210</td>
<td>2600</td>
</tr>
<tr>
<td>2</td>
<td>4450</td>
<td>2550</td>
<td>7630</td>
<td>2600</td>
</tr>
<tr>
<td>3</td>
<td>2460</td>
<td>4600</td>
<td>8560</td>
<td>2270</td>
</tr>
<tr>
<td>$\bar{X}^*$</td>
<td>3327</td>
<td>3263</td>
<td>8133</td>
<td>2489</td>
</tr>
<tr>
<td>S.E.</td>
<td>588</td>
<td>666</td>
<td>273</td>
<td>110</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=3).*
Table 18. Major ion composition of *P. putida* grown in M9/GCA medium containing \( \text{NH}_4\text{NO}_3 \) in place of \( \text{NH}_4\text{Cl} \), in chemostat culture; \( D=0.156 \text{ h}^{-1} \).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3320</td>
<td>1970</td>
<td>8570</td>
<td>2250</td>
</tr>
<tr>
<td>2.</td>
<td>7560</td>
<td>1220</td>
<td>8890</td>
<td>2200</td>
</tr>
<tr>
<td>3.</td>
<td>4030</td>
<td>1270</td>
<td>7630</td>
<td>1720</td>
</tr>
<tr>
<td>4.</td>
<td>1850</td>
<td>1060</td>
<td>8740</td>
<td>2520</td>
</tr>
<tr>
<td>5.</td>
<td>7310</td>
<td>4230</td>
<td>7730</td>
<td>1930</td>
</tr>
<tr>
<td>( \bar{X} )</td>
<td>4814</td>
<td>1950</td>
<td>8310</td>
<td>2124</td>
</tr>
<tr>
<td>S.E.</td>
<td>1127</td>
<td>591</td>
<td>263</td>
<td>138</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=5).*
Table 19. Minor ion composition of \textit{P. putida} grown in M9/GCA medium in chemostat culture; D=0.156 h\textsuperscript{-1}, T=25 \degree C.

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Fe</th>
<th>Zn</th>
<th>Sr</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>142</td>
<td>49.3</td>
<td>25.4</td>
<td>12.3</td>
</tr>
<tr>
<td>2.</td>
<td>110</td>
<td>47.9</td>
<td>24.0</td>
<td>10.1</td>
</tr>
<tr>
<td>3.</td>
<td>131</td>
<td>61.9</td>
<td>24.0</td>
<td>15.5</td>
</tr>
<tr>
<td>4.</td>
<td>114</td>
<td>27.0</td>
<td>27.1</td>
<td>6.8</td>
</tr>
<tr>
<td>5.</td>
<td>126</td>
<td>60.8</td>
<td>28.7</td>
<td>13.1</td>
</tr>
<tr>
<td>X*</td>
<td>125</td>
<td>49.4</td>
<td>25.9</td>
<td>11.6</td>
</tr>
<tr>
<td>S.E.</td>
<td>5.8</td>
<td>6.29</td>
<td>0.91</td>
<td>1.47</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=5).
Table 20. Minor ion composition of *P. putida* grown in M9/GCA medium containing $5 \times 10^{-5}$ M Cu, in chemostat culture; $D=0.096 \, \text{h}^{-1}$, $T=25 \, ^\circ\text{C}$.

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Fe</th>
<th>Zn</th>
<th>Sr</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>118</td>
<td>47.7</td>
<td>29.1</td>
<td>80.4</td>
</tr>
<tr>
<td>2.</td>
<td>130</td>
<td>44.1</td>
<td>47.1</td>
<td>368</td>
</tr>
<tr>
<td>3.</td>
<td>122</td>
<td>56.6</td>
<td>36.7</td>
<td>209</td>
</tr>
<tr>
<td>$\bar{X}^*$</td>
<td>124</td>
<td>49.4</td>
<td>37.6</td>
<td>219</td>
</tr>
<tr>
<td>S.E.</td>
<td>3.5</td>
<td>3.71</td>
<td>5.22</td>
<td>83.3</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses ($n=3$).*
Table 21. Minor ion composition of *P. putida* grown in M9/GCA medium containing NH₄NO₃ in place of NH₄Cl, in chemostat culture; D=0.156 h⁻¹, T=25 °C.

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Fe</th>
<th>Zn</th>
<th>Sr</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>160</td>
<td>80.0</td>
<td>36.0</td>
<td>23.3</td>
</tr>
<tr>
<td>2</td>
<td>134</td>
<td>83.2</td>
<td>15.2</td>
<td>13.9</td>
</tr>
<tr>
<td>3</td>
<td>137</td>
<td>60.2</td>
<td>27.7</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>192</td>
<td>66.6</td>
<td>37.0</td>
<td>28.2</td>
</tr>
<tr>
<td>5</td>
<td>136</td>
<td>44.4</td>
<td>32.3</td>
<td>18.0</td>
</tr>
<tr>
<td>X*</td>
<td>152</td>
<td>66.9</td>
<td>29.6</td>
<td>20.5</td>
</tr>
<tr>
<td>S.E.</td>
<td>11.1</td>
<td>7.03</td>
<td>3.96</td>
<td>2.44</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=5).*
Table 22. Trace element composition of *P. putida* grown in M9/GCA medium in chemostat culture; $D=0.156\ h^{-1}$, $T=25^\circ C$. Values reported are means of triplicate analyses ($n=3$).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Mn</th>
<th>Ni</th>
<th>Cd</th>
<th>Pb</th>
<th>Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.7</td>
<td>10.8(R)</td>
<td>2.2</td>
<td>2.4</td>
<td>14.2</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>3.4</td>
<td>1.4</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>2.4</td>
<td>4.2</td>
<td>2.6</td>
<td>3.6</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>4.4</td>
<td>1.4</td>
<td>3.6</td>
<td>8.3</td>
</tr>
<tr>
<td>5</td>
<td>3.0</td>
<td>3.1</td>
<td>2.4</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>$X^*$</td>
<td>3.5</td>
<td>3.8</td>
<td>2.0</td>
<td>3.2</td>
<td>5.8</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.61</td>
<td>0.28</td>
<td>0.25</td>
<td>0.36</td>
<td>2.40</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses ($n=15$).
Table 23. Trace element composition of *P. putida* grown in M9/GCA medium containing $5 \times 10^{-5}$ M Cu, in chemostat culture; D=0.096 h$^{-1}$, T=25°C. Values reported are means of triplicate analyses (n=3).

<table>
<thead>
<tr>
<th>Chemostat Run #</th>
<th>Mn (ppm)</th>
<th>Ni (ppm)</th>
<th>Cd (ppm)</th>
<th>Pb (ppm)</th>
<th>Cr (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>4.7</td>
<td>4.6</td>
<td>2.5</td>
<td>0.87</td>
<td>3.7</td>
</tr>
<tr>
<td>2.</td>
<td>5.3</td>
<td>4.8</td>
<td>1.6</td>
<td>0.13</td>
<td>9.2</td>
</tr>
<tr>
<td>3.</td>
<td>4.6</td>
<td>4.3</td>
<td>1.6</td>
<td>0.10</td>
<td>3.3</td>
</tr>
<tr>
<td>$X^*$</td>
<td>4.9</td>
<td>4.6</td>
<td>1.9</td>
<td>0.37</td>
<td>5.4</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.22</td>
<td>0.15</td>
<td>0.30</td>
<td>0.252</td>
<td>1.90</td>
</tr>
</tbody>
</table>

*Overall mean of individual analyses (n=9).*
Table 24. Trace element composition of *P. putida* grown in M9/GCA medium containing NH$_4$NO$_3$ in place of NH$_4$Cl, in chemostat culture; D=0.156 h$^{-1}$, T=25 °C. Values reported are means of triplicate analyses (n=3).

<table>
<thead>
<tr>
<th>Run #</th>
<th>Mn</th>
<th>Ni</th>
<th>Cd</th>
<th>Pb</th>
<th>Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3.2</td>
<td>3.1</td>
<td>2.6</td>
<td>5.8</td>
<td>3.4</td>
</tr>
<tr>
<td>2.</td>
<td>3.2</td>
<td>4.5</td>
<td>2.1</td>
<td>4.4</td>
<td>3.1</td>
</tr>
<tr>
<td>3.</td>
<td>4.4</td>
<td>7.0</td>
<td>1.8</td>
<td>4.6</td>
<td>3.0</td>
</tr>
<tr>
<td>4.</td>
<td>3.0</td>
<td>4.4</td>
<td>1.7</td>
<td>6.6</td>
<td>8.4</td>
</tr>
<tr>
<td>5.</td>
<td>6.0</td>
<td>NA*</td>
<td>2.2</td>
<td>4.1</td>
<td>1.7</td>
</tr>
<tr>
<td>$\bar{X}$**</td>
<td>4.0</td>
<td>4.8</td>
<td>2.1</td>
<td>5.1</td>
<td>3.9</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.57</td>
<td>0.81</td>
<td>0.16</td>
<td>0.47</td>
<td>1.16</td>
</tr>
</tbody>
</table>

*Not analyzed

**Overall mean of individual analyses (n=15).
Table 25. Comparison of Chelex-100 ion exchange and APDC-DDTC solvent extraction techniques for recovery of trace ions from 100% KSW. Values reported as ppb.

<table>
<thead>
<tr>
<th></th>
<th>Chelex-100</th>
<th></th>
<th></th>
<th>APDC-DDTC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KSW</td>
<td>KSW + spike</td>
<td>Extraction</td>
<td>KSW</td>
<td>KSW + spike</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Efficiency (%)</td>
<td></td>
<td></td>
<td>Efficiency (%)</td>
</tr>
<tr>
<td>Fe</td>
<td>bg</td>
<td>26.71</td>
<td>53.4</td>
<td>bg</td>
<td>47.70</td>
</tr>
<tr>
<td>Zn</td>
<td>3.67±0.05</td>
<td>19.91</td>
<td>81.2</td>
<td>4.18±0.41</td>
<td>23.34</td>
</tr>
<tr>
<td>Cu</td>
<td>2.89±0.05</td>
<td>11.34</td>
<td>84.5</td>
<td>2.34±0.07</td>
<td>12.10</td>
</tr>
<tr>
<td>Mn</td>
<td>2.31±0.64</td>
<td>2.57</td>
<td>5.2</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>3.04±0.13</td>
<td>6.50</td>
<td>69.2</td>
<td>2.96±0.08</td>
<td>6.87</td>
</tr>
<tr>
<td>Pb</td>
<td>1.69±0.07</td>
<td>6.18</td>
<td>89.8</td>
<td>1.30±0.17</td>
<td>5.42</td>
</tr>
<tr>
<td>Cd</td>
<td>1.16±0.12</td>
<td>3.57</td>
<td>120.5</td>
<td>1.18±0.23</td>
<td>3.56</td>
</tr>
<tr>
<td>Cr</td>
<td>0.013±0.003</td>
<td>0.024</td>
<td>0.22</td>
<td>bg</td>
<td>1.58</td>
</tr>
</tbody>
</table>

1spike values (ppb): Fe-50, Zn-20, Cu-10, Mn-5, Ni-5, Pb-5, Cd-2, Cr-5
NE = not extracted by this technique
bg = background
Table 26. Trace element composition of components of M9/GCA medium.

<table>
<thead>
<tr>
<th></th>
<th>75%KSW</th>
<th>GCA</th>
<th>NH₄Cl</th>
<th>K₂HPO₄</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>bg</td>
<td>63.3</td>
<td>2.11</td>
<td>1.33</td>
<td>66.74</td>
</tr>
<tr>
<td>Zn</td>
<td>3.27</td>
<td>36.58</td>
<td>bg</td>
<td>0.36</td>
<td>40.21</td>
</tr>
<tr>
<td>Cu</td>
<td>1.81</td>
<td>4.67</td>
<td>0.02</td>
<td>0.61</td>
<td>7.11</td>
</tr>
<tr>
<td>Mn</td>
<td>(33.31)¹</td>
<td>0.63</td>
<td>0.004</td>
<td>0.14</td>
<td>(34.09)</td>
</tr>
<tr>
<td>Ni</td>
<td>2.84</td>
<td>1.07</td>
<td>bg</td>
<td>0.97</td>
<td>4.88</td>
</tr>
<tr>
<td>Pb</td>
<td>1.41</td>
<td>1.04</td>
<td>bg</td>
<td>0.16</td>
<td>2.61</td>
</tr>
<tr>
<td>Cd</td>
<td>0.98</td>
<td>0.19</td>
<td>bg</td>
<td>0.17</td>
<td>1.34</td>
</tr>
<tr>
<td>Cr</td>
<td>bg</td>
<td>1.37</td>
<td>bg</td>
<td>0.62</td>
<td>1.99</td>
</tr>
</tbody>
</table>

¹ based on Mn extraction efficiency by Chelex-100
bg = background
Fig. 1. Growth of *P. putida* in batch culture in M9 (a) and M9/GCA (b) media.
Graph showing the Absorbance at 420 nm over Time (H)

(a) - Line graph with absorbance values remaining relatively flat.
(b) - Line graph showing an increasing absorbance over time.

Absorbance values range from 0 to 4, and Time (H) ranges from 0 to 120.
Fig. 2. Co-variance of RNA content of *P. putida* with elevated Cu content of the cells under conditions of Cu stress.
Fig. 3. Variation of Cu content of *P. putida* with reaction vessel population density in chemostat culture. $D=0.096 \, h^{-1}$, $T=25 \, ^{\circ}C$, pH 7.4. Cell count $\pm$ one standard error.