THE TOXICOLOGICAL RESPONSE OF *ANABAENA DOLIOLUM* AND *SYNECHOCOCCUS LEOPOLIENSIS* TO ALUMINUM AT SELECTED pH LEVELS

by

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Advisor: Professor Thomas E. Jensen

The influence of aluminum and pH on growth and survival was studied using two freshwater cyanobacteria, *Anabaena doliolum* and *Synechococcus leopoliensis*. The effective concentration of aluminum, or EC_{50}, which reduced growth in a population of *A. doliolum* by 50% upon exposure for 96 hours at pH 7.2 was 0.197 mM. The limiting concentration for *S. leopoliensis* was 0.145 mM. Cells were again exposed to the metal, 0.32 mM and 0.15 mM, respecitvely, for 96 hours in medium buffered to pH 5.5, 7.2, and 9.0. Morphometric analyses were carried out using transmission electron microscope (TEM) micrographs of randomly selected cells from each treatment and controls. Analysis of variance (ANOVA) revealed that the combination of aluminum and pH produced significant ultrastructural changes among *A. doliolum cells* in cell volume, cell wall volume, volumes of polyhedral and polyphosphate bodies and intrathylakoidal space as well as the numbers of polyhedral bodies, polyphosphate bodies, lipid inclusions and cyanophycin granules.

Among these cellular sectors and inclusions, more significant variations were recorded between treated and untreated cells at pH 5.5 than at pH 9.0. An increase in relative cell volume among treated cells was the single significant variation at pH 7.2.

The same statistical analysis of *S. leopoliensis* indicated significant changes in cell wall volume, polyphosphate body volume, intrathylakoidal space volume, thylakoidal surface area, and the number of polyhedral bodies among treated cells. As was true of *A. doliolum*, more changes in ultrastructure of *S. leopoliensis* occurred at pH 5.5, but differed in that one more change occurred at pH 7.2 than did at pH 9.0.

Aluminum was not consistently indicated within any cellular sector or inclusion; however, elemental analysis showed intensification of the peak for phosphorus within polyphosphate bodies of cells exposed to all concentrations of aluminum (0.037 mM, 0.185 mM, and 0.370 mM). This pattern is indicative of the detoxification response of cells exposed to heavy metals.

Neither *A. doliolum* nor *S. leopoliensis* produced detectable phytochelatins upon exposure to aluminum.

To Mama and Daddy

and Everlee

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INTRODUCTION

Acid Deposition

The Industrial Revolution which began in the mid-18th century benefitted not only the world's laborers, but also the greater population through the marked increase in machine and factory production. This widespread shift from an agricultural economy to one which was based on the use of complex machinery eventually resulted in improvements in transportation, power generation, and other advancements which required fossil fuel. Unfortunately, with the increase in use of fossil fuel came an increase in emissions of sulfur dioxide (SO_2) and nitrogen oxides (NO_x).

When fossil fuels, coal and oil, are burned, the by-products are water vapor and the common elements carbon, nitrogen, and sulfur which combine with oxygen in the air. The resultant oxides CO_x, NO_x, and SO_x ultimately react with sunlight, ozone, and moisture to produce their respective acids. Once incorporated into clouds, these pollutants not only travel great distances from the chimneys and exhausts which released the original by-products, but they may also fall to the ground as rain, mist, fog or snow, i.e., acid rain, or as gases or particles, i.e., dry deposition (Park, 1987). As precipitation becomes more acidic, it decomposes more rock and soil, thus mobilizing metals which may prove toxic as their concentrations increase.

Carbonic acid in the atmosphere makes even clean rainwater slightly acidic. Natural phenomena also contribute to the acidity of rain by releasing elements into the atmosphere: volcanoes,

hot springs, wetlands, and natural gases are sources of SO_2, while lightning is a source of NO_x (Schneck, 1981).

The controversy over whether the marked decrease in pH of soil and natural waters over the last few decades is the result of this widespread use of fossil fuels along with other anthropogenic influences (intensive animal rearing, use of lime and fertilizers), or of natural activities (ammonium from volatilization of animal excreta, nitrous oxide from seawater) has been so intense that it has prompted massive media coverage and even international conferences (Cresser and Edwards, 1987; Park, 1987).

Experiments that are designed to correlate the degree of fossil fuel usage and emissions with changes in the acidity of precipitation as single studies are limited. Research which investigates their relationship is either short-term or tends to generate controversy because of inconsistencies in techniques and sampling sites, along with failure to calculate the influence of nature or other anthropogenic activities (Dickson, 1972; Siegel *et al.*, 1973).

Sphagnum spp. is very susceptible to SO_2. While the near disappearance of the species from the Southern Pennines of England can be linked to human interference such as burning and grazing, these alone could not account for the almost total extermination of the species over large areas (Tallis, 1964). The bog moss declined under some conditions, but disappeared altogether with soot contamination, a consequence of industrialization. By comparing their data on atmospheric SO_2, rain pH, and sulfate and bisulfate levels for one year (1979-1980) with figures taken from earlier studies in this same area of England, Ferguson and Lee (1983) concluded that SO_2 concentrations in

the air and in rainfall in this area at the turn of the century could have been high enough to prove toxic to many plants. The area surrounding the Southern Pennines has been industrialized for many years.

Just as *Sphagnum spp.* monitors the terrain, diatoms are strong indicators of acidity changes in lakes and rivers. In a lake sediment study (Scotland), diatom analysis not only indicated the time at which acidification began (1840), but also tracked increases in trace metals which may be associated with industrial emissions (Battarbee *et al.*, 1985). Through comparative studies, ecological conditions such as afforestation and a decline in grazing can be eliminated as causes of increased acidity. If, when the diatom population of a locale becomes increasingly more acidobiontic after other causes of acidity have been eliminated, it lends support to the proposed link between acid deposition and lake acidification (Flower and Battarbee, 1983).

Aside from biotic indicators, environmental changes such as a two-fold increase in the mercury concentration of ice in Greenland between 1946 and 1952 (Weiss *et al.*, 1971) point to increased acid deposition. Likens and Bormann (1974) reported a striking increase in acid precipitation in northeastern Europe over two decades, along with a greater than 200-fold increase in mercury concentration of rain in some parts of Scandinavia.

A pH of 5.9 was recorded during a 1939 rainstorm in Brooklin, Maine (Likens and Butler, 1980). This reading, the earliest recorded in the United States, is only slightly less acidic than 5.6, the marker level for acid rain. The same work, however, indicates that in general, precipitation in eastern North America prior to 1930 had a pH greater than 5.6. In stark contrast to this report are those which indicate a

continuous intensification of acid precipitation in the United States and Europe (Likens and Butler, 1980; Cresser and Edwards, 1987) over the last few decades.

Following an extensive study of 20 Adirondack Park lakes in New York State, Cumming *et al.*, (1994) systematically eliminated a) natural activity and b) watershed disturbances with concomitant changes in flora and soil as the causes of acidification of 80% of the test lakes. The researchers concluded that the lakes' pH profiles were strongly consistent with previous paleolimnological and other studies which point to acid deposition from industrial sources as the cause of the acidification.

Not all of the results reported above will settle the question of the cause of increased acidity; however, they do emphasize the change in the environment since the Industrial Revolution. The conditions that are manifested by this change include the release of anions and cations into the atmosphere, soil and natural waters. The concentrations of these ions directly affect environmental acidity and alkalinity, and often become great enough to prove toxic to many organisms.

Aluminum

According to one hypothesis, organisms in their biochemical evolution have tended to select as nutrients substances which were found in abundance in the earth's crust and were readily soluble in the anaerobic environment (Driscoll and Schecher, 1990). Aluminum is the most abundant metal in the earth's crust, but was, no doubt,

unavailable to anaerobic life forms. This could explain why such an abundant element is not only non-essential, but is also found in very limited concentrations in living tissues, occurring as complex silicates (clay), oxides (bauxite), and sulphates.

This metal is too reactive to occur free in nature (Haug, 1984). It occurs, instead, as complex silicates (clay), oxides (bauxite), and sulphates. In natural waters, the metal can be found in two forms: soluble salts and colloidal compounds. The concentrations of these forms in unpolluted waters range from 0.10 mg/L to 10 m/L (Galvin, 1991). Rivers in Spain are reported to range between 0.09 mg/L and 1.86 mg/L, while North American rivers range between 0.012 mg/L and 2.250 mg/L (Galvin, 1991). In 1988 the Environmental Protection Agency (EPA) published water quality criteria for the protection of aquatic organisms exposed to aluminum. This document recommends that within any four-day period the concentration of aluminum should not exceed 87 μg/L more than once every three years where the pH is between 6.5 and 9.0 (Gostomski, 1990).

Ordinarily aluminum is trivalent, though there is evidence that it may occur in a monovalent or bivalent state. Its oxide, Al_2O_3, is amphoteric, acting as a base to form salts with acids, or as a weak acid to form salts with strong alkalies.

Aluminum is available for biological interaction only when it becomes mobilized in the soil, and even then, its activity depends upon pH, the amount of available metal, and the inorganic and organic complexing agents involved. According to Haug (1984), aluminum becomes soluble at pH $\leq$ 5.0, at concentrations between 1 ppm and 30

ppm. These values are representative of aluminum concentrations found in soils.

Although aluminum is regarded as a non-essential element, there is some consideration that, at low concentrations, the metal exerts beneficial effects on some plants (Foy *et al.*, 1978; Kinraide, 1993). In tracing the accounts of the benefits of aluminum, Foy *et al.* (1978) proposed that the metal could possibly increase iron solubility and availability in certain soils, promote the uptake of phosphorus by blocking negative charges on the cell wall, or protect calcium-deficient plants from root deterioration by slowing their growth and preventing depletion of the nutrient.

The mechanisms of aluminum's toxicity are complex (see below), but much of the research on aluminum has concentrated on recording the effects of pH and concentration on organisms which have been exposed to it (Husaini and Rai, 1992; Kinraide and Parker, 1989; Guida *et al.*, 1991; Rai, 1993). These effects can be more clearly interpreted when considered in light of the chemical behavior of the metal. In freshwater, the aluminum ion (Al^{3+}) complexes readily to form hydroxide, fluoride, sulphate and aluminum-organic matter (humus, sugars, amino acids) compounds. The ion may even occur in the free state (Lee, 1985). Within the compounds listed above, the metal may occur in a mononuclear (monomeric) state ($AlOH^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_4^{-}$), or in a polynuclear (polymeric) state ($Al_2(OH)_2^{4+}$, $Al_6(OH)_5^{13+}$, $Al_7(OH)_{17}^{4+}$) (Lee, 1985). The mononuclear forms are said to be derived from $Al(H_2O)_6^{3+}$, a form more prevalent at pH <5.0, but which yields the mononuclear species as the pH increases (Pettersson, 1989.) As solutions become neutral, the starter species becomes

$Al(OH)_3$, an insoluble form which redissolves at alkaline pH levels to become $Al(OH)_4$ (Pettersson, 1989; Guida *et al.*, 1991). Lee (1985) questions the occurrence of a dissolved $Al(OH)_3$, despite the suggestion that it may be an important species functional in the 4.5 to 8.0 pH range. The mononuclear species are determined to be stable for several days when $\{Al^{3+}\}/\{H^+\}^3 \leq 10^{8.8}$, where brackets denote activities (Kinraide and Parker, 1989).

The conversion of monomeric species to polymeric forms is not only a slow process, but is also dependent upon concentration, pH, and temperature. Because the conversion progresses so slowly, the amount of product is dependent upon the age of the solution (Pettersson, 1989). Polynuclear species are typically unstable in that they precipitate upon standing.

Cyanobacteria

The wide habitat range and large input of organic material of algae, as well as their potential for protein production, waste treatment, and chemical production (Grobbelaar, 1988) merit this group a priority position in future scientific study. As part of our aquatic ecosystems, algae are threatened by the increase in pollution which results in greater acidification. The fact that algae show greater sensitivity to environmental variation strengthens the justification for using them as test species in order to provide greater environmental protection (Hörnström, 1990). As photosynthetic, oxygen-yielding organisms, the cyanobacteria become primary producers, the first link of the food chain, or the initiators of the energy transfer system in an ecosystem.

These organisms, even in isolation, are worthy test subjects, but become even moreso in consideration of the theory that pollutants tend to accumulate as they move up the food chain. The case of the increase in residual concentrations of DDT in a Long Island, New York, salt marsh highlights the severity of the accumulation. In this study, plankton showed a total residue of 0.04 ppm of the deadly chemical (Laws, 1981). This concentration is multiplied through minnows and predatory fish up to the cormorant with 26.4 ppm, an increase of 660 times that found among plankton. If one accepts this theory of biological magnification (Laws, 1981), these autotrophs become doubly important for their responses to environmental toxins. Test results can be used to predict and to compare the effects of pollutants on organisms at higher trophic levels.

All cells have a notable capacity for taking up elements from a solution against a concentration gradient. With chlorine being totally rejected and sodium weakly rejected (in seawater), all other elements are concentrated to some extent, the order of affinity for living cells for cations being tetravalent and trivalent elements > divalent transition metals > divalent Group IIA metals > univalent Group I metals (Bowen, 1966). Organismic groups concentrate these ions to various degrees. Since concentration factors seem not to be linked to essentiality, calcium, magnesium, and sulfur are typically weakly concentrated, whereas lanthanum, lead, scrontium, thallium, titanium, and zirconium are strongly concentrated (Bowen, 1979). Microorganisms, specifically bacteria, fungi, and algae, exhibit such a capacity to accumulate cations that they have been used extensively in studies which investigate biochemical, physiological, and ultrastructural changes induced by

environmental cations, pesticides, and many other materials which are suspected of producing toxic effects. Recent considerations include using these organisms for removal of valuable and threatening ions (Lobban *et al,* 1985; Zimnik and Sneddon, 1988; Mallick and Rai, 1993).

The two species in this study belong to Phylum Cyanophyta and Class Cyanophyceae. ***Anabaena doliolum***, Order Nostocales, is an asymbiotic, aerobic nitrogen fixer with a cell diameter of approximately 7 μm. Under standard laboratory growth conditions this freshwater, filamentous species contains heterocysts throughout its growth cycle, but does not form akinetes until it enters the stationary phase. *A. doliolum* has been used in studies on urea uptake (Singh, 1990), akinete formation (Tiwari *et al*, 1989), heavy metal toxicity (Dubey and Rai, 1990), the impact of chromium and tin (Rai and Dubey, 1989), and response to bimetallic combinations of copper and nickel (Mallick and Rai, 1989).

Synecbococcus leopoliensis of Order Chroococcales is also asymbiotic and aerobic. A small cell with a length of approximately 4 μm, this species is quite common in the environment and has been the subject of extensive research (Jensen and Rachlin, 1984; Lawry and Jensen, 1986; Tang, 1993).

The focus of this study is the effect of aluminum on the ultrastructure of two aquatic, photosynthetic cells at acidic, neutral, and alkaline pH levels. Reasonably, such a study must be preceded by determination of uptake and localization of the substance in question. Actual determination of the mechanism of toxicity would require biochemical analyses which are not undertaken here; however, the

results of tests such as those to determine changes in protein composition and the effect of the metal on ultrastructural integrity are important in evaluating the toxicological responses of these species to aluminum.

Morphometric analysis may determine if certain cellular structures are targeted by aluminum.

LITERATURE REVIEW

Interest in the effects of increased acidification and release of heavy metals on biota as a focus of research dates back to the mid-1960's, prompting an international conference in 1973 (International Conference on Heavy Metals in the Environment, Hutchinson *et al.*, 1977), and even development of a new science, bioinorganic chemistry (Niebor and Richardson, 1980). The term 'heavy metals' was widely used, although little description prevailed other than the elements' capacity to induce toxicological responses, particularly at higher concentrations. Niebor and Richardson (1980), noting the lack of consistency in definition and classification of heavy metals among zoologists, botanists, and others, proposed a classification of metals into three categories. The categories, (1) class A (oxygen-seeking, i.e., aluminum, beryllium, calcium), (2) class B (nitrogen/sulphur-seeking, i.e.,), and (3) borderline (intermediate, i.e., arsenic, iron, lead), would reflect not only the elements' biological activity and toxicity, but also their chemistry. The researchers go on to substantiate their proposal through a detailed discussion of metal-ligand complex formation in solution.

Rémacle (1990) uses the same categories of ligands, but refers to the metallic ions that bind readily to oxygen-containing groups as hard ions, and those which have an affinity for nitrogen or sulfur atoms as soft ions. Hard ions, e.g., sodium, magnesium, and calcium, are nutrients of living organisms, and are generally found in large quantities. Conversely, ions such as lead, mercury, and cadmium, or

soft ions, are generally non-essential and toxic. Intermediates are those which are less toxic and may have some biological function.

The descriptive 'trace metals' has been introduced. Lobban *et al.* (1985) also suggest three categories: (1) critical, (2) toxic but very insoluble or rare, and (3) very toxic and relatively accessible. This classification places copper, manganese, iron, and zinc in category three, thus the description 'trace metals' because these are essential micronutrients which may limit growth if insufficient, but which prove toxic in greater concentrations.

Despite these and other proposals for terminology, 'heavy metals' predominates, and will be used throughout the current project, largely reflecting their toxicity.

Adsorption of metallic ions at the cell surface and their fate

Simply stated, soluble metals will inevitably reach and interact with the broad surfaces of bacteria (Beveridge, 1989). This contact and interaction are largely the result of a high surface area to volume ratio noted among small cells. Such cellular design is clearly for the benefit of bacteria since they must receive all of their nutrients through diffusion. This great capacity for diffusion calls for uptake of nearly all solutes, beneficial and harmful, that are small enough to cross the cell's barrier. Consequently, the physical and biochemical nature of the cell wall is a determinant of ionic uptake.

R. Y. Stanier is reported to have stated, after attending a conference of bacteriologists, that it is evident that no one really knows what a bacterium is (Thimann, 1968). No doubt, such a

statement resulted from the inconclusive nature of the knowledge of the "small" world at that time. Today, even basic introductory biology textbooks include notes on the breakdown of Kingdom Monera into two major groups: the archaebacteria and the eubacteria (Alberts *et al.*, 1989). For so long, the archaebacteria nearly eluded detailed study because of the difficulty of cultivation. These prokaryotes which may be distinguished on the basis of cell wall and plasma membrane include the oceanic methanogens as well as species which have adapted to more extreme environments, the halophiles and thermoacidophiles. The cell wall of these species generally lacks a murein or pseudomurein layer. A detailed analysis of two thermophilic species, *Thermoproteus tenax* and *T. neutrophilus,* revealed that their surface layer (S-layer) proteins remain intact under extreme conditions (Messner *et al.*, 1986). According to Olsen (1994) these differences are based on the nature of their RNA.

The more advanced of the prokaryotes, the eubacteria, include the rickettsiae, chlamydias, and mycoplasmas along with the more familiar photosynthetic species such as the cyanobacteria. The eubacteria have been separated into two major and one minor group based on the nature of their cell walls: gram positive describes those that take up the stain upon exposure, and gram negative those that do not. Some cells stain either negative or positive depending upon conditions such as growth phase or nutritional status and are, therefore described as gram variable (Beveridge, 1989). The cyanobacteria are found to closely resemble the gram negative bacteria with peptidoglycan as the principal wall component. Peptidoglycan is reported to have a metal-binding capacity (Beveridge *et al.*, 1982).

Microscopy shows the cyanobacterial wall to be composed of four layers which are numbered from the cell's inside as L I, L II, L III, and L IV (Sicko-Goad and Jensen, 1971). In addition to peptidoglycan this wall is structured of simple sugars, muramic and glutamic acids, diaminnopimelic acid, galactosamine, glucosamine and alanine (Trainor, 1978).

The negative charge on the cell wall and plasma membrane results from carboxyl groups, phosphate groups of membrane phospholipids, and amino acid residues of membrane proteins. These groups are the sources of oxygen, nitrogen, and sulfur for which various cations will have affinities (Nieboer and Richardson, 1980; Remacle, 1990). Cations from the extracellular environment tend to bind in the order of trivalent > divalent > monovalent. As these cations are bound, their pool is reduced and the electrical potential of the membrane changes. The reversal of the electrical charge of the cell wall is dependent upon the concentration of the metal; a certain concentration will take the charge to zero, a greater concentration can cause a positive charge (Passow, 1970).

Getting to the Cell Wall

The action of the cell surface with reference to metal ion binding has been likened unto a commercial ion-exchange resin (Lindemann *et al.*, 1990; Remacle, 1990). According to the Langmuirian theory of adsorption (Volesky, 1990), a polar or nonpolar surface will preferentially adsorb the more polar or nonpolar component of a solution in a nonpolar/polar solvent. Some ions adhere while others

return to the solution, equilibrium being established when the adsorbing surface is covered. Such is the process by which cations, including heavy metals, become associated with the cell wall of phytoplankton. When metallic ions from the aquatic environment reach the cell surface of an organism, binding can occur through displacement of hydrogen ions (Ramelow *et al.*, 1991) or by complexation with anionic ligands (Beveridge, 1989; Remacle, 1990). Organic acids such as humic (Dubey and Rai, 1990), fulvic (Pettersson, 1989), teichoic and teichuronic (Beveridge *et al*, 1982; Remacle, 1990) are known to be particularly active in metal binding. While this capacity is widespread among living cells, it has also been reported for non-living cells as well (Mullen *et al,* 1989; Maeda *et al.*, 1990). This observation underscores the passive nature of uptake.

The degree to which binding occurs is dependent not only upon the number, nature, and access of functional groups, but also upon solution pH (Mahan *et al.*, 1989). [In bacterial cells at near-neutral pH, there are only a few binding sites available; amine groups are effective at alkaline pH's (Cabral, 1992).] The decrease in metal uptake at the acidic pH levels is thought to result from the competition between metallic ions and hydrogen ions (Mallick and Rai, 1993) and from the preponderance of positive bonding sites (Ramelow *et al.*, 1991).

Once ions are bound at the cell surface, they may be immobilized through precipitation by the same ligands, or may enter the cytoplasm by either passive or active diffusion (Mullen *et al.*, 1989).

The Fate of Intracellular Heavy Metals

Fiore and Trevors (1994) detail the two-step process of entry which includes an initial, rapid but passive adsorption which occurs without temperature influence. This binding which occurs shortly after the organism encounters the ions is followed by a slow phase during which heavy metals are incorporated into the cell proper. The latter process is both temperature and metabolism dependent.

Once incorporated, heavy metals may localize in almost any cellular sector, including the nucleus, vacuoles, cell wall, or polyphosphate bodies. DNA synthesis, as measured by the incorporation of [^{3}H]thymidine, increased considerably in an aluminum-sensitive species of *Rhizobium spp.* when exposed to as little as 50 μm of the metal, while the DNA of a tolerant species was unaffected (Johnson and Wood, 1990). Speculation exists as to the biochemical bases of the increase, as well as the mechanism by which the tolerant species prevents an effect. Still, what is also important here is the localization of the metal within the nucleus.

The capacity for increased toxicity of heavy metals with an increase in acidity is well documented (Gensemer, 1990; Rosseland *et al.*, 1990; Zel, 1993). Results of the investigation of the effects of cadmium, cobalt, copper, and nickel on *Chlamydomonas reinhardtii*, however, do not support this observation. When walled and wall-less strains of the species were exposed to the four metals at pH levels ranging from 5.0 to 7.0, the walled species consistently tolerated higher levels of each metal (Macfie *et al.*, 1994). This observation agrees with others which indicate that the cell wall provides some protection against metal toxicity. The fact that the walled species exhibited

tolerance for greater concentrations of the metals at pH 5.0 than at pH 6.8 is explained as being the result of displacement of the metallic ions by hydrogen ions at the cell wall. Of course, this protective mechanism by the cell wall is operational only to the point of saturation of binding sites after which no further protection can be provided.

The spherical, electron-dense cellular bodies now more commonly known as polyphosphate bodies are a classic illustration of the arduous task of analyzing and properly characterizing a recent discovery. The fact that these inclusions were initially observable only under certain conditions of growth and nutrition created as much a question as did their composition and role in cellular activity. The suspicion that the metaphosphate was associated with RNA or DNA was eventually resolved through analyses that showed no ribose or purine (Schmidt *et al.*, 1946). Through extensive investigations with yeast, Wiame (1949) noted that not only did the metachromatic compound disappear when the cells were grown in phosphate-poor medium, but also that cells instead synthesized nucleic acid, thus dissociating any DNA or RNA associated with these granules from 'nuclear' DNA or RNA. Amid the continuing speculation as to the function of these granules as a food reserve and whether they were free or bound within an organic complex, it was reported that they form after phosphorus uptake and that they are intricately linked to oxidative phosphorylation since enzymic action on adenosine triphosphate (ATP) produces adenosine diphosphate (ADP) plus metaphosphate (Widra, 1959). While the nature and function of metaphosphate remained elusive, Jensen (1968) noted that these electron-dense bodies are visible before staining, but appear even more dense when

exposed to lead compounds. This is also known to be the response of phosphate to lead sulfide. With this observation noted, he concluded, after carefully analyzing alternate thin and thick sections, that the inclusions observed through electron microscopy were the same as those seen in light microscopy, and were morphologically similar to the polyphosphate bodies of other bacterial cells.

Jones and Chambers (1975) could not determine the function of polyphosphate in *Desulfovibrio gigas* because the inclusions could not be found among all cells examined, nor were they present among cells which had not been subcultured. Their research, however, did enable them to observe the accumulation of polyphosphate during periods of slow growth or nutritional imbalance, particularly sulfur and phosphorus depletion. This response of cells has been reported elsewhere (Sicko, 1972; Baxter and Jensen, 1986; Lawry and Jensen, 1986).

These spherical inclusions which range in size from 0.2 μm to 3.0 μm are electron dense. *Micrococcus lysodeikticus* is reported to contain polyphosphate bodies which are composed of 24% protein, 30% lipid, and 27% polyphosphate along with elemental sodium, magnesium, calcium, potassium, manganese, iron and copper (Jensen, 1990). Characteristic "holes" left by polyphosphate bodies which have sublimated under the intensity of the beam of the electron microscope can be observed among embedded cells (Sicko, 1972).

Many cyanobacteria contain polyphosphate bodies (Jensen *et al.*, 1977; Jensen *et al.*, 1982a; Rachlin *et al.*, 1984; Jensen, 1993; Jensen and Corpe, 1994). There are number and size variations, depending upon the cells' environment and nutrition. Composition of these

inclusions, however, is more uniform consisting of phosphorus (P), magnesium (Mg), potassium (K), and calcium (Ca) with sulfur (S), chlorine (Cl), sodium (Na), and iron (Fe) occurring less often or in lower concentrations (Jensen, 1990). A small fraction is thought to be composed of protein-bound lipid as well as RNA (Widra, 1959).

The phosphate component of polyphosphate bodies points to an energy storage mechanism (Jones and Chambers, 1975). However, that polyphosphate bodies increase in number and mass under adverse growth conditions and nutrition status may help explain the capacity of these inclusions to trap heavy metals (Sicko-Goad and Stoermer, 1979; Baxter and Jensen, 1980a, 1980b; Rachlin *et al.*, 1984, 1985; Rai *et al.*, 1990). Jensen *et al.* (1982) were able to determine heavy metal uptake by air-dried cells of *Plectonema boryanum*, using energy dispersive x-ray (EDX) microanalysis. In all cases of uptake the metals (cadmium, cobalt, copper, mercury, nickel, lead, and zinc) were sequestered in greater concentrations in polyphosphate bodies than in cell sectors which did not contain these inclusions. The researchers propose that these inclusions, by trapping heavy metals, function in detoxification. *Anabaena cylindrica* sequesters aluminum in its cell walls and polyphosphate bodies (Pettersson *et al.*, 1985b). This EDX study showed that under conditions of high phosphorus content in the cell wall, the uptake of aluminum was high, leading the research team to consider that the phosphate-rich environment increases the cell's capacity for uptake or binding of aluminum.

The discovery of the group of low molecular weight, metal-binding polypeptides known as phytochelatins is one that is related to the discovery of similar substances in animals. Phytochelatins, so

the discovery of similar substances in animals. Phytochelatins, so named because they occur only in species of Kingdom Phyta (Rauser, 1990), were initially thought to be similar to those produced by animals (Margoshes and Vallee, 1957). The plant products are, however, characterized by the general structure $(r\text{-Glu-Cys})_n Gly$, where n = 2 to 11 (Grill *et al.*, 1988) are synthesized only when the cells are in the presence of heavy metals. It was important that these proteins be distinguished from metallothioneins that animals and fungi produce, by gene action, in response to metals (Gekeler *et al.*, 1988).

When *Chlorella fusca* as well as other members of six of the ten classes of Phycophyta synthesized phytochelatins upon exposure to cadmium, lead, silver, copper, zinc, and mercury, it was determined that the cysteine-rich polypeptides also had r-glutamyl linkages. These linkages indicate that phytochelatins, in contrast to metallothioneins, are not primary gene products, but may be synthesized from glutathione or r-glutamyl-cysteine purcursors (Grill *et al.*, 1985; Gekeler *et al.*, 1988). Further characterization could be established from the study with metal-sensitive *Acer pseudoplatanus* and a resistant species, *Silene cucubalis*. When grown in soil enriched with zinc, the two species sequestered similar amounts of the metal in their root systems and leaves, and synthesized substantial amounts of phytochelatins. Control samples, however, neither synthesized phytochelatins nor registered appreciable amounts of zinc within the tissues (Grill *et al.*, 1988). Thus, the team could conclude that the plants produced the polypeptides in response to the metal.

Metal thiolate polypeptides are divided into classes based on their structures: (a) Class I: polypeptides with locations of cysteine closely related to those in equine renal metallothionein;
(b) Class II: polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein; and (c) Class III: atypical, nontranslationally synthesized (Rauser, 1990). The polypeptides of Class III have now been detected in algae and in flowering plants up to orchids (Grill *et al.*, 1988). These compounds are small, heat-stable compounds composed largely of three (3) amino acids: cysteine, glutamic acid, and glycine, but also with a high content of cadmium, copper, or zinc (Reddy *et al.*, 1990). The metal binding capacity was confirmed by the high metal content.

Phytochelatins resemble metallothioneins most in function, metal detoxification by formation of thiolate complexes. Analyses (via polyacrylamide gel electrophoresis, reverse-phase high-performance liquid chromatography (HPLC), and amino acid analysis) of the plant products reveal Cd:Cys ratios of 1:2 to 1:3, while those of Cu:Cys range from 1:2 to 1:6 (Rauser, 1990). Such ratios are matched with a range of 10% to 90% of cellular metal being bound.

The research on phytochelatins among the cyanobacteria is limited (Olafson, 1991). If found, however, they would prove advantageous to these cells to have yet another mechanism for coping with widespread environmental pollution.

Heavy metal toxicity

Although aluminum is generally regarded as a non-essential element, studies show that, at low concentrations, the metal affords beneficial effects on some plants. In tracing the accounts of the benefits of aluminum Foy (1974) reports on the catalytic activity of aluminum in photosynthesis and its inducement to germination in various species. The effective concentrations of aluminum generally ranged from 0.1 ppm to 5 ppm with large potato tubers responding positively to 20 ppm. In a later work Foy *et al.* (1978) speculated that while the mechanisms of aluminum activity in plants were not yet clear, the responses observed may not necessarily result from the metal's effects *per se.* Rather, the presence of the metal might have physiological effects such as displacing bound iron to overcome a deficiency, promoting phosphorus uptake by blocking negative charges on the cell wall, and retarding root deterioration in the presence of calcium deficiency by slowing the growth rate to prevent calcium depletion. As might be expected, these benefits are sometimes greater in species which tolerate higher concentrations of aluminum than in those which are more sensitive.

While aluminum at low concentrations may yield some photosynthetic or growth benefits, increased concentrations, acidic or alkaline pH levels, ligands or chelators and certain aspects of the environment all contribute to a toxicological response in many photosynthetic species. Toxic concentrations are particularly likely in light of the increased pollution of soil and natural waterways discussed above.

Studies on the toxicological effects of heavy metals, particularly cadmium, zinc, lead, and copper abound (Jensen *et al.*, 1982a; Jensen *et al.*, 1982b; Rachlin *et al.*, 1984; Rachlin *et al.*, 1985; Andersson, 1992; de Lima and Copeland, 1994; Lindberg and Griffiths, 1993). The results of these and other studies have greatly elucidated organisms' growth, photosynthetic, biochemical, physiological and morphological responses to these pollutants. Still, many of the specifics of metal toxicity remain unclear.

Les and Walker (1984) present an extensive parallel study of their work and that of others on the effects of copper, zinc, and cadmium on cyanobacteria. The concentrations of 0.2 ppm, 1.0 ppm, and 2 ppm for copper, cadmium, and zinc respectively, which inhibited growth in *Chroococcus paris*, indicate not only the order of toxicity of the three metals, but also agree with results of tests which showed growth of *Anabaena sp.* to be totally inhibited at concentrations between 0.6 and 6 ppm of copper and by 1 ppm to 11 ppm of cadmium. Another species, *Anabaena inequalis,* showed significant growth inhibition at 0.02 ppm cadmium and complete inhibition at 0.06 ppm. Acetylene reduction and photosynthesis in this species proved to be keenly sensitive to cadmium, being significantly inhibited by 1 ppm. Further comparison is shown in a study in which 1 mM had only a slight effect on the growth rate of *Chlorella sp.*, but caused a net gain in calcium, a response that is largely attributed to the failure of treated cells to transport soluble carbon compounds.

A location in the natural environment in which a heavy metal occurs singly is rare; consequently, it is important to study the effect of metals in combination. The fact that these studies provide no

knowledge of the effects of a single metal in isolation makes both types of study important. In a study involving the combination of copper and nickel, Mallick and Rai (1989) observed that not only was growth among exposed cells of *Anabaena doliolum* more retarded than that shown by control cells, but also severe inhibition of carbon fixation. This drastic response was thought to be the result of disruption of photosynthetic membranes. The proposed involvement of photosynthetic membranes is strengthened by a concomitant reduction in nitrate and ammonia uptake and nitrogen fixation. Cyanobacteria characteristically generate the reducing equivalents for nitrogen fixation via photosynthesis. The inhibition of glutamine synthetase and urease is linked to the observed reduction in ATP, a product of both cyclic and non-cyclic photophosphorylation.

The introduction of the current study included a discussion of the role of pH in determining the toxifying species of aluminum and the degree of toxicity. *Escherichia coli*, when exposed to concentrations of aluminum from 0.9 mM to 2.25 mM showed a greater growth sensitivity at pH 5.4 than at pH 6.6 to 6.8 (Guida *et al.*, 1991). The growth inhibition was thought to be markedly dependent upon pH, with $Al(H_2O)_6^{3+}$. The research team further concluded that since toxicity was greater in the absence of iron than when it was included, that possibly localization of the metal in polyphosphate granules and the cell wall pointed to involvement of iron transport pathways.

Mercury is reportedly one of the most toxic of the heavy metals (Bowen, 1966; Hongve *et al.*, 1980). The report that this metal intensified fluorescence in the cyanobacterium, *Spirulina platensis* (Murthy *et al*, 1989), points not only to localization of the metal in

phycobilisomes, but also a myriad of changes resulting from inhibition of Photosystems I and II. The shift in fluorescence of phycocyanin was accompanied by partial inhibition of oxygen evolution via para-benzoquinone as well as whole-chain electron transport activity (H_2O ---> methylviologen). It seems reasonable to conclude that if electron transport in phycobilisomes is severely interrupted, chlorophyll is not adequately activated and even if thylakoids and their associated proteins are intact, photosynthesis does not proceed with efficiency.

The level of concentration is particularly critical in the case of metals that are micronutrients. Dosages greater than the metabolic requirements become toxic, as in the case of selenium. *Anabaena flos-aquae* is reported to utilize and regulate up to 1 to 3 mg/L of inorganic forms of selenium without growth inhibition or a decrease in chlorophyll a (Kiffney and Knight, 1990). Both characters, however, were significantly reduced by 5 mg/L. The presence of selenoproteins among microorganisms is an indication of the metal's necessity in certain enzymes. Additionally, its stimulatory effect on the growth of some cells underlines its benefits. *A. flos-aquae*, as shown by this study, is capable of concentrating very high levels of selenium from the surrounding medium over several days, but the capability is severely reduced when the concentrations in the medium are greatly increased. The researchers go on to suggest that the toxicity is caused by dysfunctional proteins which disrupt biochemical activity.

The capacity for growth at pH 4.7 demonstrated by several diatom and desmid species in a study by Pillsbury and Kingston (1990) is an example of a greater tolerance for low pH than for aluminum. Control cells grown at pH 5.7 and pH 4.7 showed no significant difference in

growth, but growth in some of the more aluminum-sensitive cells, i.e., *Asterionella ralfsii* v. *americana, Arthrodesmus octocornus,* and *Stenokalyx monilifera*, declined in as little as 50 μg of the metal. These species, as well as those which tolerated higher concentrations, displayed a greater nitrogen requirement over the span of the experiment. While such results help to identify those species which would be found in ecosystems that are simulated in the experiment, they are also valuable in that they identify ions which, at certain concentrations, enhance growth. The increase in growth observed in *Dinobryon bavaricum* and *Stenokalyx monilifera* with 100 μg of aluminum agrees with the results of this study in which 1.8 ppm promoted growth in both *A. doliolum* and *S. leopoliensis.*

Heavy Metal Tolerance

It is reasonable that the cellular nature of cyanobacteria would be suited for the accumulation and inactivation of heavy metals considering that these organisms are known to have this pronounced capacity. The role of some of those inclusions was discussed earlier in this work. Additionally, some species even secrete an extracellular mass which affords some protection. As a result of increased investigation, there is an impressive list of mechanisms by which algae tolerate the metal-contaminated environments which they inhabit. The most commonly reported techniques include a) extracellular binding or precipitation; b) internal detoxification and metal transformation; and c) intracellular binding via proteins or polysaccharides (Maeda and Sakaguchi, 1990; Fiore and Trevors, 1994). The species

investigated in the current research demonstrate cellular changes such as dense internal deposits and granular cytoplasm upon exposure to high concentrations of aluminum.

Determining the metallic concentrations which can be used to distinguish tolerant species from sensitive ones remains an issue. Extensive research is required to determine organisms' response at various pH levels and metal speciation at any given pH level. On the other hand, the positive results of recent metal detoxification research include determining many indicator organisms for various environments and the genetic bases of heavy metal tolerance. The gene for tolerance is often, though not always, carried on a plasmid. When present, this 'foreign' DNA can render a species tolerant by providing an efflux or by-pass mechanism, by coding for enzymes which transform metals, or by making the cell wall impermeable to certain metals (Trevors *et al,* 1985).

Morphometry

One has only to recall the basics of plane geometry for a beginner's approach to stereology. Planimetry seeks to measure the area of a plane figure by tracing the perimeter of that figure. One of the most widely known uses of two-dimensional observation is in the art of cartography, map making, in which a specified line length corresponds to an actual distance. The application was extended when Delesse, a geologist, developed what has come to be known as the Delesse principle (Weibel *et al.*, 1966), according to which, the volume fraction V_{Vi} of a component i in tissue can be estimated by measuring

the area fraction A_{Ai} of a random section occupied by transections of *i*: $V_{Vi} = A_{Ai}$ (Weibel *et al.*, 1966). In a similar fashion, surface area of a membranous cellular inclusion can be determined. In this case, the surface density, S_{Vi}, of a given inclusion is equivalent to the relationship of its total surface, S_i, to the volume of the cell, V_T. When a lattice of fixed length is used as an overlay with a micrograph, these dimensions are calculated from 'counts' (points at which the lines and structures touch). These techniques allow for measurement of cells photographed from the light microscope, and even from the electron microscope, with techniques for correcting accuracy which may be lost along with the structure's third dimension because of the limitation of photography.

The morphometric technique allows for a greater range of data collection based on comparative studies (Jensen, 1993; Travis *et al.*, 1993) as well as the analyses of a single type observed under natural or laboratory conditions (Zancanaro *et al.*, 1993; Ding *et al.*, 1988; Hama *et al.*, 1989). Organismic response to heavy metal is also one area that has been widely investigated using morphometric techniques (Bennet *et al.*, 1985; Rachlin *et al.*, 1985; Rai *et al.*, 1990; de Lima and Copeland, 1994).

The use of a two-dimensional image of a three-dimensional figure as well as irregularities such as cellular inclusions of varying shapes and sizes builds in biases that result in inaccurate interpretations. These techniques for cellular measurement must, understandably, include devices for corrections in order to minimize bias. Bertram and Bolender (1990) determined that similar results could be obtained from random sectioning (used in electron micrsocopy) and serial

sectioning (used in light microscopy), but only if correction were made for the bias created from compression of sections and from the variety in nuclear shape. Much of their work involved finding ways to make those corrections.

Using an extension of the Delesse principle (see above), Sicko-Goad *et al.* (1977) set forth several guidelines for correcting some of the errors that are inherent in making measurements among components which vary widely even while falling within a range. It is understandable that wide differences would occur between cultures from the natural environment and those grown in the laboratory. These correcting techniques can be used to analyze such cultures. In addition, species from a single growth environment will also show differences in response to various influences. The research team reported that it is important to determine that adequate sampling has been done. This can be accomplished by examination of coefficients of variation and plots of cumulative means and variances. Among the species, corrections are accomplished by a step such as retrimming the (epoxy) block after collecting a sample from the surface. This practice prevents repeated sampling of a single cell. Other corrections include using only one cell of a filament and omitting cellular masses which appear to be mostly or only cell wall from the count.

The role of nutrient status is often the focus of toxicological studies, as in the investigation conducted by Lazinsky and Sicko-Goad (1990) which involved morphometric analyses of phosphorus-rich and phosphorus-deficient diatom cells exposed to chromium. After noting changes such in chloroplast number and (relative) volume, as well as volumes of vacuoles, polyphosphate bodies, lipid bodies, and nuclei,

the researchers concluded that these and other changes in volume and number of cellular inclusions point strongly toward a relationship between phosphate nutrient status and metal toxicity. The nature of the relationship, however, cannot be established on the basis of limited research.

Jensen (1985) has presented an extensive report of the cellular inclusions of the cyanobacteria. Allen (1984) detailed the changes resulting from environmental influence observed in polyhedral bodies, phycobilisomes, cyanophycin granules, and polyphosphate bodies as well as other inclusions. Often the environmental influence is a combination of heavy metals that induce changes not only in the numbers of cellular inclusions, but also in their volumes. The findings of Rachlin *et al.*, (1985) were determined using selected concentrations of zinc on two cyanophycean species, *Anabaena flos-aqua* and *Anabaena variabilis.* Both species showed significant increase in thylakoidal surface area as well as the number of lipid bodies. Other responses differed, however, with *A. flos-aquae* showing a reduction in cyanophycin granules while *A. variabilis* experienced a significant reduction in cell and cell wall volumes.

In an earlier comparative study Rachlin *et al* (1982) employed stereological techniques to quantify the changes in *Plectonema boryanum* after exposure to eight heavy metals (manganese, zinc, mercury, cadmium, lead, copper, cobalt, nickel). The changes observed were increases in the thylakoidal surface area induced by lead, manganese, cobalt, zinc, cadmium and nickel, and decreases caused by copper. Intrathylakoidal spaces were reduced significantly by zinc, mercury, cobalt and nickel. Also observed were a decrease in

lipid inclusions by all eight metals and a decrease in polyphosphate bodies by all of the metals except copper and nickel.

Studying thin sections of three algal species (*Melosira granulata, Fragilaria capucina,* and *Anacystis cyanea*), Sicko-Goad (1982) reported ultrastructural responses to short-term, low doses of copper and lead. While the species were generally more sensitive to copper than to lead, they responded differently to each of the cations. The cyanobacterium, *Anacystis cyanea*, showed statistically significant changes, specifically an increase in the number of cyanophycin granules and poly-ß-hydroxybutyric acid granules as well as a constant decrease in thylakoidal surface area.

The study by Rachlin *et al.* (1984) in which *Anabaena flos-aquae* was exposed to several concentrations of cadmium (1.18 μM, 11.8 μM, 118.33 μM) is one which demonstrates cells' (and individual inclusions') varied responses to different concentrations of a single metal. The lowest concentration caused a significant decrease in intrathylakoidal space and the number of lipid inclusions. The latter inclusion conversely showed no response at 11.8 μM. This concentration, however, produced not only a reduction in the number of polyphosphate bodies, but also the percentage of the cell that was occupied by these bodies. A similar decrease was observed in layers II, III, and IV of the cell wall. The greatest concentration expectedly disintegrated polyphosphate bodies to the extent that few could be definitively identified, but caused an increase in layer I of the cell wall.

In addition to physiological changes (culture density, nitrogenase activity, and CO_2 fixation) *Anabaena cylindria* showed changes in

ultrastructure upon exposure to aluminium at pH 6.0 (Pettersson *et al.*, 1985). The most notable changes included an accumulation of cyanophycin granules, degradation of the thylakoidal membranes and a decrease in inter-thylakoidal electron density. Interestingly, heterocysts remained unchanged.

MATERIALS and METHODS

Culture Maintenance

Cultures of the freshwater cyanobacteria, *Anabaena doliolum* (Bharadwaja) (UTEX B2094) and *Synechococcus leopoliensis* (Racib.) Komarck (UTEX LB2434) were obtained from the Starr Culture Collection (Starr and Zeikus, 1987). All stock and experimental cultures were grown in modified Fitzgerald's medium (see Appendix A) in a Sherer Controlled Growth Chamber (Model Cel B) at 25°C on a 12 hour light/darkness schedule. Cool white fluorescent bulbs were used to set the illumination at 500 foot candles, along with two 25W incandescent bulbs. The cells were agitated daily. Cell growth was determined by optical density (turbidity technique) with a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 440 nm, which was determined by dilution curves (Sorokin, 1975). Cell count (cell number per ml) was determined with a Spencer Bright Line hemocytometer counting chamber under a light microscope. Aluminum chloride ($AlCl_3 \cdot 6H_2O$) from Sigma (Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178) was used in the study. Protein quantification was also used to determine growth after exposure to the metal (see below).

Sublethal Aluminum Exposure

Cells from rapidly growing cultures of both species were transferred through sterile technique to fresh growth medium to achieve a concentration of 1 x 10^6 cells per ml. The optical density was

taken at 440 nm against a standard of the growth medium after which cells were exposed to aluminum according to Rachlin and Farran (1974) and Rachlin *et al.* (1984).

A 3.7 mM stock solution of aluminum in distilled water was used to make test solutions of 0.37 mM, 0.23 mM, 0.14 mM, 0.067 mM, and 0.037 mM in modified Fitzgeralds's medium. Five (5) ml of cells from the adjusted cultures were centrifuged and the supernatant replaced by 5 ml of the respective test solutions. Each treatment, as well as a control, was set up in triplicate in sterile 30 ml Falcon tissue culture flasks (Becton Dickinson and Company, 2 Bridgewater Lane, Lincoln Park, New Jersey 07035). Aluminum solutions were prepared just prior to use.

After a 96-hour exposure in the chamber under the growth conditions given above, the optical density was taken on each replicate. The preparation for protein analysis was done according to a method developed by Dr. J. S. Poindexter of the Department of Biological Sciences, Barnard College of Columbia University, New York, New York.

The three replicates of each test solution were combined and centrifuged. The supernatant was replaced with 5%trichloroacetic acid (TCA) for 30 minutes. This supernatant was removed, the cells resuspended in 5% TCA, and boiled in covered test tubes in a water bath at 100°C for 30 minutes. The tubes were cooled in an ice bath.

The supernatant was removed after centrifugation. The sediment was resuspended in 0.2N NaOH and boiled in covered test tubes in a water bath at 100°C for 30 minutes. The sediment was discarded and the supernatant was analyzed by the Lowry (1951) test for protein.

The Lowry Test

Anabaena doliolum

The following reagents were made:

A: 2% Na_2CO_3 in 0.1N NaOH

B: 1% $CuSO_4 \cdot 5H_2O$

C: 2% Na K tartrate

D: 0.5 ml of Reagent B + 0.5 ml of Reagent C + 50 ml of Reagent A

E: Phenol reagent (Fisher) The stock reagent is diluted 1:2 before use.

For the standard, a Stock Solution A of bovine serum albumin (BSA) was made with a concentration of 7 mg BSA to 7 ml distilled water. Stock Solution B was made with 1 ml of Stock Solution A and 4 ml of distilled water. Seven (7) standard concentrations were made from Stock Solution B:

Standard	Stock B	H_2O	Concentration
1	0.6 ml	0.0 ml	120 µg/0.6 ml
2	0.5 ml	0.1 ml	100 µg/0.6 ml
3	0.4 ml	0.2 ml	80 µg/0.6 ml
4	0.3 ml	0.3 ml	60 µg/0.6 ml
5	0.2 ml	0.4 ml	40 µg/0.6 ml
6	0.1 ml	0.5 ml	20 µg/0.6 ml
7	0.0 ml	0.6 ml	0 µg/0.6 ml (Blank)

To 0.6 ml of each concentration of standard was added 3.0 ml of Reagent D for 10 minutes at room temperature. The contents of each tube were combined with 0.3 ml of Reagent E, mixed thoroughly, and allowed to react for 20 minutes at room temperature. The optical density of each standard was taken at 650 nm against the blank. Each value was plotted against its corresponding protein concentration.

Each test culture was subjected to the same procedure as was the protein standard (BSA). To 0.6 ml of each test culture was added 3.0 ml of Reagent D for 10 minutes at room temperature. The contents of each tube were combined with 0.3 ml of Reagent #, mixed thoroughly, and allowed to react for 20 minutes at room temperature. The optical density of each test concentration was plotted against the calibration curve of the standard.

The Lowry Test

Synechococcus leopoliensis

The tests were identical except for a single variation. The standard had to be increased because the highest protein yield (from control cells) was greater than the original standard. Solution A (7 mg BSA to 7 ml distilled water) was used undiluted (This eliminated Solution B.) Eight (8) standard concentrations were made from Solution A to accommodate the higher yield of this species.

Eight (8) standard concentrations:

Standard	Solution A	H_2O	Concentration
1	0.16 ml	0.44 ml	160 μg/0.6 ml
2	0.14 ml	0.46 ml	140 μg/0.6 ml
3	0.12 ml	0.48 ml	120 μg/0.6 ml
4	0.10 ml	0.50 ml	100 μg/0.6 ml
5	0.08 ml	0.52 ml	80 μg/0.6 ml
6	0.06 ml	0.54 ml	60 μg/0.6 ml
7	0.04 ml	0.56 ml	40 μg/0.6 ml
8	0.02 ml	0.58 ml	20 μg/0.6 ml

Confirmation runs were made by exposing rapidly growing cells to a new range of concentrations based on the calculated EC_{50} for each species. The confirmation concentrations for *A. doliolum* were 0.00 mM, 0.14 mM, 0.18 mM, and 0.32 mM. Those for *S. leopoliensis* were 0.00 mM, 0.119 mM, 0.130 mM, and 0.145 mM.

Aluminum Uptake by Whole Cells for Energy Dispersive X-ray Microanalysis

Cells were grown for 14 days under the conditions described for growth response. Trace minerals (Gaffron's) were omitted from the growth medium in order to assure that all aluminum taken up by the cells came from the test solutions.

Cells were exposed to 0.04 mM, 0.19 mM, and 0.37 mM aluminum for five hours. Distilled water was used for exposure of control cells.

All were centrifuged and washed three times in distilled water before being placed in single droplets onto formvar-coated copper grids (Baxter and Jensen, 1980b). After air drying, cells were analyzed with the Hitachi H-7000 electron microscope equipped with a PGT Integrated Microanalyzer and Imaging X-Ray (IMIX) computer. With the microscope in the STEM mode (spot setting), x-ray collections were made for 100 seconds with the probe placed on either polyphosphate bodies, cell wall, or cytoplasm. These readings were subtracted from a single formvar collection for final spectra of each of the cellular sectors.

Aluminum Exposure at Selected pH Levels

Cells of both cultures were exposed to aluminum as described above for growth response to the metal. *A. doliolum* was adjusted to achieve a concentration of 1 x 10^6 cells/ ml and exposed to the metal in medium buffered with (2-[N-morpholino]ethanesulfonic acid) MES (Wehr *et al.*, 1986), to pH 7.2, pH 5.5, and pH 9.0. At the alkaline and acid pH levels, cells were exposed to 0.32 mM aluminum, the EC_{50}. At pH 7.2, cells were treated with 0.32 mM as well as with 0.032 mM and 3.15 mM. These concentrations were selected based on preliminary work (not reported here) whose results showed that *A. doliolum* could tolerate higher levels of the metal than indicated by the EC_{50}.

In addition to the exposure at the EC_{50}, 0.197 mM, cells were also exposed to 0.00037 mM, 0.085 mM, and 0.74 mM. These three concentrations were based on a documented environmental range of the metal (Galvin, 1991). Control studies were conducted at all pH levels.

S. leopoliensis was adjusted to 7 x 10^6 cells/ml and exposed to the metal in buffered medium at concentrations of 0.145 mM, the EC_{50}, at the acid and alkaline pH levels. Cells were treated at pH 7.2 with a concentration range which included 0.014 mM, 0.145 mM, and 1.45 mM. As with *A. doliolum*, the second species was exposed to the environmental concentrations.

At the end of the 96-hour exposure, cells of both species were harvested by centrifugation and fixed according to Pankratz and Bowen (1963). The 3-hour fixation was accomplished with 1% osmium tetroxide (see Appendix B) at room temperature after which cells were dehydrated in a 50%, 70%, 95%, and 100% ethanol series. The cells were conditioned for embedding in three steps with propylene oxide. This conditioning was followed by a propylene oxide-Epon series (25%, 50%, 75% Epon) after which the cells were embedded in 100% Epon and left at room temperature overnight. Over a five-day period, the embedments were heated in ovens at 35°, 45°, and 60° for polymerization of Epon (Luft, 1961).

The Reichert-Jung Ultracut E Ultramicrotome was used with a diamond knife for thin sectioning. Sections were collected onto 300-mesh grids, poststained with uranyl acetate (Stampak and Ward, 1964), and rinsed in a methanol-ethanol-distilled water series. Grids were analyzed in the Hitachi H-7000 electron microscope and cells of only a single square per grid were photographed to assure that no cell was considered more than once (Sicko-Goad and Stoermer, 1979). If more cells were required, either another block was sectioned or the previously sectioned area was cut away and the block retrimmed.

Morphometric Analysis

Morphometric analyses (Weibel and Bolender, 1973; Sicko-Goad *et al.*, 1977; Sicko-Goad and Stoermer, 1979; Jensen and Corpe, 1988) were made from micrographs of thirty (30) cells of *A. doliolum* at a total magnification of 54,400 x. Each micrograph was overlayed with a 1.0 cm acetate lattice (Figure 1) and dot counts were converted to relative values of cell volume, cell wall volume, polyphosphate bodies, polyhedral bodies, cyanophycin granules, membrane limited crystalline inclusions, and mesosomes and volume of intrathylakoidal space. Relative surface area was calculated from counts of thylakoids.

S. leopoliensis was analyzed at a total magnification of 60,000 x for values of cell volume, cell wall volume, first layer of cell wall volume, polyphosphate bodies, mini polyphosphate bodies, polyhedral bodies, and nucleoplasm and volume of intrathylakoidal space. Relative surface area was calculated from counts of thylakoids.

Descriptive statistics and single classification and two-factor analyses of variance (ANOVA) were completed using the Statview 512$^+$ Program from Macintosh.

Phytochelatins

The tests for isolation and analysis of proteins to determine if the cells synthesized phytochelatins either before or after exposure to aluminum were conducted by Dr. Meinhart H. Zenk of Pharmazeutische Biologie, Universität München, Müchen, Germany, according to Grill *et al.* (1985) and Gekeler *et al.* (1988).

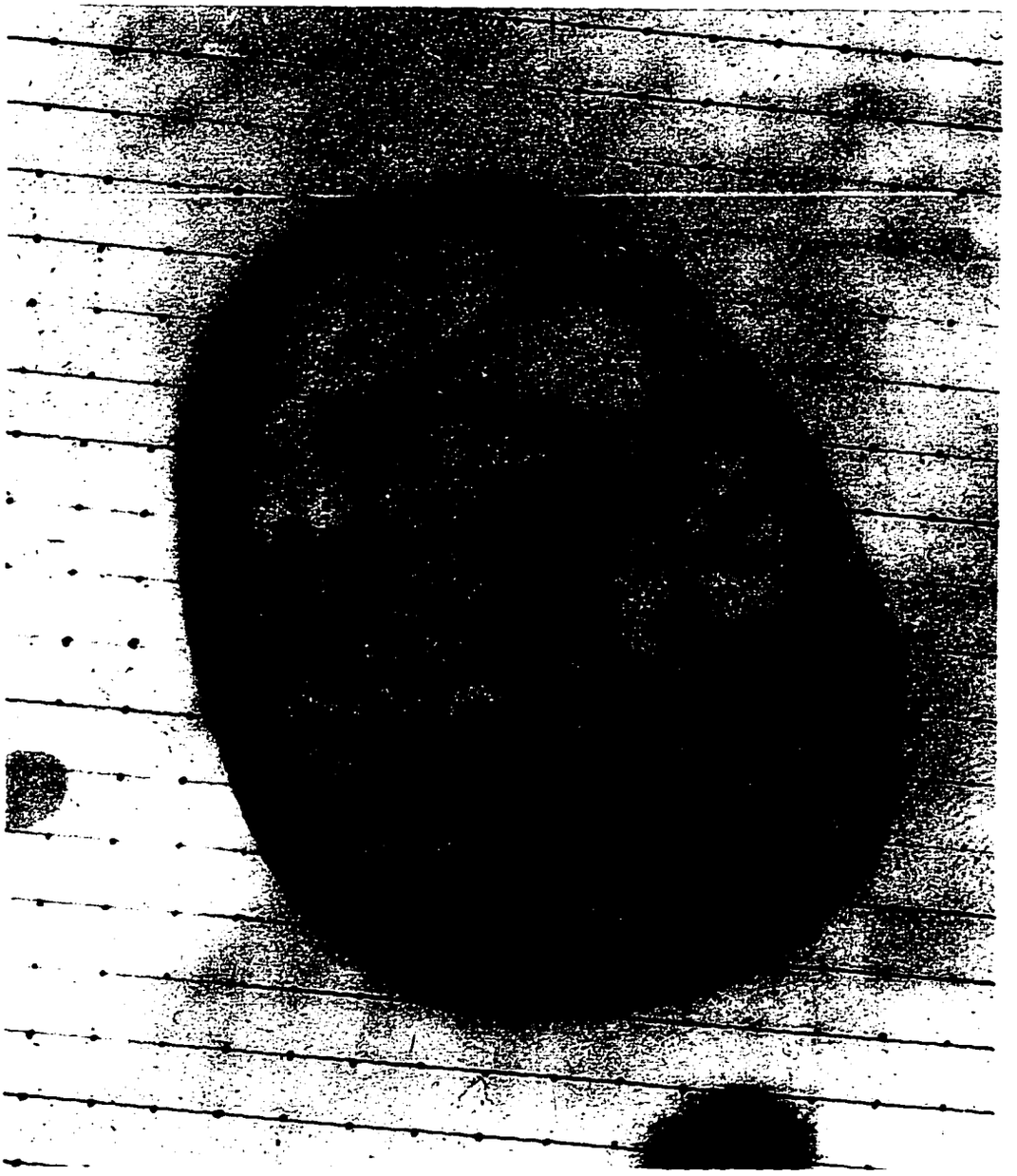

Figure 1. Micrograph of a cell overlaid with a counting grid used throughout morphometric analysis of cellular inclusions and sectors. The cell was magnified 34,000 X.

Twenty (20) mg (dry weight) of *A. doliolum* exposed to 0.32 mM, 0.74 mM, and 3.2mM aluminum were sonicated in a solution containing 200 µl 1N NaOH with 1 mg/ml $NaBH_4$, and 100 µl of water. After centrifugation 200 µl of the supernatant were acidified with 50 µl 3.6 N HCl. A sample of the acidified solution, 100 µl, was analyzed by HPLC (post-column-derivation with Elman's Reagent). The tests were repeated on cells of *S. leopoliensis* which had been exposed to 0.15 mM and 0.74 mM aluminum. All treatments were matched with control samples.

RESULTS

Determination of Protein Concentration of *A. doliolum* After Exposure

A growth curve was developed over a 35-day period during which growth rate was determined through optical density. This process revealed that the species entered a stage of rapid growth about day 7 and one of near exponential growth about day 9. This rate was maintained up to about day 21 when growth began to slow until a stationary phase was entered about day 31. Day 14 was selected as a time of exponential growth and all subsequent tests were conducted when the species was at this stage.

The culture adjusted to 1 x 10^6 for the growth response test showed an optical density of 1.1 at pH 7.5. The average optical density values for triplicates at the end of the 96-hour exposure are not reported. Table 1. shows the determination of the protein concentrations of treated cells as determined from the optical densities and protein concentrations of the standard.

The cells showed a consistent decrease in protein concentration and in optical density as the aluminum concentration increased. The final protein concentration ranged was 55 μg to 45 μg, with corresponding optical density values which ranged from 0.110 to 0.080. Untreated cells contained 58 μg of protein with a spectrophotometrical reading of 0.120. The protein concentrations of cells at the test concentrations and optical densities are given in Table 1.

Table 1. The protein concentrations of *A. doliolum* with corresponding optical densities of combined triplicates after the 96-hour exposure.

Al Concentration (mM)	Protein Concentration (μg)	O. D.$_{650}$
0.000	58	0.120
0.037	55	0.110
0.067	50	0.095
0.120	45	0.080
0.230	----	0.000
0.370	----	0.000

Table 2. The EC_{50} value of aluminum calculated from the percent response of *A. doliolum* after 96-hour exposure, with respective regression equation (Data based on triplicate runs).

Conc. (mM)	Log Conc.	Protein Conc. (μg)	O.D.$_{650}$	% Control Growth	Empirical Probit
0.000	----------	58	0.120	100	----------
0.037	-1.4318	55	0.110	91.7	6.3852
0.067	-1.1739	50	0.095	79.2	5.8134
0.120	-0.9208	45	0.080	66.7	5.4316

$Y = -1.867x + 3.682$; $r^2 = 0.988$; log $EC_{50} = -0.705945366$; $EC_{50} = 0.197$ mM ±0.074

The protein concentrations of the samples were converted to a percentage of response in comparison to control cells. Key Stat, an IBM-compatible computer program (Eckblad, 1986) generated the regression equation from two variables, the log of the concentration and the empirical probit value which corresponds to each percentage response. The estimated EC_{50} is 0.197 mM ± 0.074 (Table 2.)

Effect of Aluminum on the Ultrastructure of *Anabaena doliolum* at Different pH Levels

Relative Volumes

Cell Volume

A. doliolum is a bacillus shape cell with thylakoids distributed throughout the cytoplasm. Polyhedral bodies are often in clusters near the cell's center, while lipid droplets are found closer to the plasma membrane (Figure 2). Polyphosphate bodies may be found dispersed within the cytoplasm. Fewer cells possess cyanophycin granules; seldom will more than one occur in a cell. Membrane-bound crystalline inclusions and mesosomes occur even less often. The comparative data for the effect of pH and aluminum on the ultrastructure of *A. doliolum* are presented in Table 3 through Table 11.

Two-factor ANOVA indicated a significant interaction between pH and aluminum concentration with respect to total cell volume ($P<0.05$) with a stronger influence of pH ($r^2=0.13$). The profile clearly shows a parallel increase among control cells and among treated cells over the pH range (Figure 3). At pH 5.5 a trend toward decrease was observed among treated cells, but at only 11.31%, was not significant according to the single classification ANOVA (Table 6). The cytoplasm in many of these cells appeared granular and detached from the plasma membrane (Figure 4). A trend toward increase in cell volume was detected among cells treated with the metal at pH 9.0. The increase at pH 7.2, however, was significant with addition of aluminum ($P<0.05$) (Table 8).

Cell Wall Volume

The effect of pH and 0.32 mM aluminum across the pH range proved to be significant ($P<0.05$). Untreated cells varied little, but addition of the metal resulted in a sharp decrease in the cell wall volume from pH 5.5 to pH 9.0 (Figure 5). The 32.07% increase shown by treated cells at pH 5.5 reflects the change from an average wall thickness of 7 nm in control cells to an average of nearly 10 nm in cells exposed to the metal.

Polyhedral Body Volume

The influence of pH ($r^2=0.03$) as well as that of 0.32 mM

Table 3. Summary of morphometric data for *A. doliolum* cells exposed to selected concentrations of aluminum at pH 5.5. Values reported are means (standard error in parentheses) of a sample size of 30 sections of cells.

	N	$[Al3^{+}]$ mM	0.00	0.32
Cell volume	30		204.17 (12.07)	181.07 (9.03)
Cell wall Vv	30		7.39 (0.41)	9.76 (1.11)
Polyhedral bodies Vv	30		1.93 (0.31)	0.37 (0.19)
Polyphosphate bodies Vv	30		0.08 (0.07)	1.55 (0.49)
Intrathylakoidal space Vv	30		4.12 (0.79)	16.97 (2.49)
Thylakoidal surface area Sv	30		1.24 (0.15)	1.22 (0.15)

Table 4. Summary of morphometric data for *A. doliolum* cells exposed to selected concentrations of aluminum at pH 7.2. Values reported are means (standard error in parentheses) of a sample size of 30 sections of cells.

	N	$[Al3^{+}]$ mM	0.00	0.32
Cell volume	30		211.53 7.99)	242.47 (10.88)
Cell wall Vv	30		7.67 (0.32)	6.78 (0.44)
Polyhedral bodies Vv	30		1.61 (0.33)	2.07 (0.47)
Polyphosphate bodies Vv	30		0.36 (0.13)	0.27 (0.11)
Intrathylakoidal space Vv	30		2.28 (0.36)	3.1 (0.68)
Thylakoidal surface area Sv	30		0.84 (0.12)	1.10 (0.13)

Table 5. Summary of morphometric data for *A. doliolum* cells exposed to selected concentrations of aluminum at pH 9.0. Values reported are means (standard error in parentheses) of a sample size of 30 sections of cells.

	N	$[Al3^{+}]$ mM	0.00	0.32
Cell volume	30		237.9 (9.56)	259.93 (13.7)
Cell wall Vv	30		6.92 (0.29)	6.73 (0.32)
Polyhedral bodies Vv	30		2.42 (0.32)	1.19 (0.22)
Polyphosphate bodies Vv	30		0.41 (0.19)	0.18 (0.06)
Intrathylakoidal space Vv	30		6.19 (1.01)	8.61 (0.95)
Thylakoidal surface area Sv	30		1.19 (0.13)	1.81 (0.16)

Figure 2. Micrograph of *A. doliolum* grown at pH 7.2 showing polyhedral bodies (Pb) clustered near the cell's center and lipid inclusions (L) near the periphery. X 34,000.

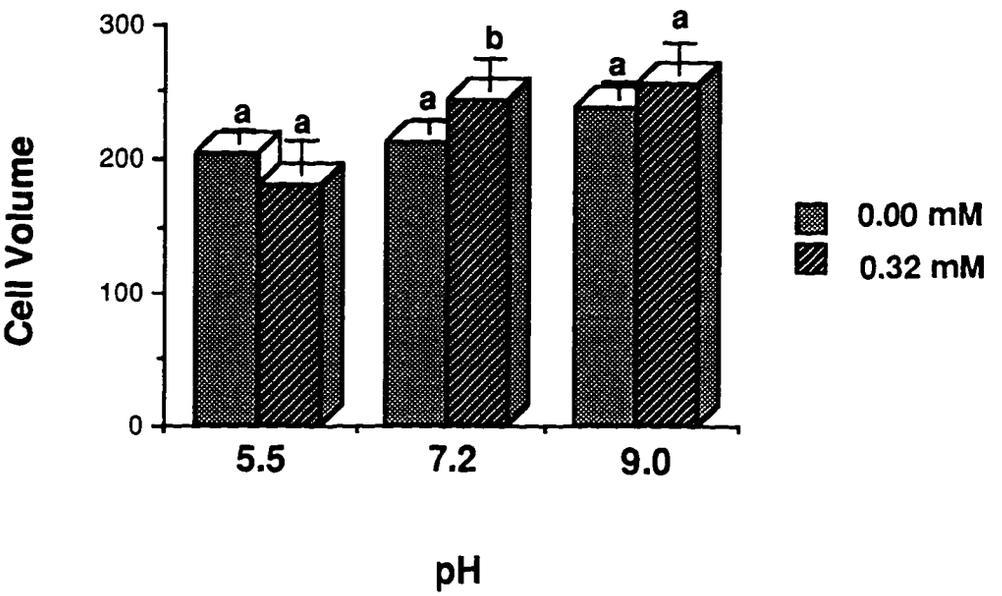

Figure 3. Effect of 0.32 mM aluminum on cell volume Vv of *A. doliolum* at different levels of pH (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

Figure 4. Micrograph of *A. doliolum* exposed to 0.32 mM aluminum at pH 5.5. This exposure resulted in granular cytoplasm which is detached from the plasma membrane. Note the extensive loss of thylakoids (Th). X 34,000.

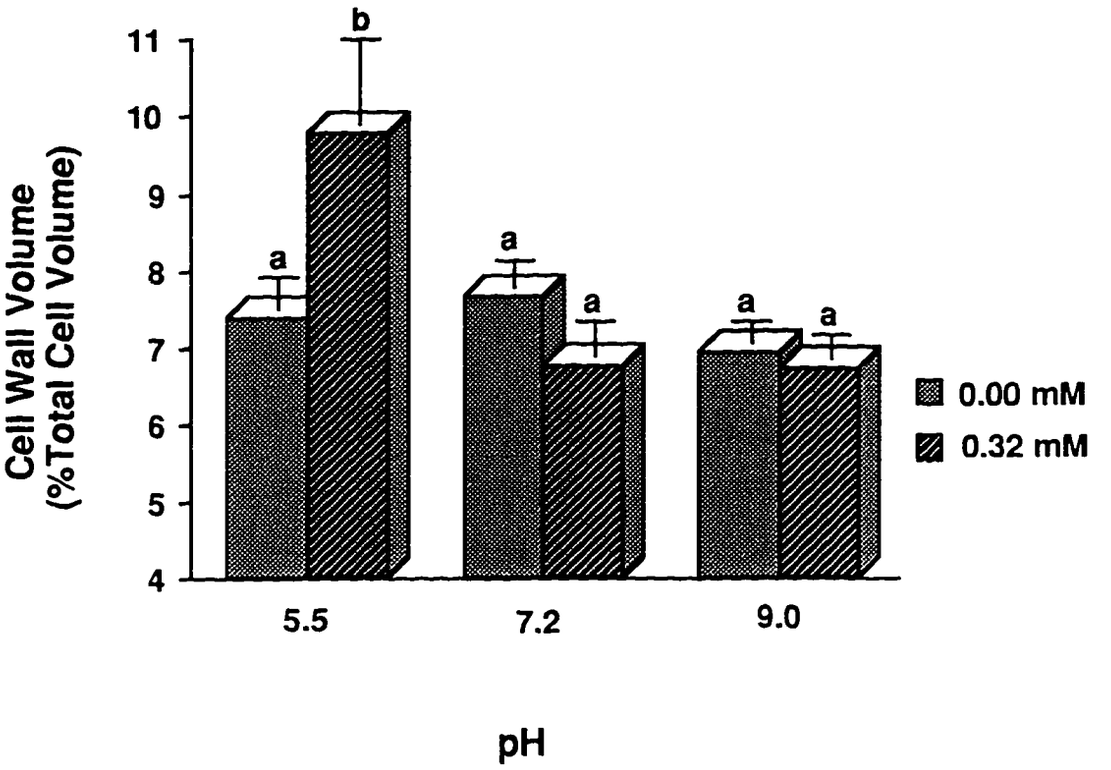

Figure 5 . Effect of 0.32 mM aluminum on the cell wall Vv of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity)
P<0.05)

Table 6. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 5.5, by single classification ANOVA.

$[AL^{3+}]$ mM	Cell volume	Cell wall Vv	Polyhedral body Vv
0.00	204.17_a	7.39_a	1.93_a
0.32	181.07_a	9.76_a	0.37_b
N	60	60	60
F	2.35	4.00	18.38
P	0.1308	0.0503	0.0001***

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

*** = $P < 0.001$

aluminum (r^2=0.05) individually on the relative volume ofpolyhedral bodies was strong; however, the combined influence was not as effectual. The significant decrease by 80.82% in volume of polyhedral bodies within cells exposed to 0.32 mM aluminum at pH 5.5(P<0.001) (Table 6) is among the most pronounced changes in ultrastructure observed among the treatments (Figure 6). The response of this inclusion at pH 9.0 likewise proved to be a significant decrease (P<0.01) (Table 10). Cells exposed to the metal at pH 7.2, however, did not respond as strongly.

Polyphosphate Body Volume

The profile of polyphosphate body volume (Figure 7) shows a relationship between pH and the metal with a 350% increase in relative volume of polyphosphate bodies among untreated cells from pH 5.5 to pH 7.2, and a 13.98% increase from pH 7.2 to pH 9.0. Cells treated with 0.32 mM aluminum demonstrated an 82.58% decrease in polyphosphate body volume from pH 5.5 to pH 9.0 (P<0.001).

Cells treated with 0.32 mM aluminum at pH 5.5 showed a highly significant increase (P<0.001) (Table 7) in volume of polyphosphate bodies over cells grown with no metal added. This marked increase (1838%) is demonstrated by an average volume of 0.084 cm^2 in control cells to one of 1.546 cm^2 in those exposed to the metal.

Table 7. Comparison of relative volumes (Vv) of polyphosphate bodies and intrathylakoidal space and thylakoidal surface area (Sv) among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 5.5, by single classification ANOVA.

$[AL^{3+}]$ mM	Polyphosphate body Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
0.00	0.08_a	4.12_a	1.24_a
0.32	1.55_b	16.97_b	1.22_a
N	60	60	60
F	8.76	24.21	0.006
P	0.0045*	0.0001***	0.9407

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P < 0.05$

*** = $P < 0.001$

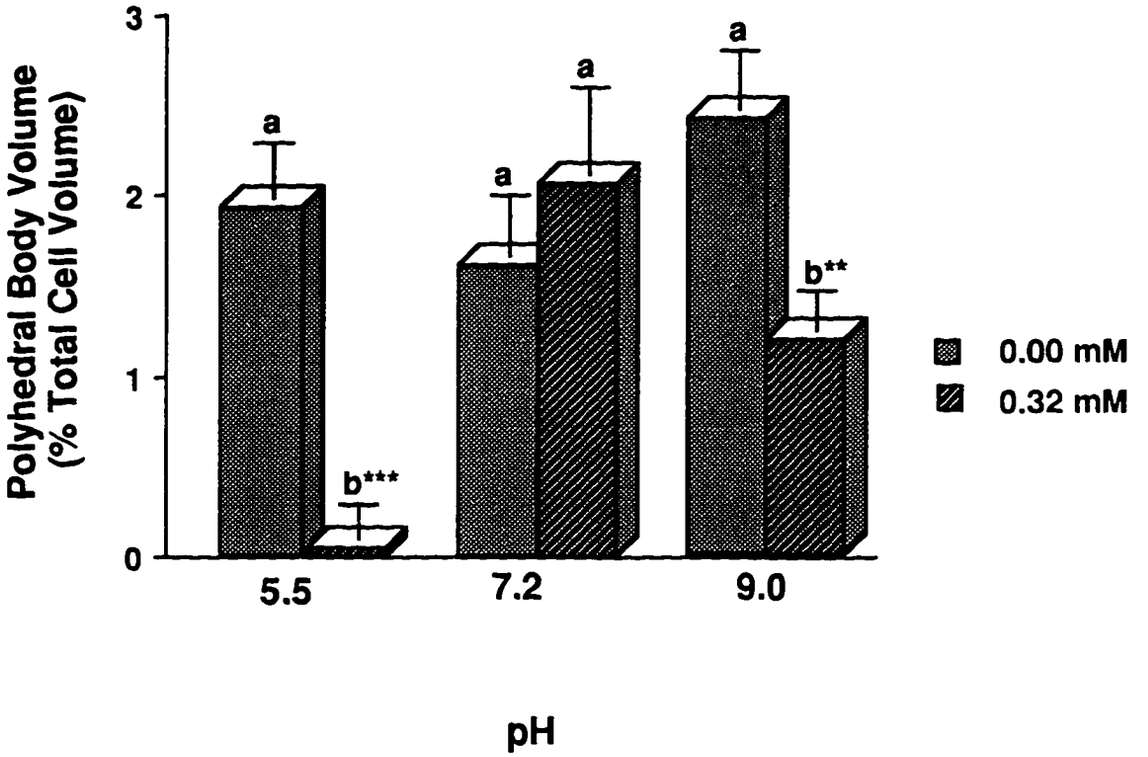

Figure 6. Effect of 0.32 mM aluminum on polyhedral body volume Vv of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

** = P<0.01

*** = P<0.001

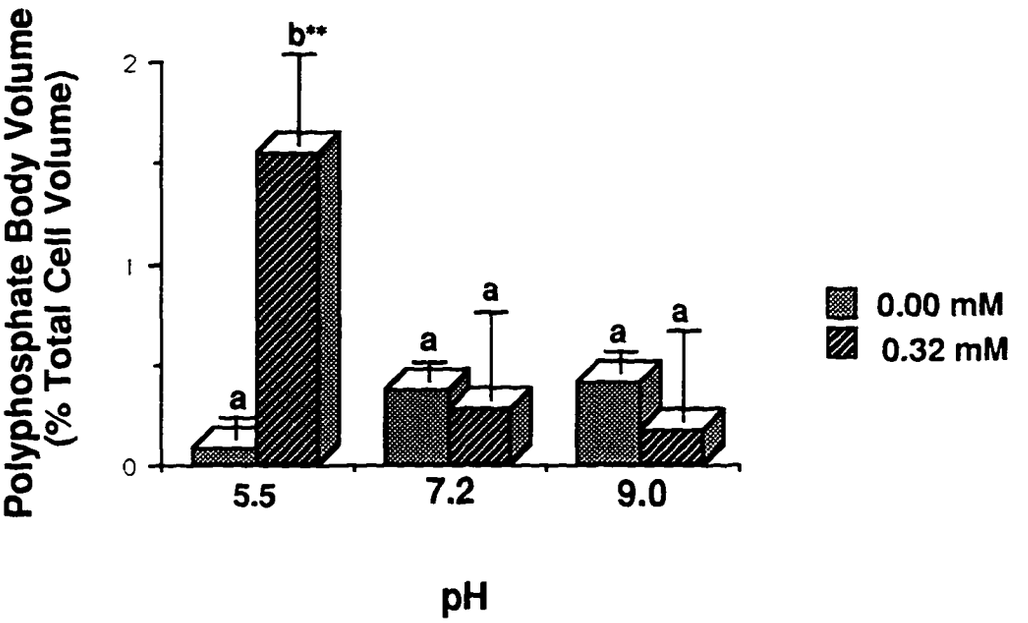

Figure 7. Effect of 0.32 mM aluminum on polyphosphate body volume Vv at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
** = P<0.01

Intrathylakoidal Space Volume

Both the significance ($P<0.0001$) and the degree of variation ($r2=0.10$) reflect the extent of interaction on this cellular sector. The extensive increase in intrathylakoidal space ($P<0.001$) (Figure)) with a loss of thylakoids upon exposure to the metal at pH 5.5 can be seen in Figure 8. The increases at pH 7.2 (Table 9) and pH 9.0 (Table 11), though not significant, are consistent with the responses in thylakoidal surface area.

Thylakoidal Surface Area

Using the near-neutral pH as the starting point and moving to pH 9.0, the profile (Figure 10) shows a positive correlation between pH and the metal as both treated and untreated cells increase in relative thylakoidal surface area. Comparison between cells at pH 7.2 and those at pH 5.5, however, shows a repeat of the increase among controls, along with a decrease among treated cells. The increase by 50.83% in relative thylakoidal surface area among cells exposed at pH 9.0 proved to be significant ($P<0.01$) (Table 11) when compared to controls. Thylakoids are prominent in Figure 11.

Relative Counts

Polyhedral Body Number

The response of polyhedral body number to pH and 0.32 mM

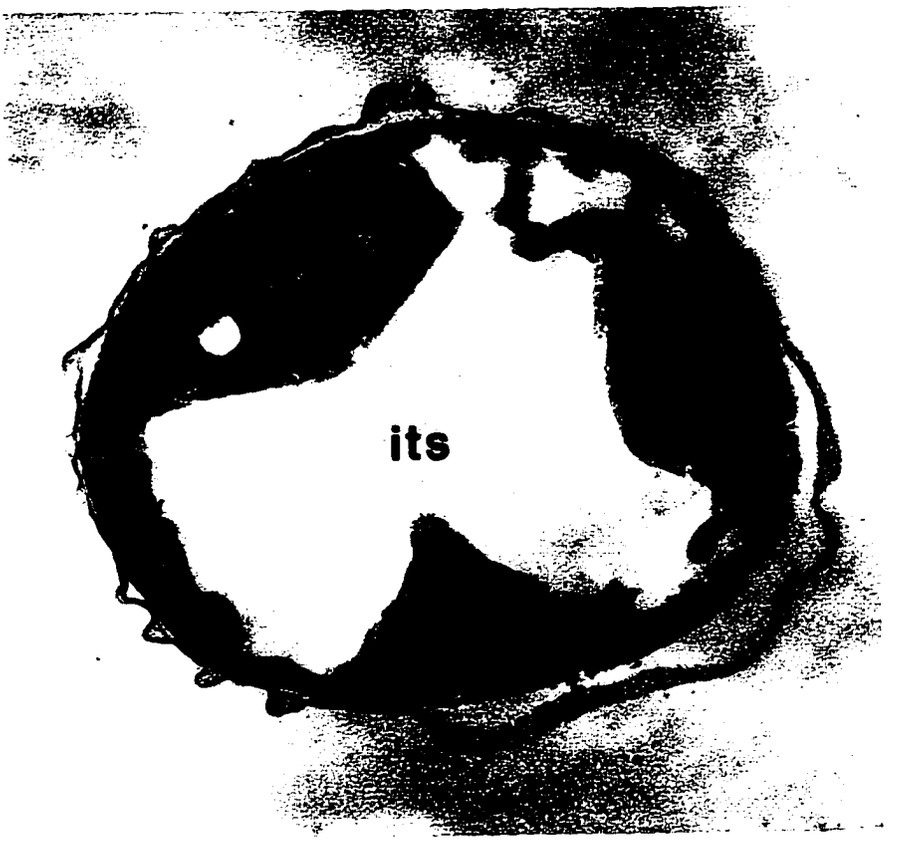

Figure 8. Micrograph of *A. doliolum* exposed to 0.32 mM aluminum at pH 5.5 showing the increase in intrathylakoidal space (its). The cytoplasm is granular and devoid of thylakoids. X 34,000.

Table 8. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 7.2, by single classification ANOVA.

$[AL^{3+}]$ mM	Cell volume	Cell wall Vv	Polyhedral body Vv
0.00	211.53_a	7.67_a	1.61_a
0.32	242.47_b	6.78_a	2.07_a
N	60	60	60
F	5.25	2.66	0.64
P	0.0256*	0.1082	0.4262

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P < 0.05$

Table 9. Comparison of relative volumes (Vv) of polyphosphate bodies and intrathylakoidal space and thylakoidal surface area (Sv) among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 7.2, by single classification ANOVA.

$[AL^{3+}]$ mM	Polyphosphate body Vv	Intrathylakoidal Space Vv	Thylakoidal surface area Sv
0.00	0.36_a	2.28_a	0.84_a
0.32	0.27a	3.10_a	1.10_a
N	60	60	60
F	0.29	1.14	2.27
P	0.5889	0.2900	0.1370

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

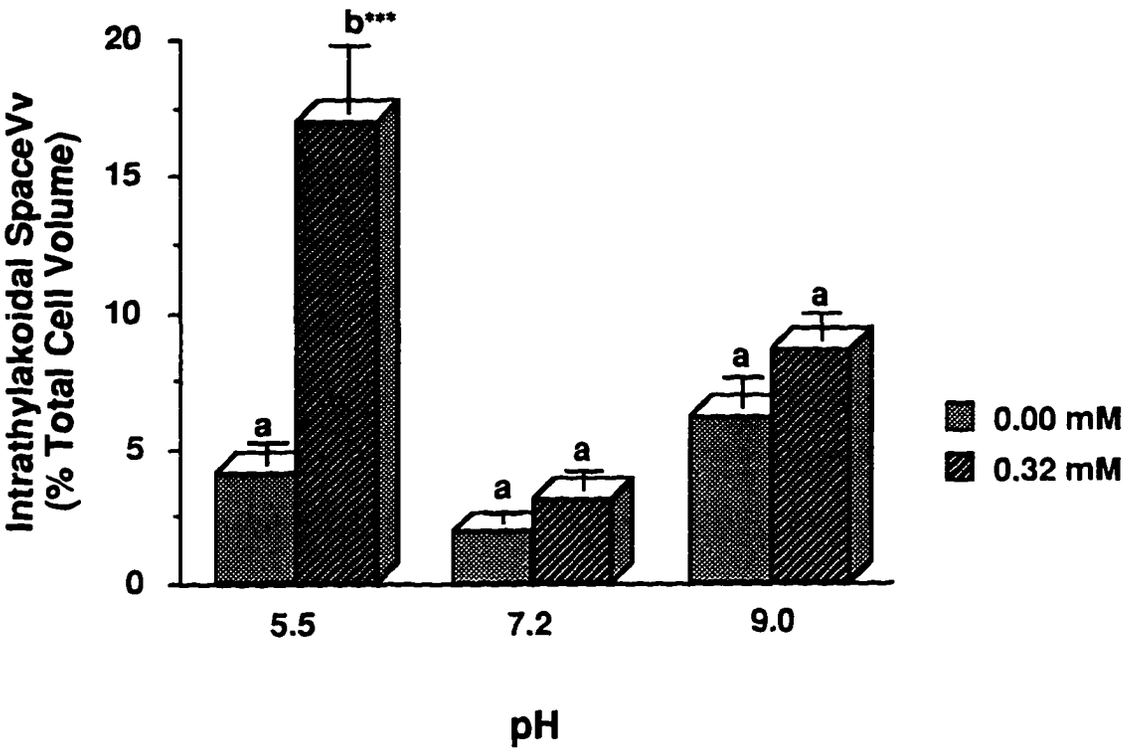

Figure 9. Effect of 0.32 mM aluminum on intrathylakoidal space Vv at different pH levels (errorbar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
*** = P<0.001

Table 10. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 9.0, by single classification ANOVA.

$[AL^{3+}]$ mM	Cell volume	Cell wall Vv	Polyhedral body Vv
0.00	237.90_a	6.92_a	2.42_a
0.32	259.93_a	6.73_a	1.19_b
N	60	60	60
F	1.729	0.197	9.854
P	0.1937	0.6585	0.0027**

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

** = $P < 0.01$

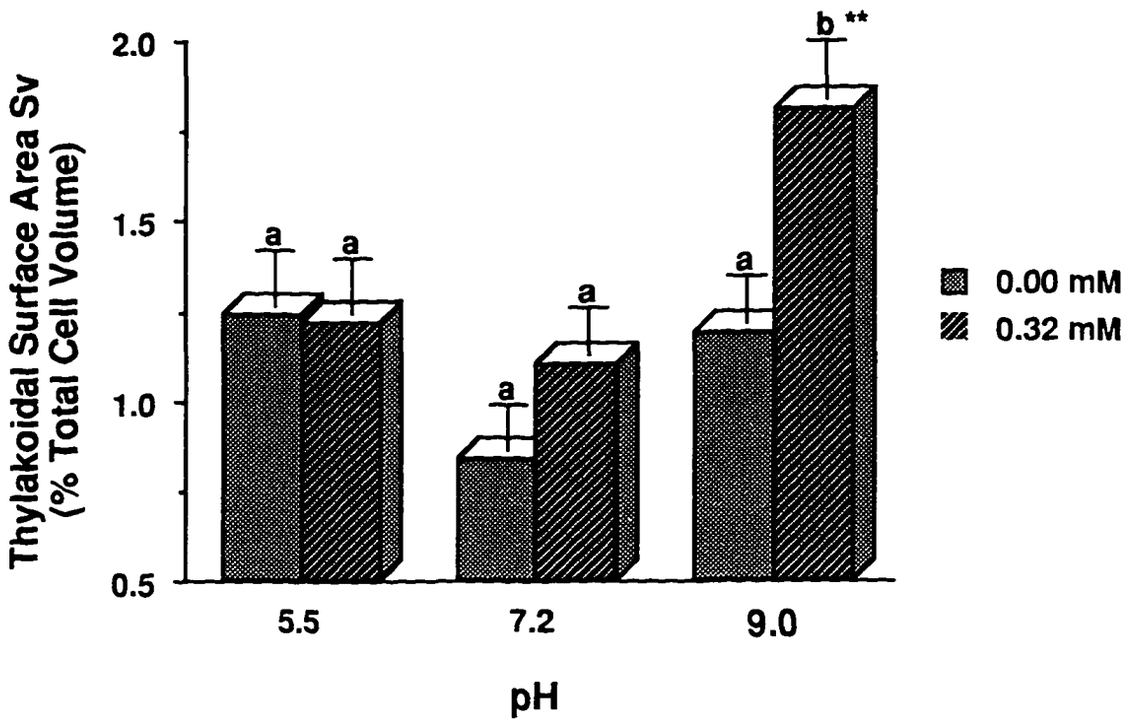

Figure 10. Effect of 0.32 mM aluminum on thylakoidal surface area Sv at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
** = P<0.01

Table 11. Comparison of relative volumes (Vv) of polyphosphate bodies and intrathylakoidal space and thylakoidal surface area (Sv) among cells of *A. doliolum* exposed to selected aluminum concentrations at pH 9.0, by single classification ANOVA.

$[AL^{3+}]$ mM	Polyphosphate body Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
0.00	0.41_a	6.19_a	1.19_a
0.32	0.18_a	8.61_a	1.81_b
N	60	60	60
F	1.254	3.064	9.316
P	0.2675	0.0854	0.0034**

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

** = $P < 0.01$

Figure 11. Micrograph of *A. doliolum* grown at pH 7.2. Thylakoids (Th) are prominent. X 34,000.

aluminum indicates significant interaction ($P<0.01$) (Figure 12). The increase among cells exposed to the metal at pH 7.2 was not significant; however, the response marks a contrast to the significant decrease at both pH 5.5 ($P<0.001$) and pH 9.0 ($P<0.05$), and emphasizes the degree of significance of interaction. The significance levels produced through two-factor ANOVA indicate nearly equal influence of pH ($r^2=0.06$), the metal concentration ($r^2=0.07$), and their interaction ($r^2=0.05$).

Polyphosphate Body Number

The significant interaction ($P<0.05$) indicated through the response of this cellular inclusion is strengthened by the 875% increase among cells exposed to 0.32 mM aluminum at pH 5.5 ($P<0.01$) (Figure 13), a response which was consistent with the increase in polyphosphate body volume. These dense bodies were found among 36.6% of the treated cells compared to 6.67% of cell grown with no aluminum. The significant increase in the relative number at pH 9.0 ($P<0.01$), however, is opposite the response exhibited by relative volume. Further analysis revealed that the relative number of polyhedral bodies decreased with treatment at the acid and alkaline pH levels, while the relative number of polyphosphate bodies increased at the same pH levels (Figures 12 and 13).

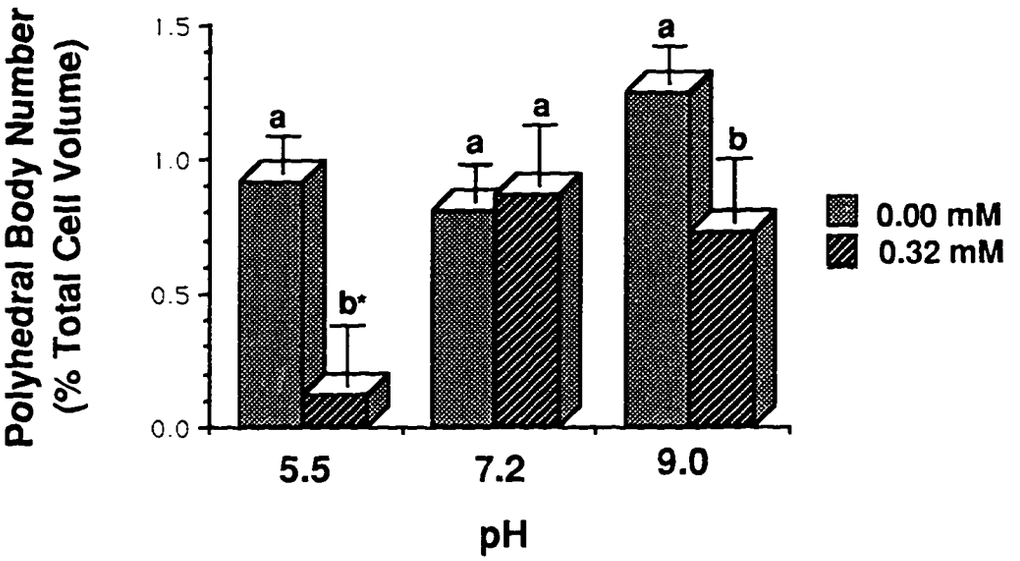

Figure 12. Effect of 0.32 mM aluminum on polyhedral body number of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
*** = P<0.05**

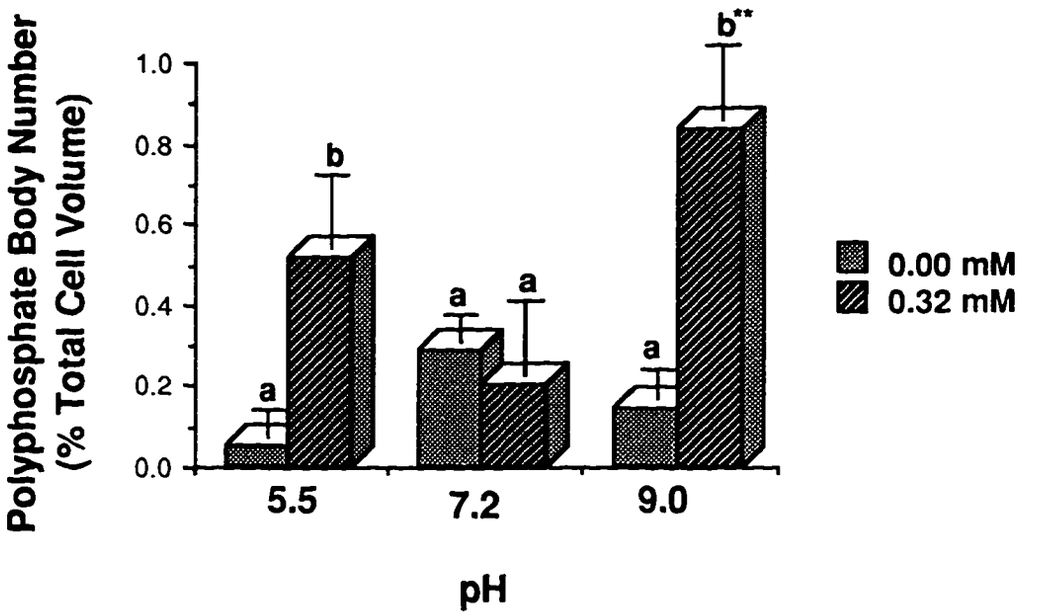

Figure 13. Effect of 0.32 mM aluminum on polyphosphate body number of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
** = P<0.01

Lipid Inclusion Number

The interaction of pH and 0.32 mM aluminum proved to be significant ($P<0.01$) (Figure 14). Control cells at pH 5.5 had three and one-half (3.5) times more lipid inclusions than did cells exposed to the metal at the same pH. This significant reduction ($P<0.001$) is opposite the cells' response at pH 9.0 (Figure 14) where a significant increase occurred.

Cyanophycin Granule Number

The response of this inclusion is expressed through a significant interaction between pH and the treatment ($P<0.01$) (Figure 15). The change in the number of cyanophycin granules at pH 5.5 was a significant reduction ($P<0.01$).

Mini Polyphosphate Bodies, Special Lipid Bodies, Membrane-Bound Crystalline Inclusions and Mesosomes Numbers

There was no significance at any pH level or aluminum concentration among these inclusions. They appeared in few cells and at low counts.

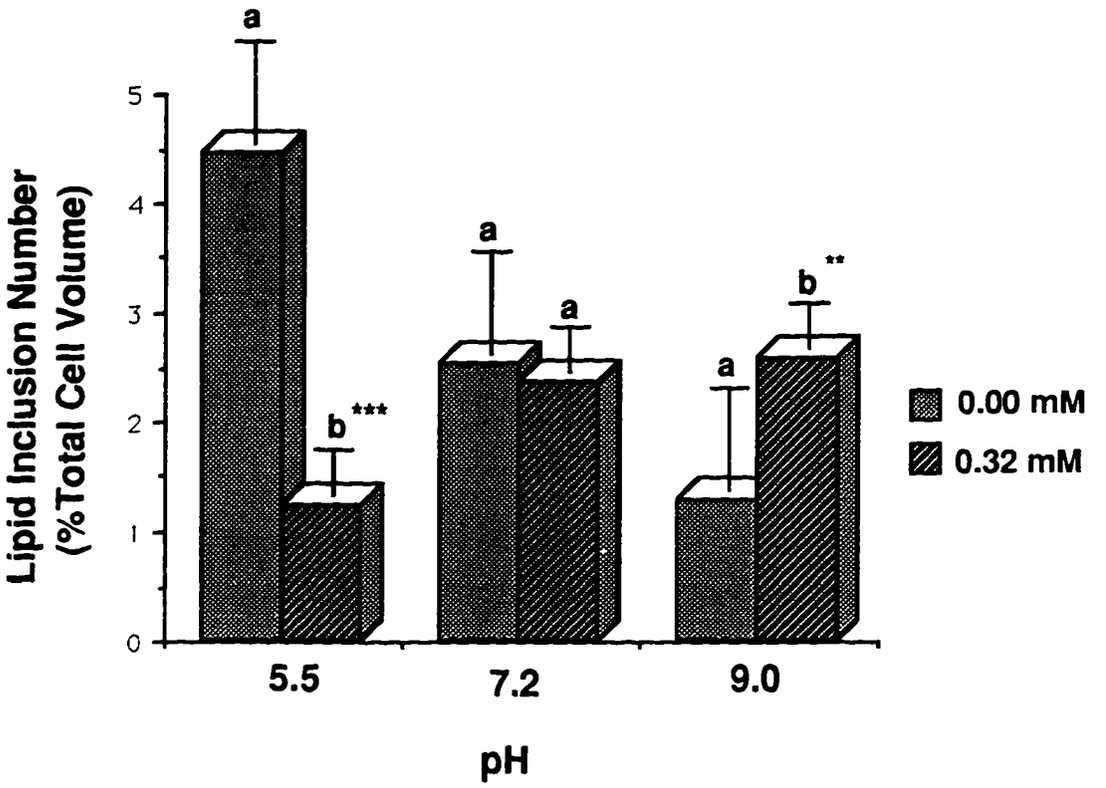

Figure 14. Effect of 0.32 mM aluminum on lipid inclusion number of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
** = P<0.01
*** = P<0.001

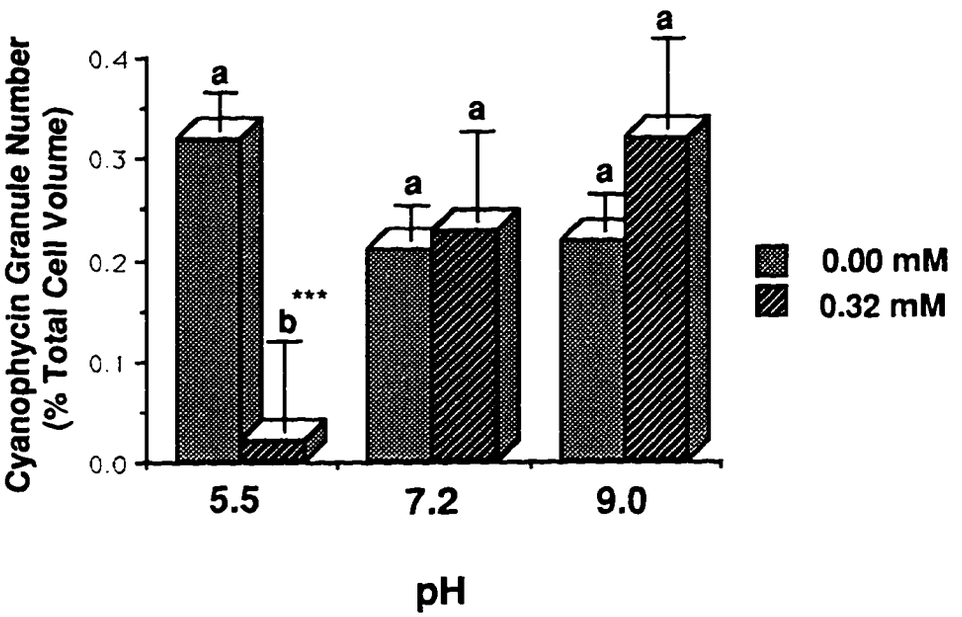

Figure 15. Effect of 0.32 mM aluminum on cyanophycin granule number of *A. doliolum* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
*** = P<0.001

Effect of pH on the Ultrastructure of *A. doliolum*

Analyis of variance (ANOVA) revealed that pH significantly influenced variation in cell volume ($P<0.05$) and in intrathylakoidal space volume ($P<0.01$).

Relative Volumes

Further analysis through the Fisher paired least significant difference (PLSD) indicated that cell volume increased significantly($P<0.05$) (Table 12) in cells treated with 0.32 mM aluminum at pH 7.2. Neither the decrease among cells treated at pH 5.5 nor the increase at pH 9.0 was as strongly influenced. Significant increase in intrathylakoidal space was recorded for cells treated at pH 5.5 (Table 13), but only slight increases were detected at pH 7.2 and pH 9.0.

Relative Counts

Significant variation as a result of pH was observed for numbersof polyphosphate bodies ($P<0.05$) (Table 14) and for lipid inclusions ($P<0.001$) (Table 15). The Fisher analysis confirmed a significant increase in the relative numbers of these two components in cells exposed to 0.32 mM aluminum at pH 5.5 and at pH 9.0.

Table 12. Effect of different pH levels on cell volume and relative volumes (Vv) of other cellular components among cells of *A. doliolum* (single classification ANOVA).

pH	Cell volume	Cell wall Vv	Polyhedral body Vv
5.5	204.17_{a}	7.39_{a}	1.93_{ab}
7.2	211.53_{ab}	7.67_{a}	1.61_{a}
9.0	237_{b}	6.92_{a}	2.42_{b}
N	30	30	30
F	3.15	1.19	1.57
P	0.047*	0.310	0.213

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

*** = $P<0.05$**

Table 13. Effect of different pH levels on thylakoidal surface area (Sv) and relative volulmes (Vv) of other cellular components among cells of *A. doliolum* (single classification ANOVA).

pH	Polyphosphate body Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
5.5	0.08_a	4.12_{ab}	1.24_a
7.2	0.36_a	2.28_a	0.84_b
9.0	0.41_a	6.19_b	1.19_{ab}
N	30	30	30
F	1.51	6.46	2.79
P	0.227	0.002**	0.067

Means followed by different letters denote statistical dissimilarity (P<0.05, using Fisher PLSD)

** = P<0.01

Table 14. Effect of different pH levels on polyphosphate body number among cells of *A. doliolum* (single classification ANOVA).

pH	Polyphosphate Body Number
5.5	0.053_a
7.2	0.285_b
9.0	0.147_{ab}
N	30
F	4.064
P	0.0205*

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P<0$

Table 15. Effect of different levels of pH on lipid inclusion number among cells of *A. doliolum* (single classification ANOVA).

pH	Lipid Inclusion Number
5.5	4.457_a
7.2	2.535_b
9.0	1.285_c
N	30
F	14.350
P	0.0001***

Means followed by different letters denote statistical dissimilarity (P<0.05, using FIsher PLSD)

*** = P<0.001

Use of Energy Dispersive X-Ray Microanalysis to Study the Uptake of Aluminum by *A. doliolum*

The spectra in Figure 16 of the cytoplasm and Figure 17 of the cell wall of untreated cells show only minor peaks for phosphorus (P), sulfur (S), and chlorine (Cl), potassium (K) and calcium (Ca) ions that are so strongly associated with polyphosphate bodies and cellular exposure to heavy metals. The modest preparation of air-dried cells does not allow for cellular detail (Figure 18); however, this pattern of elemental composition is consistent when the probe is positioned for analysis of these two cellular sectors. Analysis of polyphosphate bodies of control cells shows not only an increase in the height of the phosphorus peak (Figure 19), but also generation of small peaks for calcium and potassium. Occasionally, cells showed a minor iron (Fe) peak as well.

When cells were exposed to as little as 0.037 mM aluminum, there was only a slight change in the ionic composition of the cell margin and the cytoplasm. Among cells of the latter, the phosphorus, sulfur, and chlorine peaks increased only slightly (Figure 20), but these increases were even greater than those generated by the cell wall (Figure 21). The polyphosphate bodies of these cells, however, produced tremendous phosphorus peaks, along with peaks for magnesium (Mg), sulfur, chlorine, potassium, and calcium that were stronger than those of other cellular sectors. Figure 22 is a spectrum of a polyphosphate body exposed to this low concentration of aluminum which is only slightly detectable.

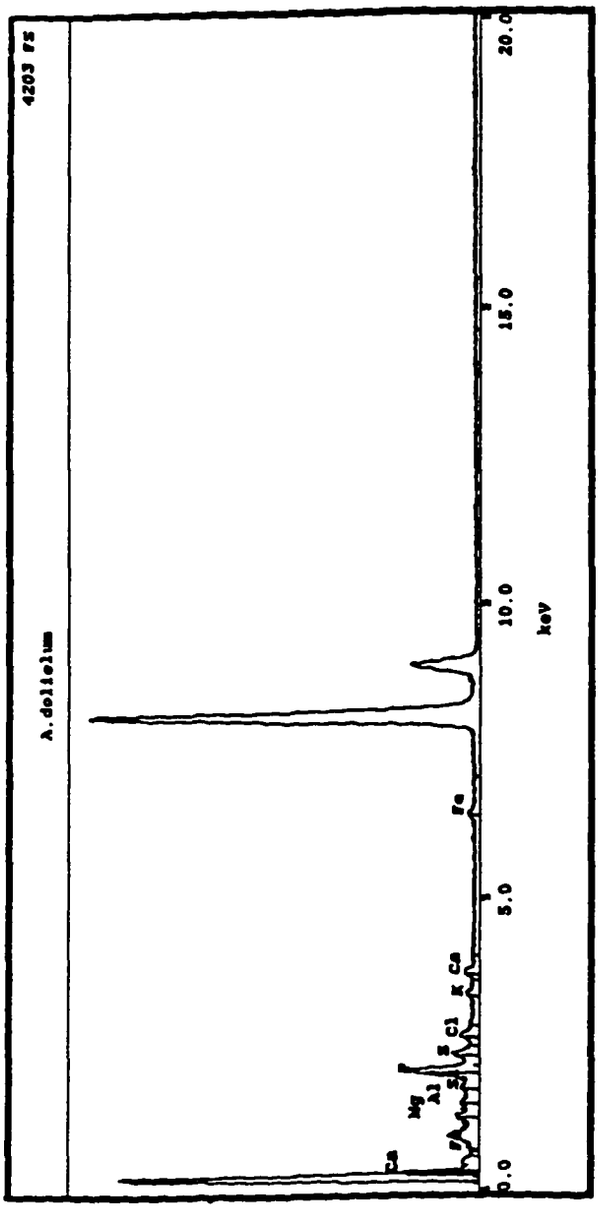

Figure 16. Spectrum of the cytoplasm of a control air-dried cell of *A. doliolum*. Identifiable peaks are Mg, P, S, and Cl.

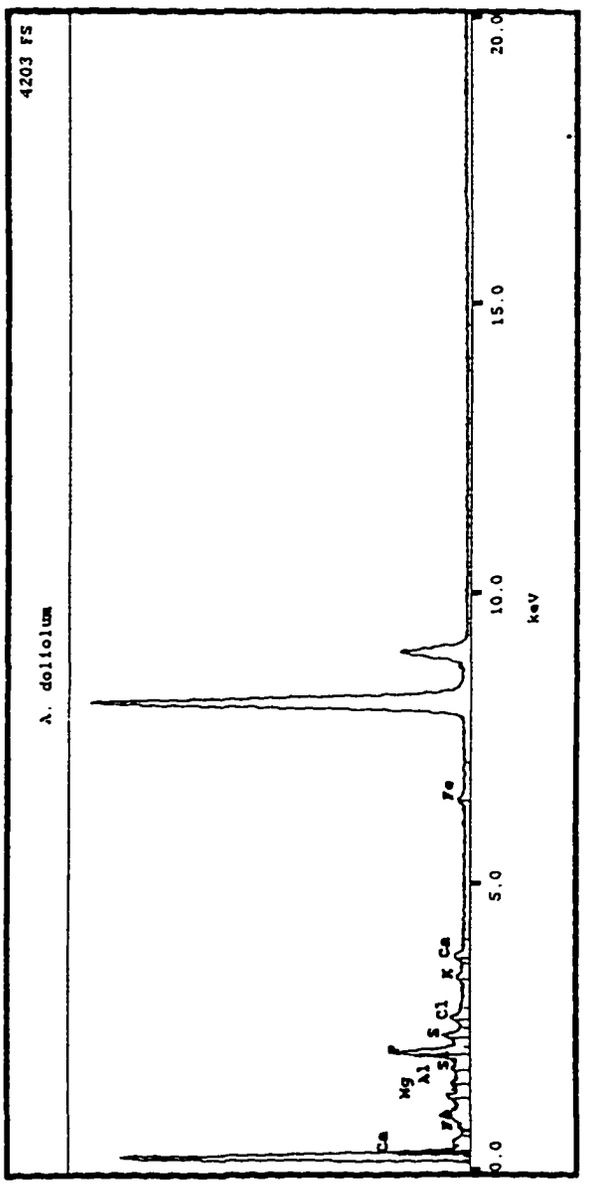

Figure 17. Spectrum of the cell wall of a control air-dried cell of *A. doliolum*. Identifiable peaks are Mg, P, S, and Cl.

Figure 18. TEM image of an air dried *A. doliolum* cell. All the dense bodies in the cell are polyphosphate bodies (arrow).

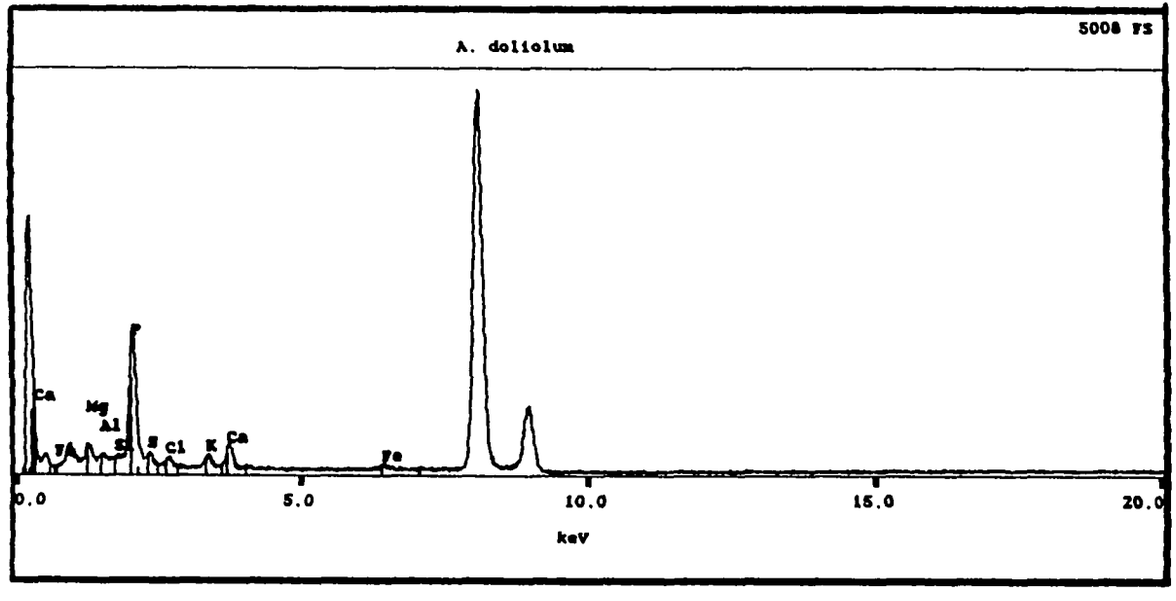

Figure 19. Spectrum of a polyphosphate body of a control air dried cell of *A. doliolum*. Note the peak for P. Other identifiable peaks are Mg, S, Cl, K, and Ca.

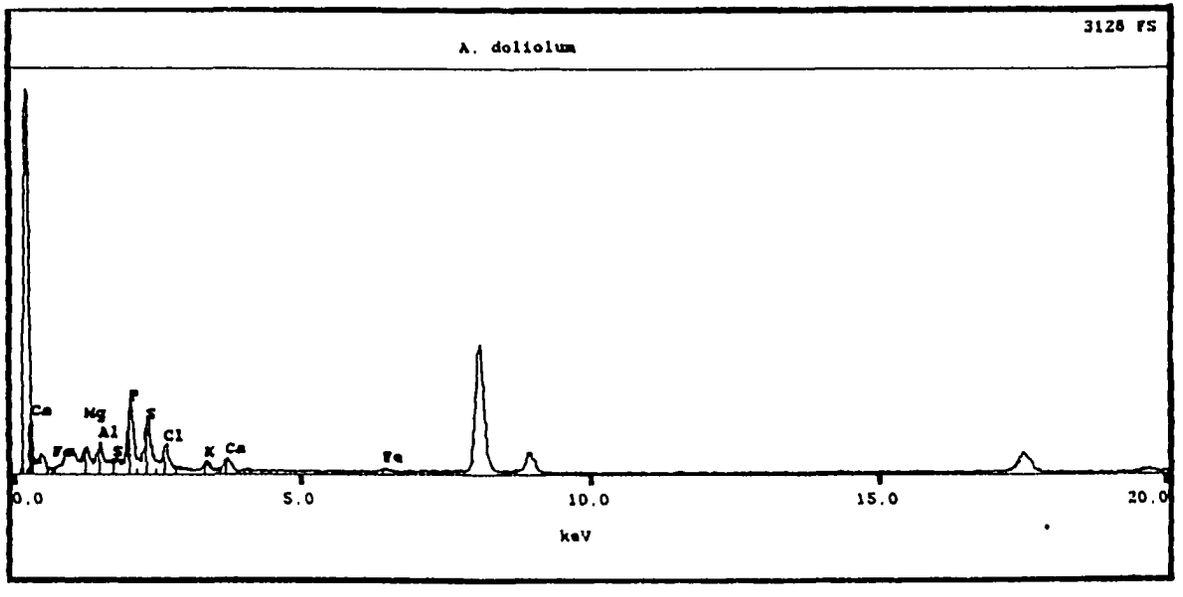

Figure 20. Spectrum of the cytoplasm of an air dried cell of *A. doliolum* exposed to 0.037 mM Al. Identifiable peaks are Mg, P, S, Cl, and Ca. Note the small peak for Al.

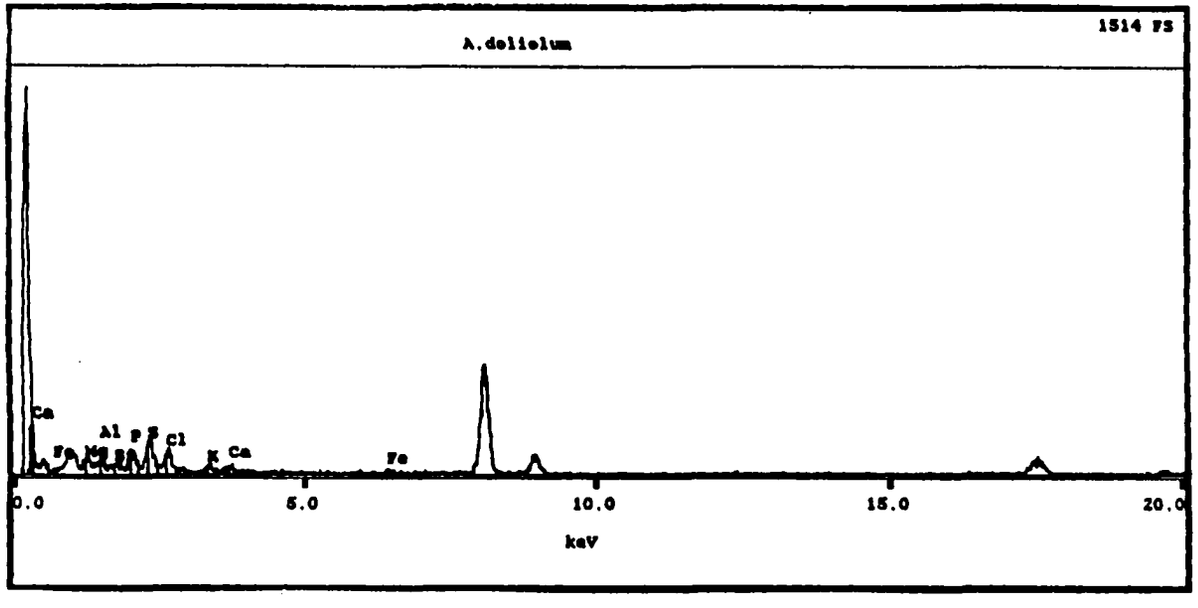

Figure 21. Spectrum of the cell wall of an air dried cell of *A. doliolum* that has been exposed to 0.037 mM Al. The peaks indicate that P, S, and Cl are barely detectable.

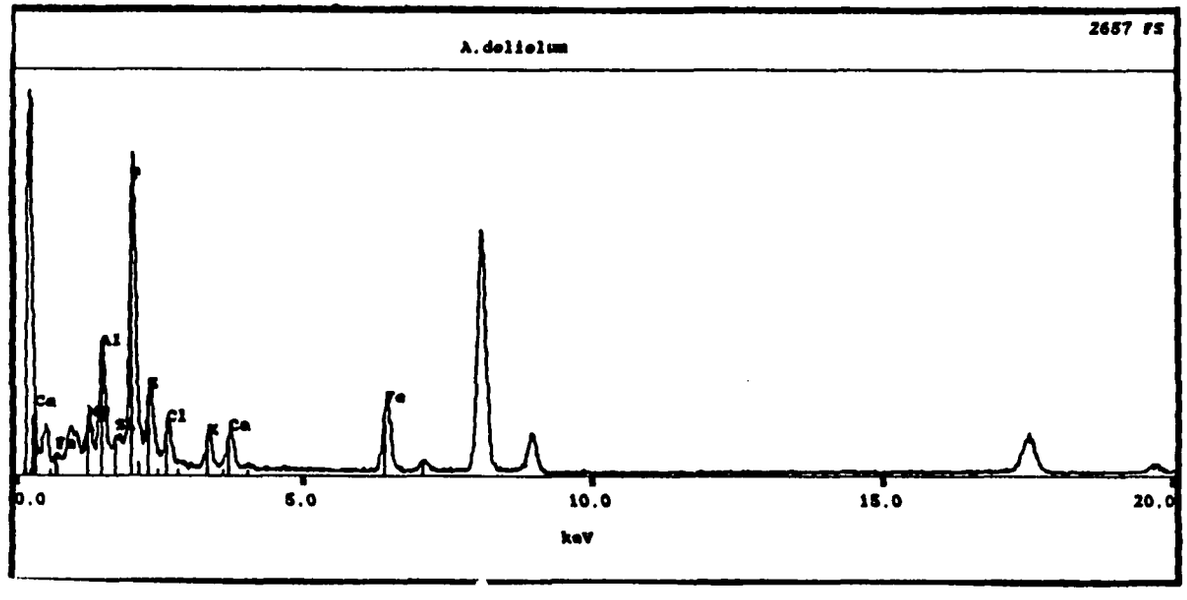

Figure 22. Spectrum of a polyphosphate body of an air dried cell of *A. doliolum* that was exposed to 0.0337 mM Al. The peak for P is prominent. Those for Mg, S, Cl, K, and Ca are identifiable. Note the peak for Al.

Exposure to 0.185 mM aluminum produced constancy in the response of cellular sectors to the metal in that the phosphorus peak of the cytoplasm and the cell wall was pronounced (moreso in the cytoplasm (Figure 23) than in the cell wall), while those of magnesium, sulfur and chlorine remained small. Response is more marked within polyphosphate bodies all of which produced a prominent phosphorus peak (Figure 24). The peaks for magnesium, sulfur, chlorine and calcium of polyphosphate bodies are consistent with those of the cytoplasm and the cell wall. In a few cases, potassium was detected, but at this concentration, no cellular area that was analyzed produced an aluminum peak.

The cytoplasm of cells exposed to the greatest concentration used in this study, 0.370 mM aluminum, generated peaks for phosphorus, sulfur, and calcium (Figure 25). Chlorine increased considerably, but magnesium did not register. The cell wall, however, showed little response. The phosphorus, sulfur and chlorine peaks are visible, but only slightly above the spectra's baselines (Figure 26). Magnesium was negligible; the potassium was lost. Polyphosphate bodies exposed to this aluminum concentration generated peaks for phosphorus and sulfur that, in some cases, approached the full scale line (Figure 27), while chlorine and calcium remained strong. Magnesium was lost. No cellular sector at this exposure showed the presence of aluminum.

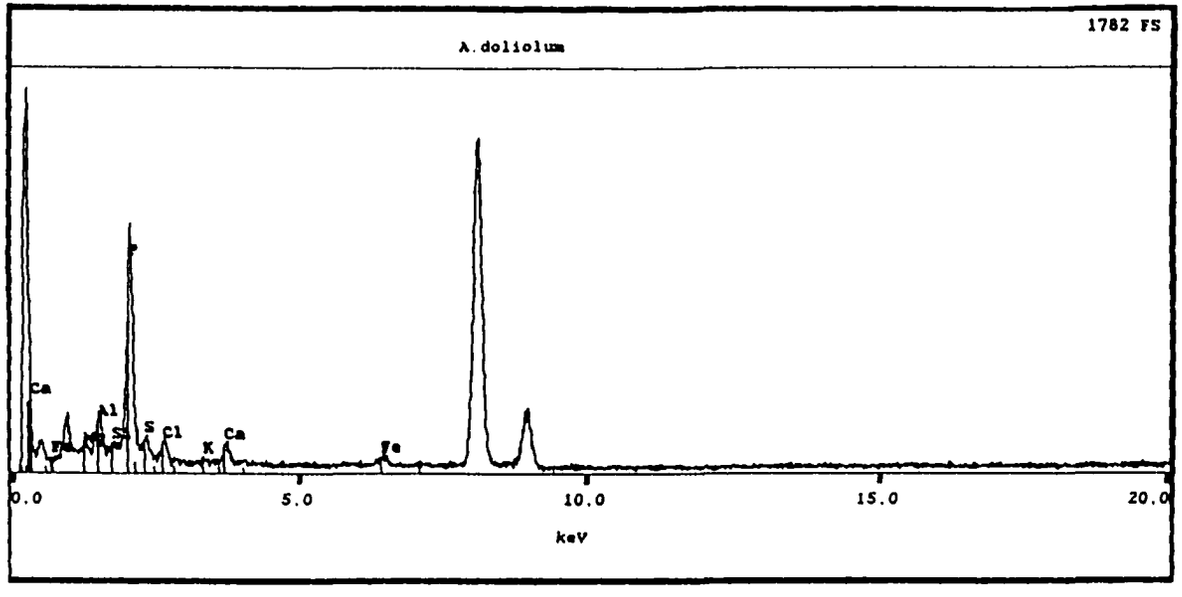

Figure 23. Spectrum of the cytoplasm of an air dried cell of *A. doliolum* exposed to 0.185 mM Al. Identifiable peaks are for Mg, P, S, and Ca. The peak for P is prominent.

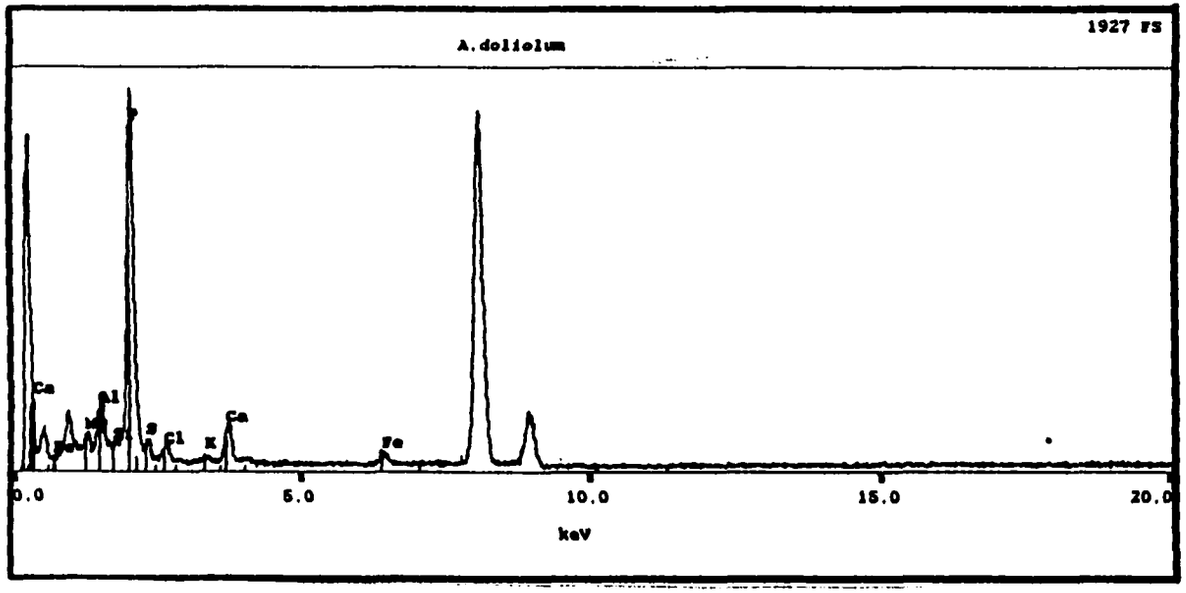

Figure 24. Spectrum of a polyphosphate body of an air dried *A. doliolum* cell exposed to 0.185 mM Al. Identifiable peaks are Mg, S, Cl, and K. Note the prominent peak for P. A peak for Al is distinguishable.

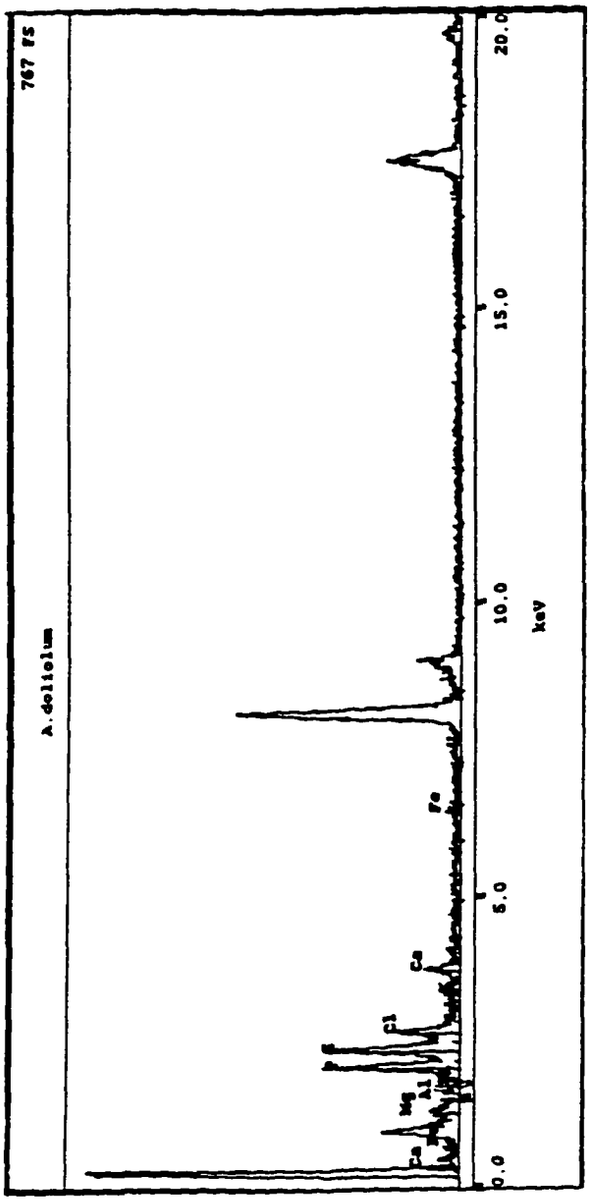

Figure 25. Spectrum of the cytoplasm of an air dried cell of *A. dolicholum* exposed to 0.370 mM Al. Identifiable peaks are for P, S, Cl, and Ca.

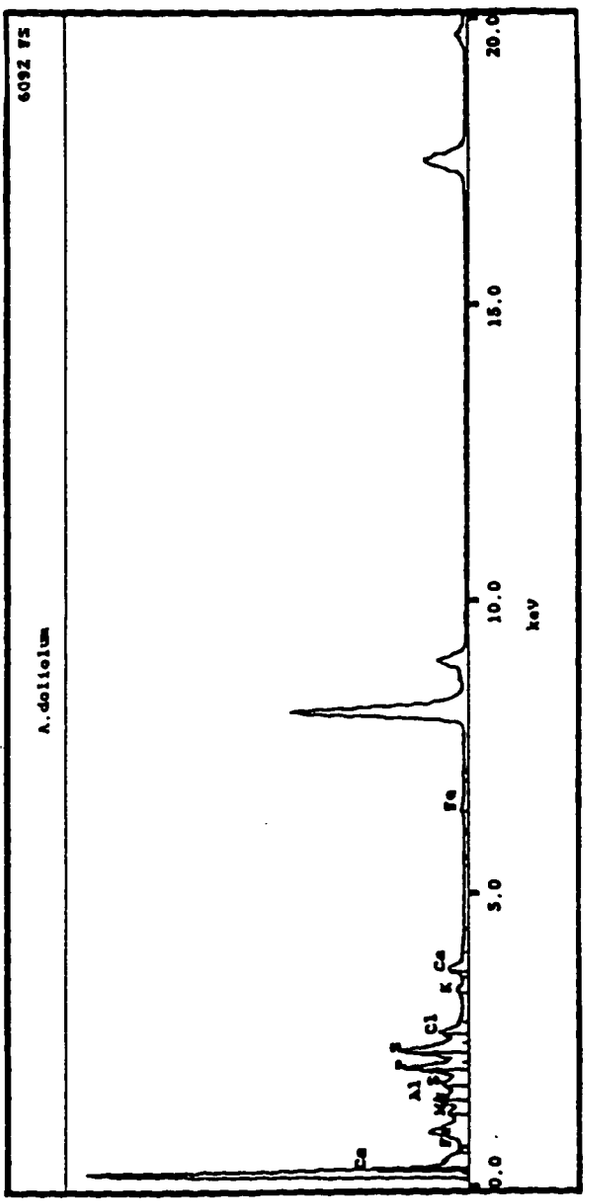

Figure 26. Spectrum of the cell wall of an air dried cell of *A. dolliolum* exposed to 0.370 mM Al. Identifiable peaks are for Mg, P, S, Cl and Ca.

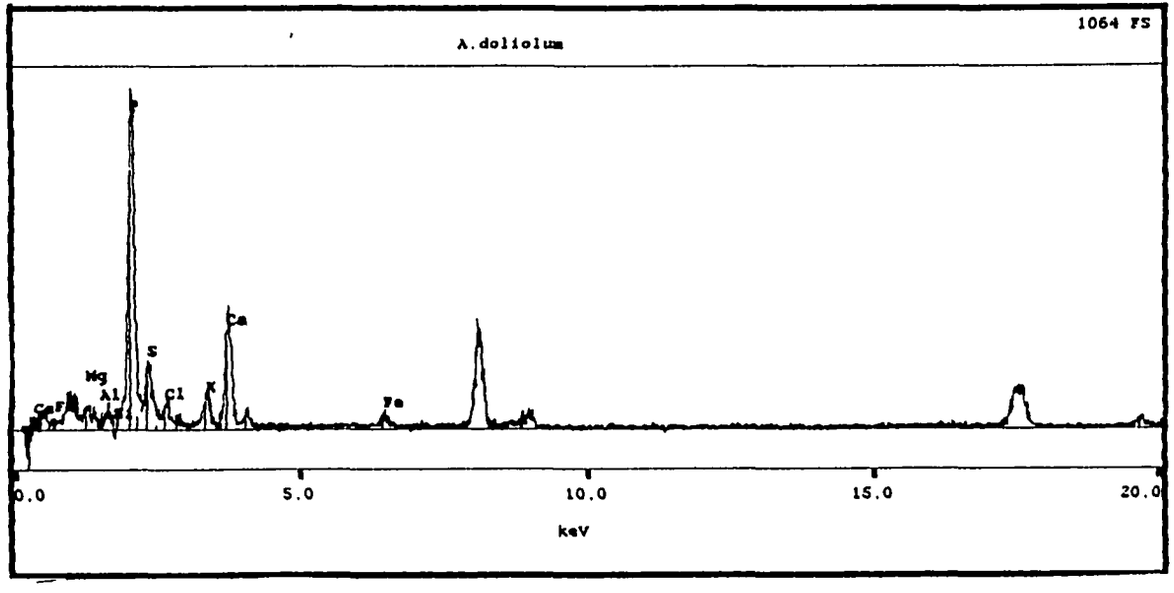

Figure 27. Spectrum of a polyphosphate body of an air dried cell of *A. doliolum* exposed to 0.370 mM Al. Note the more prominent peaks for P, S, Cl, and Ca in comparison to the control cell in Figure 19.

Phytochelatins

Phytochelatins were not detected in *A. doliolum*.

Determination of Protein Concentration of *S. leopoliensis* After Exposure

The procedure reported for *A. doliolum* was repeated. The difference in optical density of the culture for days 1 and 3 was only 0.01. From day 3 to day 7 the species grew rapidly, then accelerated its growth to the exponential level. This rate was maintained until day 25 when the cells entered a stationary phase. As with *A. doliolum* all subsequent tests were performed when the cells were undergoing exponential growth.

The optical density of the culture adjusted to 3×10^6 for protein determination was 0.050 at pH 7.5. Protein concentrations of control and treated cells after a 96-hour exposure as determined from their optical densities and protein concentrations of the standard are given in Table 16.

As the aluminum concentration increased, the protein concentrations of exposed cells decreased. The final protein concentration range was 122 μg to 112 μg, with corresponding optical density values which ranged from 0.530 to 0.480. Control cells contained 124 μg of protein with an optical density of 0.540. The estimated EC_{50} of 0.145 μM ± 0.90 (Table 17) was calculated by the method as that for *A. doliolum* reported above.

Effect of Aluminum on the Ultrastructure of *S. leopoliensis* at Different pH Levels

Relative Volumes

Cell Volume

S. leopoliensis is a bacillus shape cyanobacterium in which the four layers of the cell wall are often quite visible (Figure 28). Thylakoids are located just inside the plasma membrane and are generally separated from the cytoplasm at the core by an intrathylakoidal space (Figure 28). In many cells, nucleoplasm, an area of concentrated DNA, is visible as well as polyhedral bodies and polyphosphate bodies (Figure 29). The comparative data for the effect of pH and aluminum on the ultrastructure of *S. leopoliensis* are presented in Table 18 through Table 26.

Interaction of pH and aluminum on total cell volume did not prove to be significant through two-factor ANOVA (Figure 30), despite the strong influence of pH (r^2=0.06). At pH 5.5, treated cells showed only a 3.77% decrease from control cells, a change which showed no significance. The increase among cells treated with aluminum at pH 9.0 likewise was not significant. At pH 7.2, however, cells treated with the metal showed a significant decrease ($P<0.05$) (Table 23).

Table 16. The protein concentrations of *S. leopoliensis* with corresponding optical densities of combined triplicates after the 96-hour exposure.

Al Concentration (mM)	Protein Concentration (μg)	O. D. $_{650}$
0.000	124	0.54
0.037	122	0.53
0.067	120	0.52
0.120	112	0.48
0.230	-----	0.03
0.370	-----	0.02

Table 17. The EC_{50} value of aluminum calculated from the percent response of *S. leopoliensis* after 96-hour exposure, with respective regression equation (Data based on triplicate runs).

Conc. (mM)	Log Conc.	Protein Conc. (μg)	O. $D._{650}$	%Control Growth	Empirical Probit
0.000	----------	124	0.54	100	---------
0.037	-1.4318	122	0.53	98.1	7.0749
0.067	-1.1739	120	0.52	96.3	6.7866
0.120	-0.9208	112	0.48	88.9	6.2212
0.230	-0.6383	-----	0.03	5.6	3.4107

$Y = -4.44X + 1.25$; $r^2 = 0.808$; log $EC_{50} = 0.844594594$; $EC_{50} = 0.145$ mM ± 0.90

Figure 28. Micrograph of *S. leopoliensis* at pH 7.2 showing detail of cell wall layers #1, #2, #3, and #4. X 51,428

Cell Wall Volume

The interaction of pH and 0.15 mM aluminum on cell wall volume proved to be highly significant ($P<0.001$) (Figure 31), moreso than with any other cellular component. Neither the 18.56% increase among treated cells at pH 7.2 nor that of 13.59% at pH 9.0 was significant. The variation among cells treated with the metal at pH 5.5, however, proved to be a significant decrease ($P<0.001$) (Figure 31). This change in cell wall thickness from an average of 16.60 nm in control cells to one of 11.46 nm among treated cells is marked. The cell in Figure 32 has a thick cell wall.

Cell Wall Layer #1

This cellular sector was not strongly influenced by the interaction of pH and the metal (Figure 33). At no pH level was there significant variation (Figure 33), although among the treatments pH proved to be significant ($P<0.001$) ($r^2=0.12$).

Polyhedral Body Volume

As with layer #1 of the cell wall, there was no significant interaction of pH and aluminum (Figure 34).

Polyphosphate Body Volume

There was significant interaction of pH and 0.15 mM aluminum

(P<0.01) (Figure 35). The response of cells at pH 7.2 (Table 21) involved only a slight increase among cells exposed to the metal. At pH 9.0, even a 345.83% increase among treated cells did not prove significant. However, this volume among cells treated with the metal at pH 5.5 decreased significantly (P<0.01) (Table 19).

Nucleoplasm

Here the interaction of the metal and pH was not significant. Instead, pH singly exerted a strong influence (r^2=0.14). The decrease among cells exposed at pH 5.5 (23.17%) paralleled the increase among treated cells at pH 7.2 (34. 68%) (Figure 36). The response of cells treated to the metal at pH 9.0 proved significant (P<0.05) with an increase of 67.6%.

Intrathylakoidal Space Volume

This is the only cellular sector in which not only was the interaction of the metal and pH significant (P<0.01), but pH also proved significant individually (P<0.001) as did 0.15 mM aluminum (P<0.01). At pH 5.5 the interaction resulted in only a slight decrease among treated cells over the control cells, and at pH 9.0 treated cells responded by slightly increasing this volume (Figure 37). Treated cells at pH 7.2 showed a significant increase (P<0.05) (Table 24).

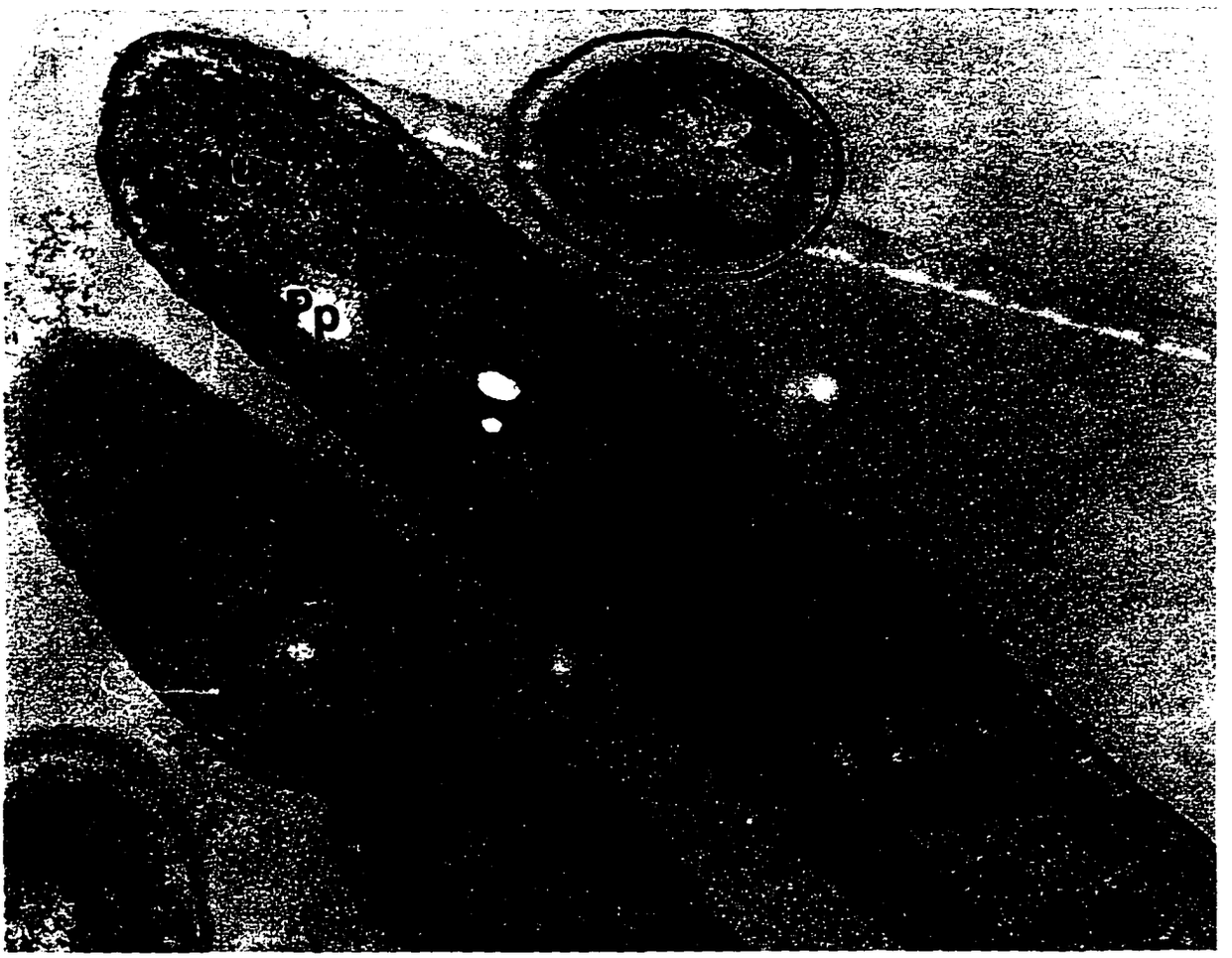

Figure 29. Micrograph of a cell exposed to 0.15 mM aluminum at pH 5.5 Nucleoplasm (Nu), polyhedral Bodies (Pb). and polyphosphate bodies (Pp) are visible. X 51,428

Table 18. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 5.5.
Values reported are means (standard error in parentheses) of a sample size of 30 sections of cells.

	N	$[Al^{3+}]$mM	0.00	0.15
Cell volume	30		58.30 (5.10)	56.10 (4.50)
Cell wall Vv	30		16.60 (0.90)	11.50 (0.66)
Cell wall Layer #1 Vv	30		5.59 (1.40)	7.13 (1.73)
Polyhedral bodies Vv	30		1.52 (0.42)	2.33 (0.42)

Table 19. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 5.5. Values reported are means (standard error in parenthesess) of a sample size of 30 sections of cells.

	N	$[Al^{3+}]$ mM	0.00	0.15
Polyphosphate bodies Vv	30		1.18 (0.33)	0.14 (0.08)
Nucleoplasm Vv	30		2.46 (0.72)	1.89 (0.75)
Intrathylakoidal space Vv	30		0.17	0.10
Thylakoidal surface area Sv	30		1.50 (0.35)	4.05 (0.62)

Table 20. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 7.2. Values reported are means (standard error in parentheses) of a sample size of 30 sections of cells.

	N	$[Al^{3+}]$ mM	0.00	0.15
Cell volume	30		51.77	40.00
			(4.65)	(3.53)
Cell wall Vv	30		10.83	12.84
			(0.69)	(0.78)
Cell wall	30		0.89	2.17
Layer #1 Vv			(0.55)	(0.85)

Table 21. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 7.2. Values reported are means (standard error in parenthesis) of a sample size of 30 sections of cells.

	N	$[Al^{3+}]$ mM	0.00	0.15
Polyhedral bodies Vv	30		2.77 0.47)	3.25 (0.54)
Polyphosphate bodies Vv	30		0.18 (0.13)	0.27 (0.19)
Nucleoplasm Vv	30		1.74 (0.52)	2.33 (0.64)
Intrathylakoidal space Vv	30		1.55 (0.43)	3.86 (0.78)
Thylakoidal surface area Sv	30		1.88 (0.54)	2.03 (0.26)

Table 22. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 9.0. Values reported are means (standard error is in parentheses) of a sample size of 30 sections of cells.

	N	$[Al^{3+}]$ mM	0.00	0.15
Cell volume	30		42.03 (3.30)	47.10 (3.93)
Cell wall Vv	30		12.58 (0.94)	14.29 (0.80)
Cell wall Layer #1 Vv	30		2.38 (0.93)	1.05 (0.74)
Polyhedral bodies Vv	30		2.79 (0.72)	2.01 (0.37)

Table 23. Summary of morphometric data for *S. leopoliensis* cells exposed to selected concentrations of aluminum at pH 9.0. Values reported are means (standard error in parenthesis) of a sample size of 30 sections of cells.

	N	$[Al^{3+}[$	0.00	0.15
Polyphosphate bodies Vv	30		0.17 (0.14)	0.75 (0.32)
Nucleoplasm Vv	30		4.33 (0.95)	7.26 (0.95)
Intrathylakoidal space Vv	30		0.36 (0.19)	0.88 (0.30)
Thylakoidal surface area Sv	30		4.16 (0.61)	1.07 (0.30)

Table 24. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 5.5, by single classification ANOVA.

$[Al^{3+}]$ mM	Cell volume	Cell wall Vv	Cell Wall layer #1 Vv	Polyhedral body Vv
0.00	58.30_a	16.60_a	5.59_a	1.52_a
0.15	56.10_a	11.46_b	7.13_a	2.33_a
N	60	60	60	60
F	0.1050	21.13	0.475	1.8490
P	0.7469	0.0001***	0.4932	0.1792

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

***** = $P < 0.001$**

Table 25. Comparison of relative volumes (Vv) of polyphosphate bodies and other cellular components and thylakoidal surface area (Sv) among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 5.5, by single classification ANOVA.

$[Al^{3+}]$ mM	Polyphosphate body Vv	Nucleoplasm Vv	Intrathylakoidal space VV	Thylakoidal surface area Sv
0.00	1.18_a	2.46_a	0.17_a	1.50_a
0.15	0.14_b	1.89_a	0.10_a	4.05_a
N	60	60	60	60
F	9.36	0.294	0.1810	12.895
P	0.0034**	0.5895	0.6718	0.0007***

Means followed by different letters denote statistical dissimilarity (P<0.05, using Fisher PLSD)

** = P < 0.01

*** = P < 0.001

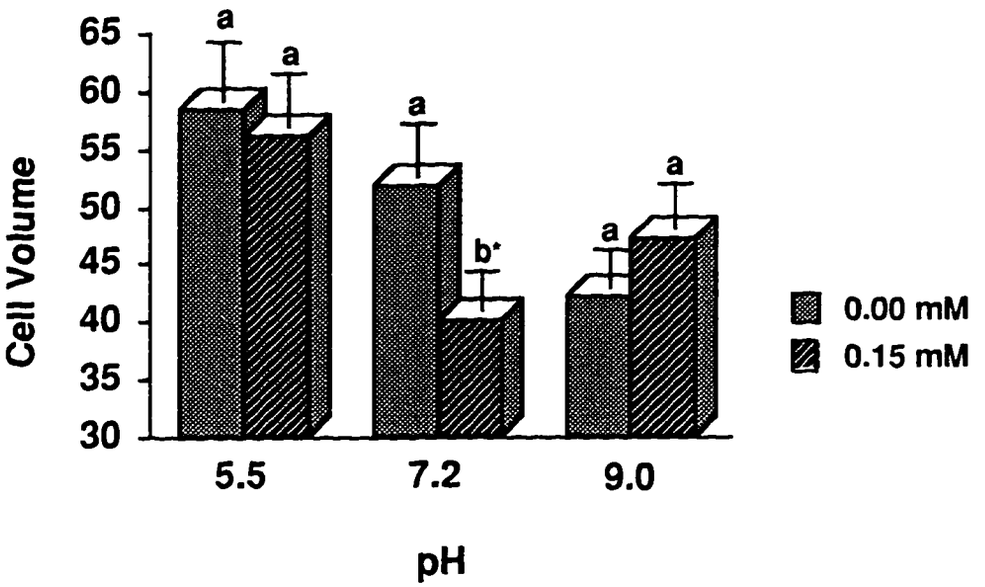

Figure 30. Effect of 0.15 mM aluminum on cell volume Vv of *S. leopoliensis* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

*** = P < 0.05**

Table 26. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 7.2, by single classification ANOVA.

$[Al^{3+}]$ mM	Cell volume	Cell wall Vv	Cell wall layer #1 Vv	Polyhedral body Vv
0.00	51.77_a	10.83_a	0.89_a	2.77_a
0.15	40.00_b	12.84_a	2.17_a	3.25_a
N	60	60	60	60
F	4.063	3.716	1.589	0.455
P	0.0485*	0.0588	0.2125	0.5025

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P < 0.05$

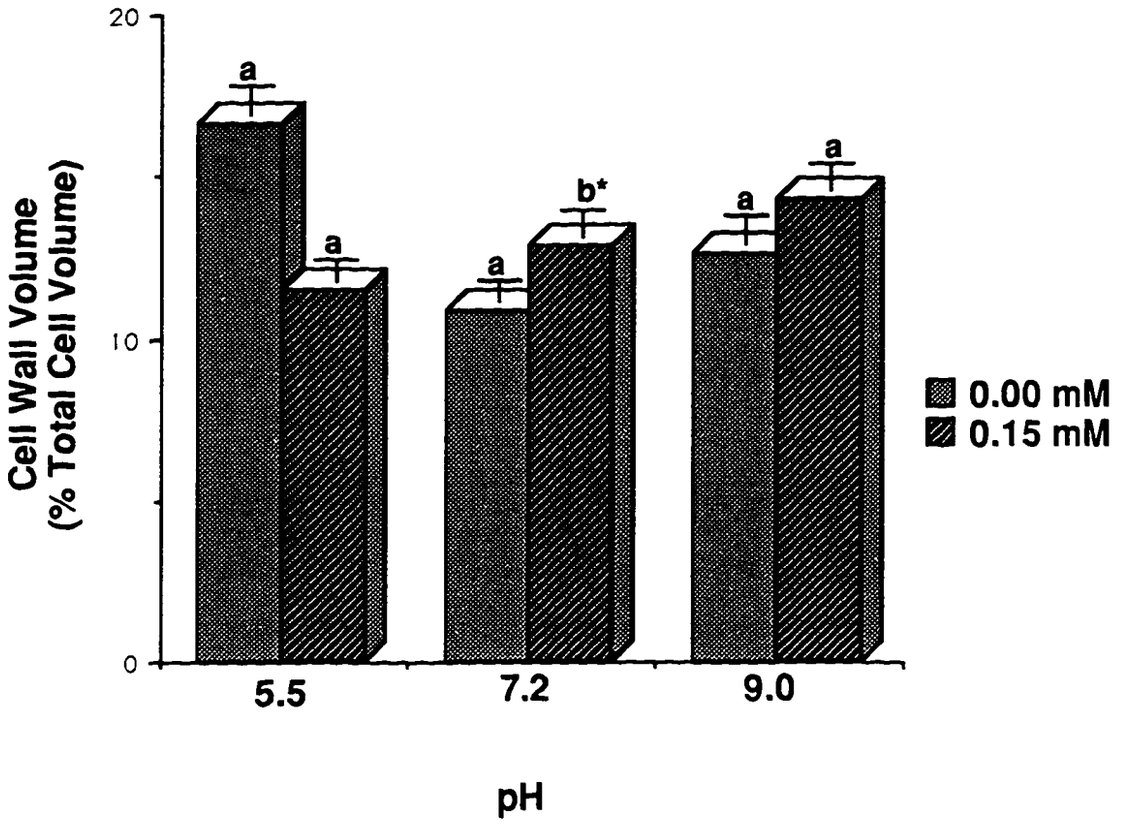

Figure 31. Effect of 0.15 mM aluminum on cell wall volume Vv of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
*** = P<0.001

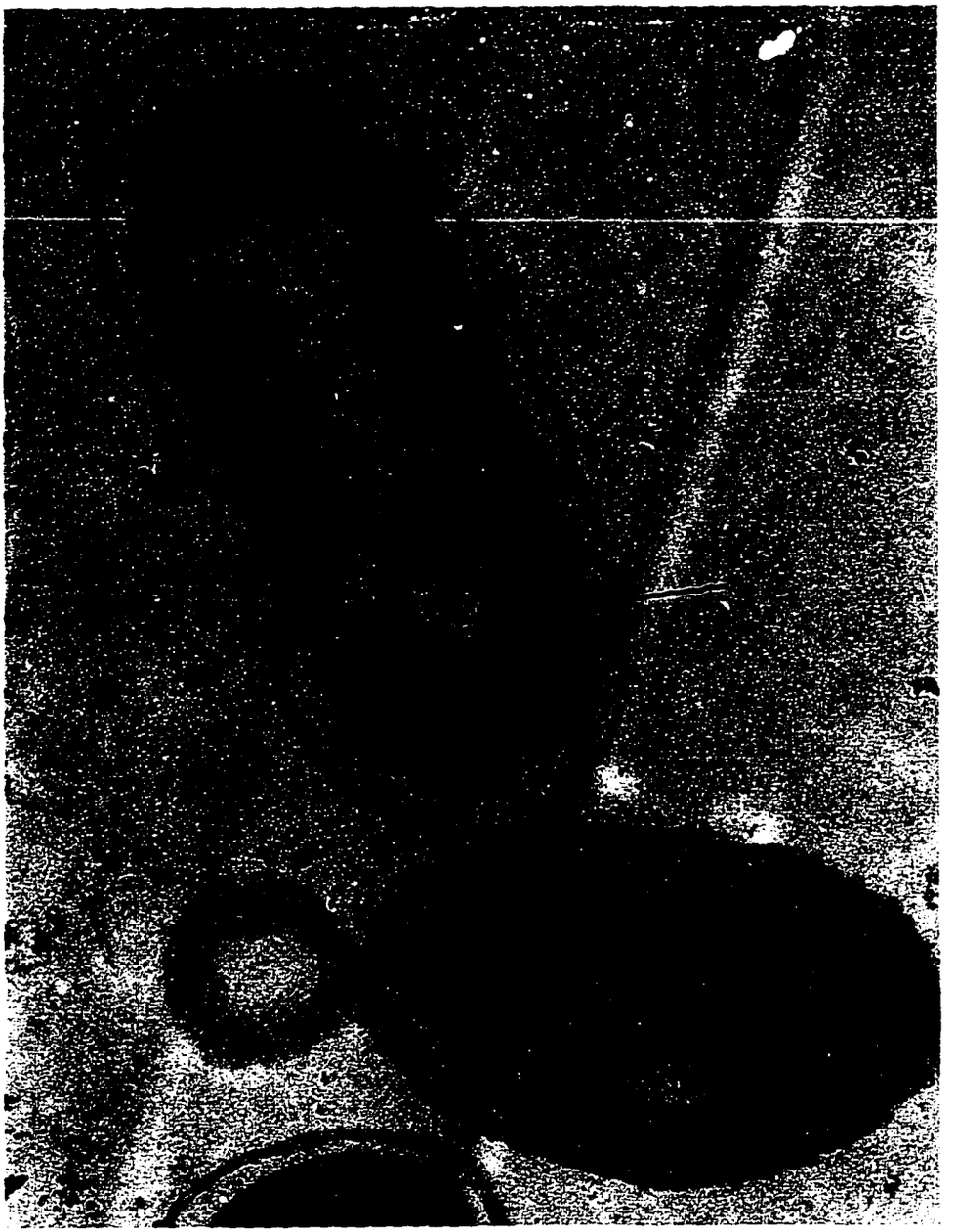

Figure 32. Micrograph of *S. leopoliensis* exposed to 0.15 mM Al at pH 5.5. Note the thickness of cell wall layer #1.

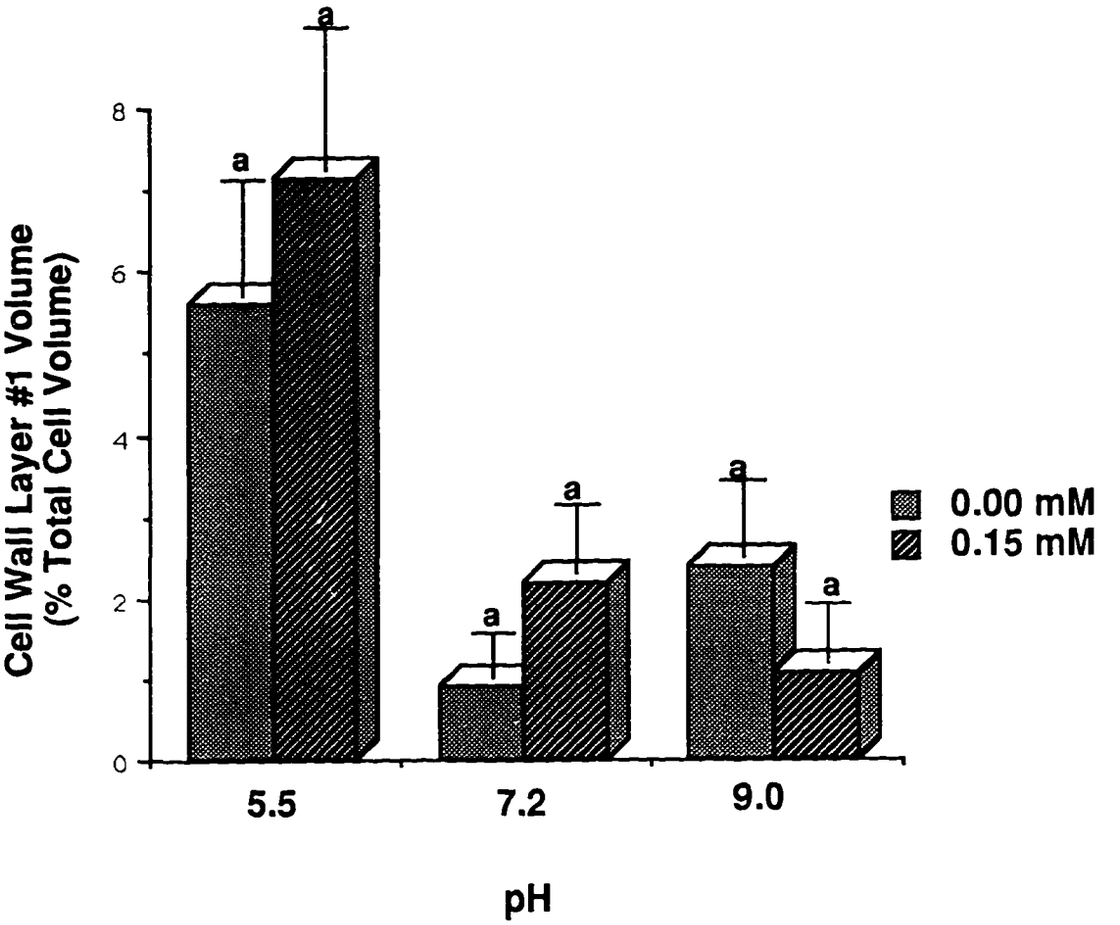

Figure 33. Effect of 0.15 mM aluminum on cell wall layer #1 volume Vv of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical disimilarity P<0.05).

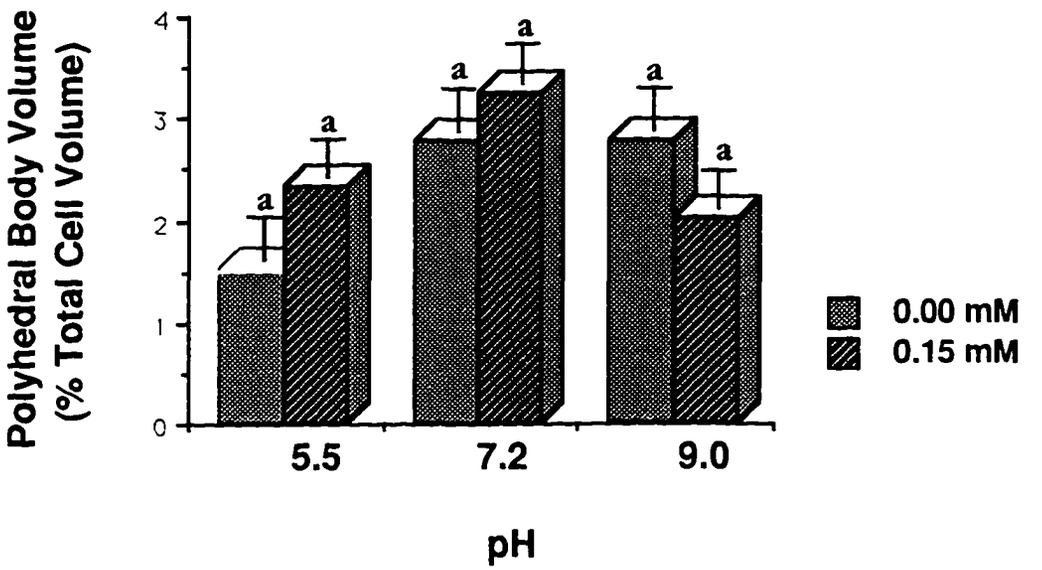

Figure 34. Effect of 0.15 mM aluminum on polyhedral body volume Vv of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

Thylakoidal Surface Area

The interaction of pH and 0.15 mM aluminum was highly significant ($P<0.001$). At pH 7.2 a usual pattern is repeated in that the variation between control cells and those exposed to the metal was not significant. At the extreme pH levels, however, metal treatment induced notable variation. At pH 5.5, the increase in surface area among treated cells (170%) proved to be highly significant ($P<0.001$) (Figure 38). The inverse was observed among treated cells at pH 9.0 where the decrease (74.37%) was also significant ($P<0.001$)(Table 25).

Relative Counts

Polyhedral Body Number

The response of polyhedral body number to pH and 0.15 mM aluminum indicates significant interaction ($P< 0.05$) (Figure 39). The decrease among cells exposed to the metal at pH 9.0 was not significant, nor was there significant difference among untreated cells at the various pH levels. However, the percentage of cell volume taken up by these inclusions increased significantly at both pH 5.5 ($P<0.01$) and pH 7.2 ($P<0.05$). The significance levels produced through two-factor ANOVA indicate nearly equal influence of pH ($r^2=0.02$) and the aluminum ($r^2=0.03$), and their interaction ($r^2=0.04$).

Polyphosphate Body Number

The response of these inclusions to pH and 0.15 mM aluminum was not detected in any other inclusion. The interaction proved to be less than significant. This failure to affect a significant change is also observed with each variable independently, despite a 151.19% increase among cells treated with the metal at pH 9.0 (Figure 40).

Mini Polyphosphate Bodies

The data for these inclusions were incomplete. No values were recorded at pH 7.2.

Effect of pH on the Ultrastructure of *S. leopoliensis*

Analysis of variance (ANOVA) revealed that pH significantly influenced the variation in cell volume ($P<0.05$), in cell wall volume ($P<0.001$), volume of layer #1 of the cell wall ($P<0.01$), polyphosphate body volume ($P<0.01$), volume of nucleoplasm ($P<0.05$), intrathylakoidal space volume ($P<0.01$), and thylakoidal surface area ($P<0.001$).

Relative Volumes

Further analysis through the Fisher paired least significant

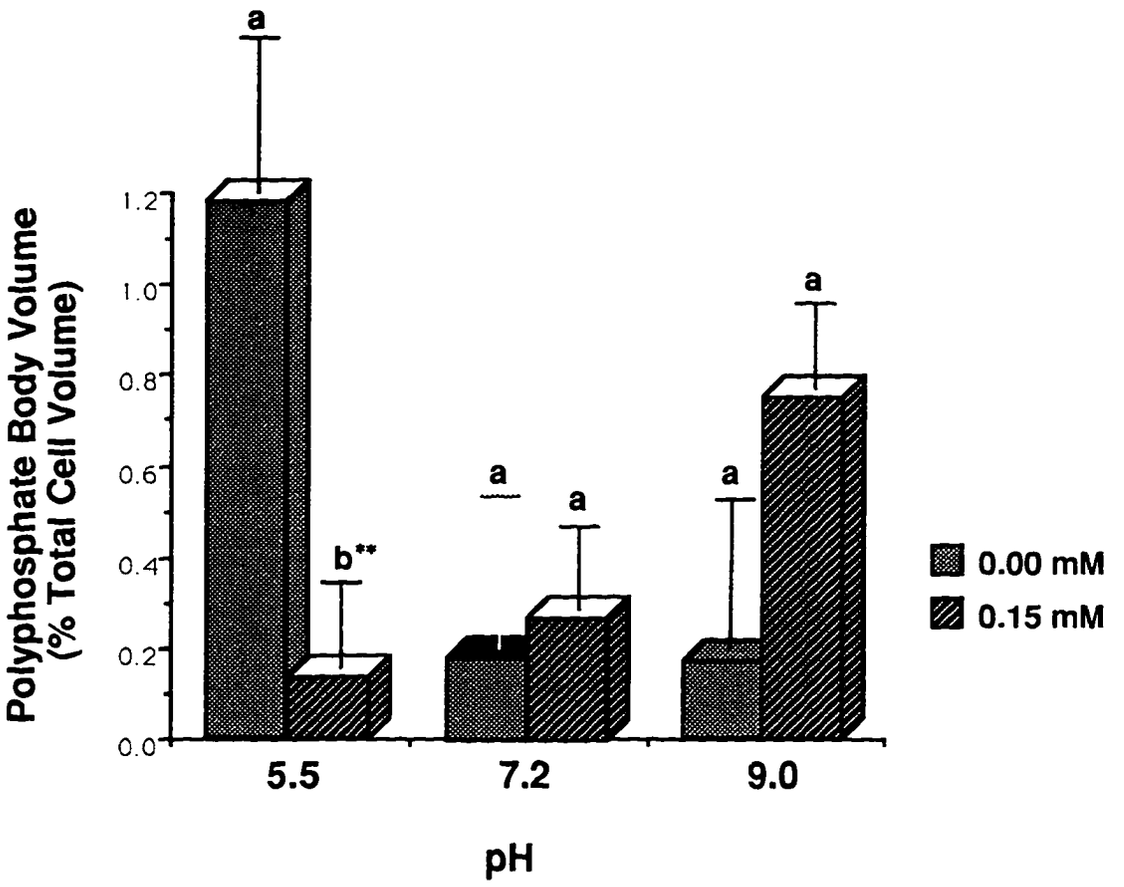

Figure 35. Effect of 0.15 mM aluminum on polyphosphate body volume Vv of *S. leopoliensis* at different pH levels (error bar = SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)
** = P < 0.01

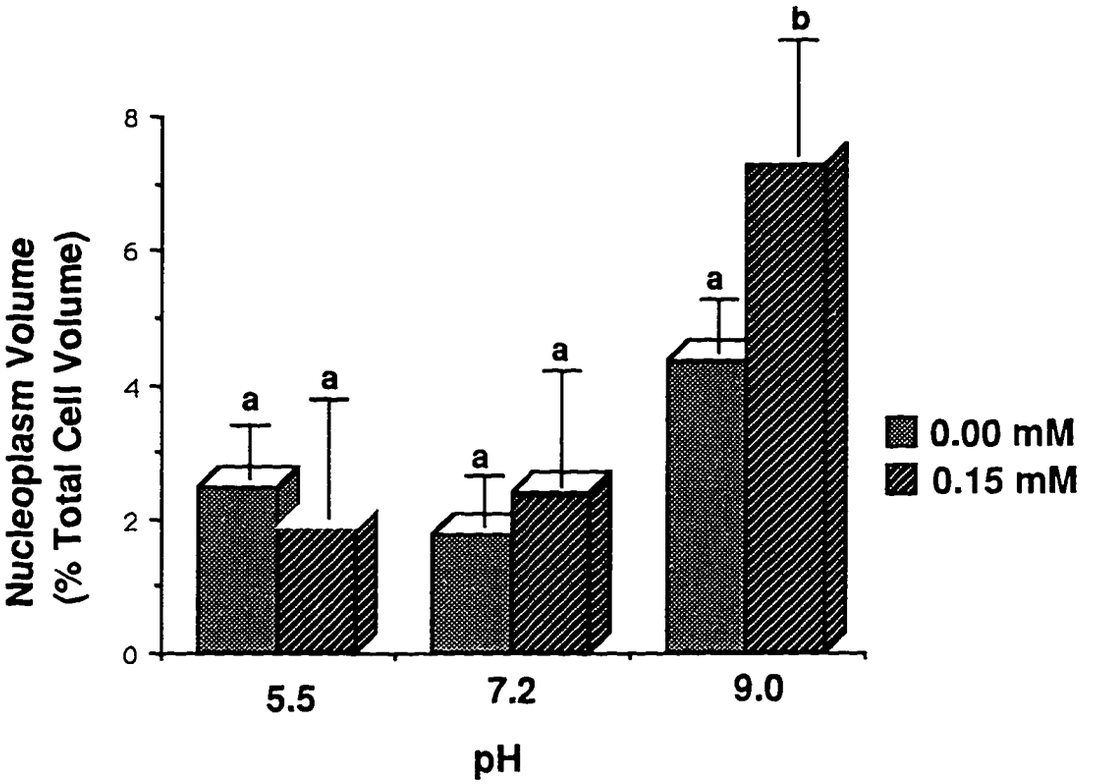

Figure 36. Effect of 0.15 mM aluminum on nucleoplasm volume Vv of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity $p<0.05$)

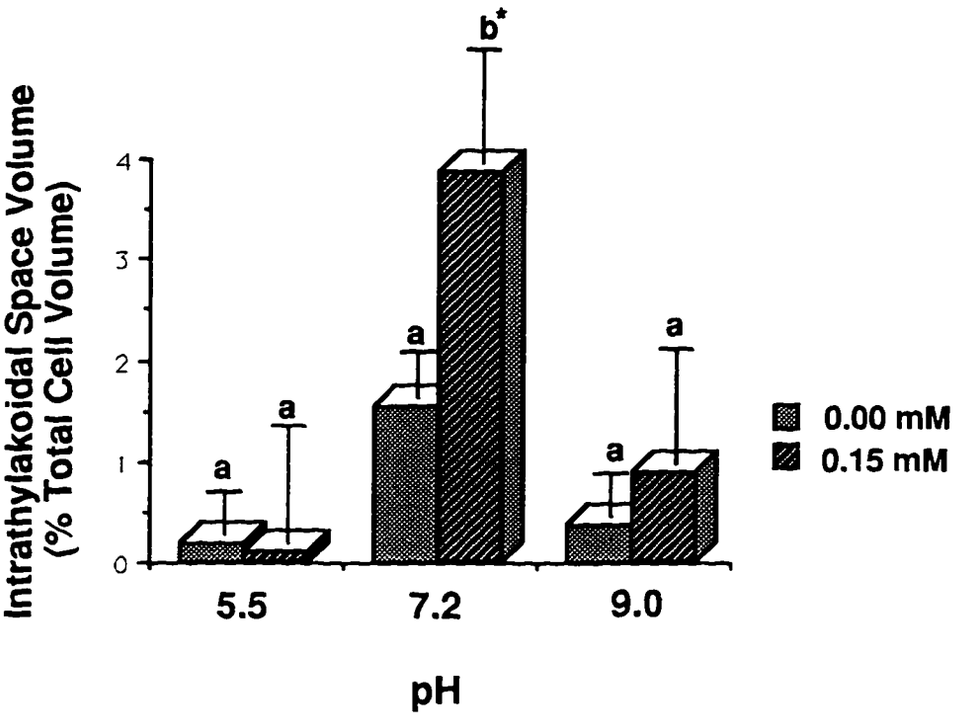

Figure 37. Effect of 0.15 mM aluminum on intrathylakoidal space volume Vv of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH statistical dissimilarity P<0.05)
*** = P<0.05**

Table 27. Comparison of relative volumes (Vv) of polyphosphate bodies and other cellular components and thylakoidal surface area (Sv) among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 7.2, by single classification ANOVA.

$[Al^{3+}]$ mM	Polyphosphate body Vv	Nucleoplasm Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
0.00	0.18_a	1.73_a	1.55_a	1.88_a
0.15	0.27_a	2.33_a	3.86_b	2.03_a
N	60	60	60	60
F	0.159	0.515	6.737	0.064
P	0.6914	0.476	0.0119*	0.8018

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P < 0.05$

Table 28. Comparison of cell volume and relative volumes (Vv) of other cellular components among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 9.0, by single classification ANOVA.

Al^{3+} mM	Cell volume	Cell wall Vv	Cell wall layer #1 Vv	Polyhedral body Vv
0.00	42.03_a	12.58_a	2.38_a	2.78_a
0.15	47.10_a	14.29_a	1.05_a	2.01_a
N	60	60	60	60
F	0.9750	1.9270	1.2610	0.9110
P	0.3274	0.1704	0.2660	0.3437

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

Table 29. Comparison of relative volumes (Vv) of polyphosphate bodies and other cellular components and thylakoidal surface area (Sv) among cells of *S. leopoliensis* exposed to selected aluminum concentrations at pH 9.0, by single classification ANOVA.

Al^{3+} mM	Polyphosphate body Vv	Nucleoplasm Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
0.00	0.17_a	4.33_a	0.36_a	4.16_a
0.15	0.75_a	7.26_b	0.88_a	1.07_b
N	60	60	60	60
F	2.7670	4.728	2.2830	20.8410
P	0.1016	0.0338*	0.1363	0.0001***

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

** = $P < 0.05$

*** = $P < 0.001$

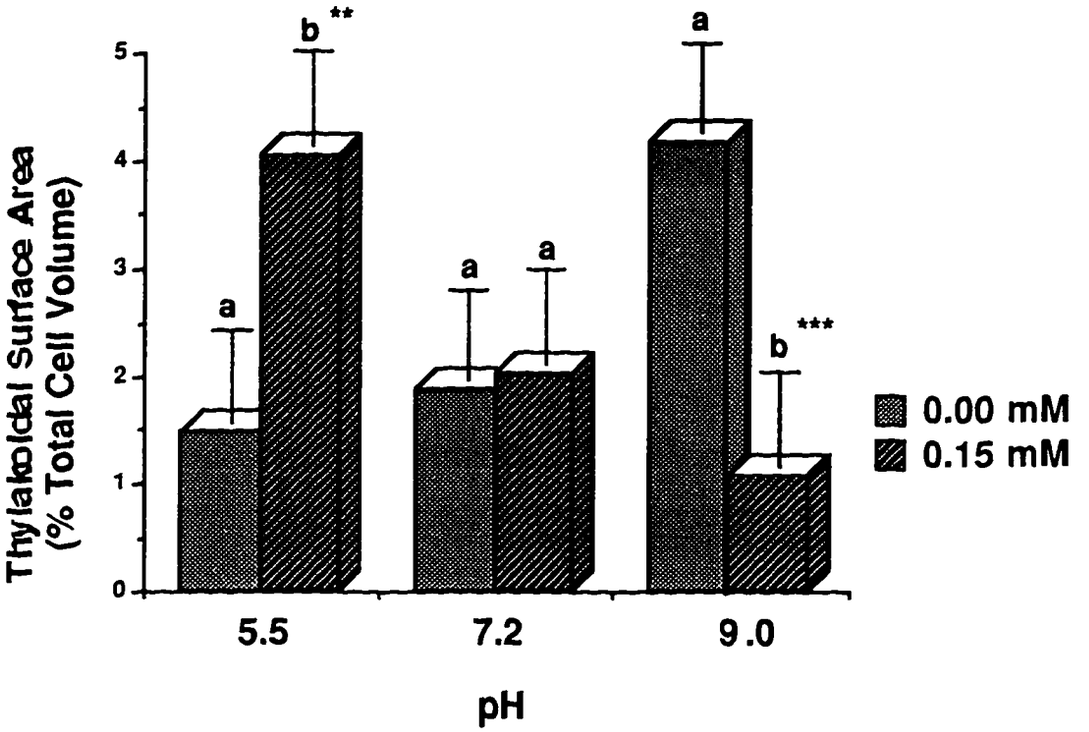

Figure 38. Effect of 0.15 mM aluminum on thylakoidal surface area SV of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

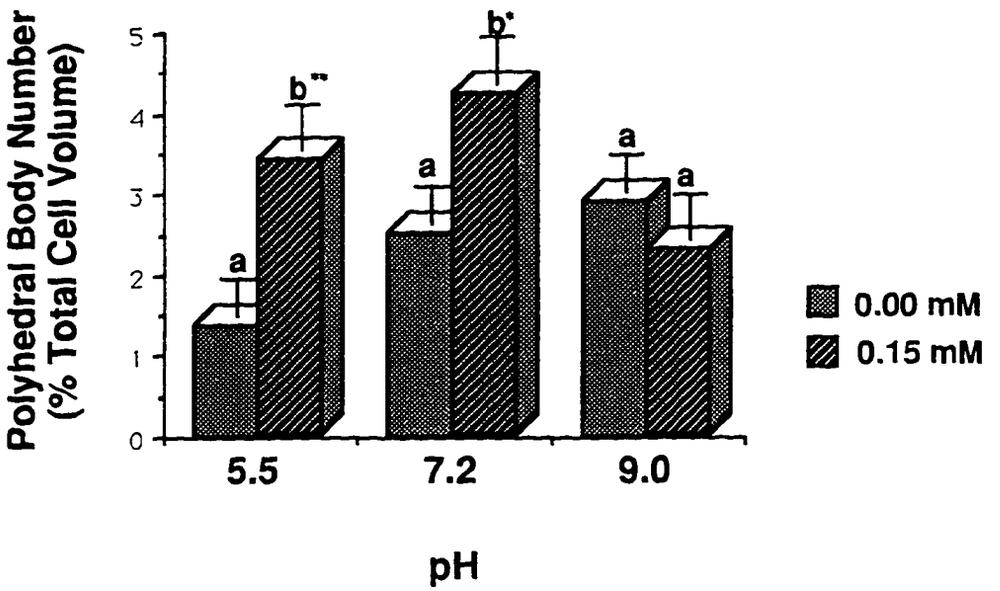

Figure 39. Effect of 0.15 mM aluminum on polyhedral body number of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

*** = P<0.05**

**** = P<0.01**

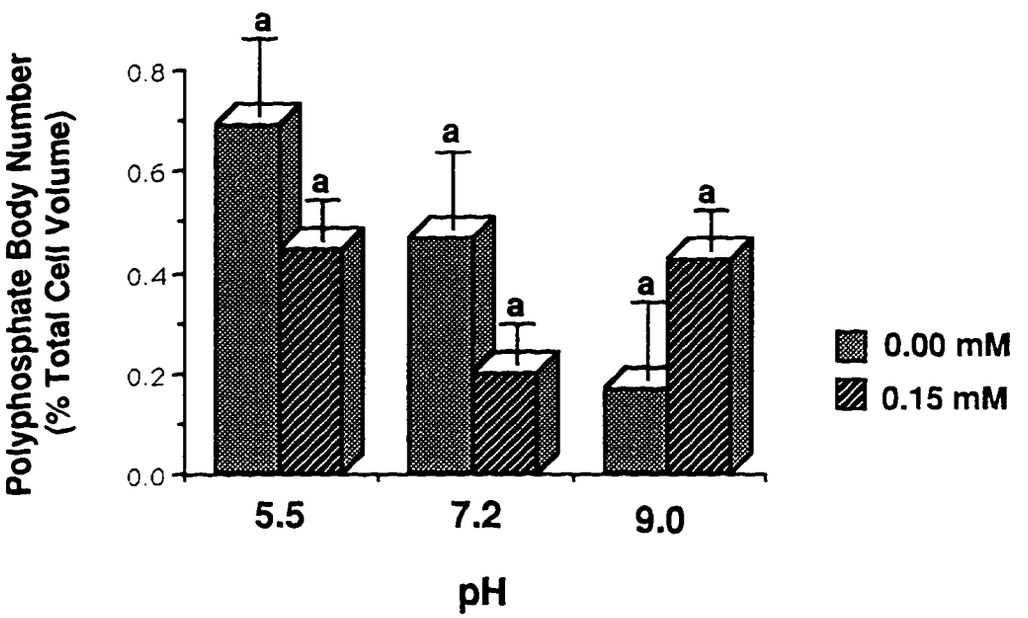

Figure 40. Effect of 0.15 mM aluminum on polyphosphate body number of *S. leopoliensis* at different pH levels (error bar=SE; different letters on each bar at a given pH denote statistical dissimilarity P<0.05)

difference (PLSD) indicated that cell volume decreased significantly when cells were grown as pH 9.0 compared to those grown at pH 5.5 and pH 7.2 (Table 27). The same analysis indicated a highly significant decrease in cell wall volume among cells subjected to pH 7.2 and pH 9.0 in comparison to those grown at the acid pH. This trend was also observed in layer #1 of the cell wall in that the decrease among cells at pH 7.2 and pH 5.5 was significant in comparison to cells grown at pH 5.5, and in polyphosphate body volume where there was a decrease in cells at pH 7.2 and pH 9.0 (Table 28).

A new trend was observed in the volume of nucleoplasm and surface area of thylakoids. In both, significant increase was expressed among cells grown at pH 9.0 compared to those at pH 5.5 and pH 7.0. Yet another pattern was observed in volume of intrathylakoidal space where the increase among cells grown at pH 7.2 was significant compared to the cells at pH 5.5 and pH 9.0.

Relative Counts

Significant variation as a result of pH was observed for mini polyphosphate bodies; however, these data were not reported here because no values were recorded at pH 7.2.

Table 30. Effect of different pH levels on cell volume and relative volumes (Vv) of other cellular components among cells of *S. leopoliensis* (single classification ANOVA).

pH	Cell volume	Cell wall Vv	Cell wall layer #1 Vv	Polyphos-phate body Vv
5.5	58.30_{a}	16.60_{a}	5.59_{a}	1.178_{a}
7.2	51.77_{ab}	10.83_{c}	0.89_{c}	0.177_{b}
9.0	42.03_{b}	12.58_{bc}	2.38_{bc}	0.178_{bc}
N	30	30	30	30
F	3.435	12.08	5.49	6.998
P	0.037*	0.0001***	0.0057**	0.0013**

Means followed by different letters denote statistical dissimilarity (P<0.05, using Fisher PLSD)

* = P<0.05

** = P<0.01

*** = P<0.001

Table 31. Effect of different pH levels on volumes of nucleoplasm and intrathylakoidal space and surface area of thylakoids among cells of *S. leopoliensis* (single classification ANOVA).

pH	Nucleo-plasm Vv	Intrathylakoidal space Vv	Thylakoidal surface area Sv
5.5	2.457_{ab}	0.173_{b}	0.750_{b}
7.2	1.737_{b}	1.550_{a}	0.940_{b}
9.0	4.327_{a}	0.356_{b}	2.080_{a}
N	30	30	30
F	3.156	7.186	7.937
P	0.0475*	0.0013**	0.0007***

Means followed by different letters denote statistical dissimilarity ($P<0.05$, using Fisher PLSD)

* = $P<0.05$

** = $P<0.01$

*** = $P<0.001$

Use of Energy Dispersive X-Ray Microanalysis to Study the Uptake of Aluminum by *S. leopoliensis*

The spectra generated by the cytoplasm of control cells showed peaks for most of the ions being investigated, i.e., chlorine (Cl), magnesium (mg), phosphoruss (P), potassium (K), and sometimes even iron (Fe). In all cases, peaks were modest (Figure 41). The pattern produced by the cytoplasm is repeated almmosty identically in the cell wall (Figure 42). Among these cells, even polyphosphate bodies--which typically generate a strong phosphorus (P) peak--produced spectra (Figure 43) that are very similar to those produced by the other cellular sectors under investigation.

Exposure to 0.037 mM aluminum produced no variation in either cytoplasm (Figure 44) or in the cell wall (Figure 45). Polyphosphate bodies, however, proved to be slightly more sensitive to this low concentration of the metal. Figure 46, a spectrum of a polyphosphate body, shows the characteristic phosphorus (P) peak, as well as an increase in the strength of the peaks for magnesium (Mg) and potassium (K).

Among cells exposed to 0.185 mM aluminum, absence of the iron (Fe) peak from the cytoplasm is notable. The peaks for the ions identified are almost uniform (Figure 47); however, in a few cases, the phosphorus (P) peak was stronger (Figure 48). Analysis of the cell wall at this concentration revealed very low signals for all ions (Figure 49). The polyphosphate bodies created a marked contrast in that all

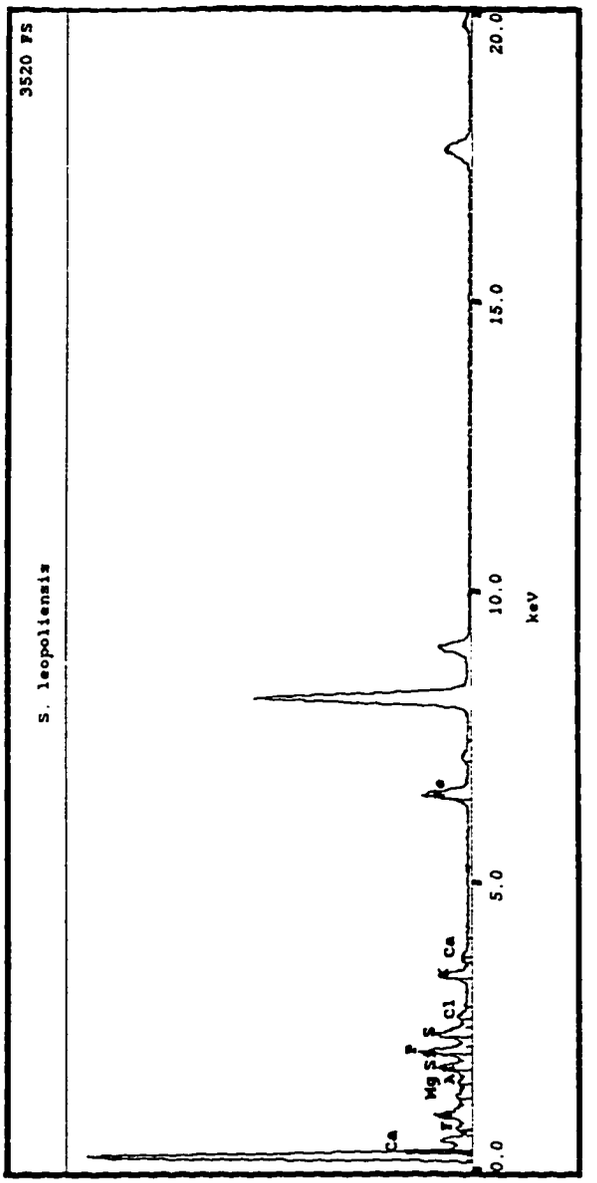

Figure 41. Spectrum of the cytoplasm of an air dried control cell of *S. leopoliensis* .

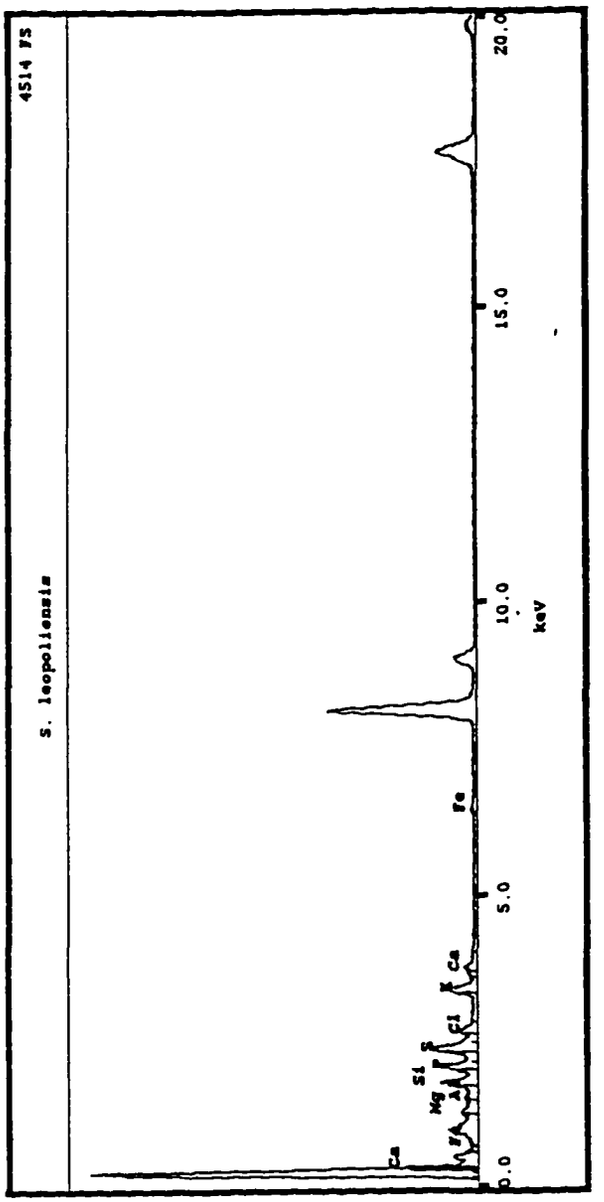

Figure 42. Spectrum of the cell wall of a control *S. leopoliensis* cell.

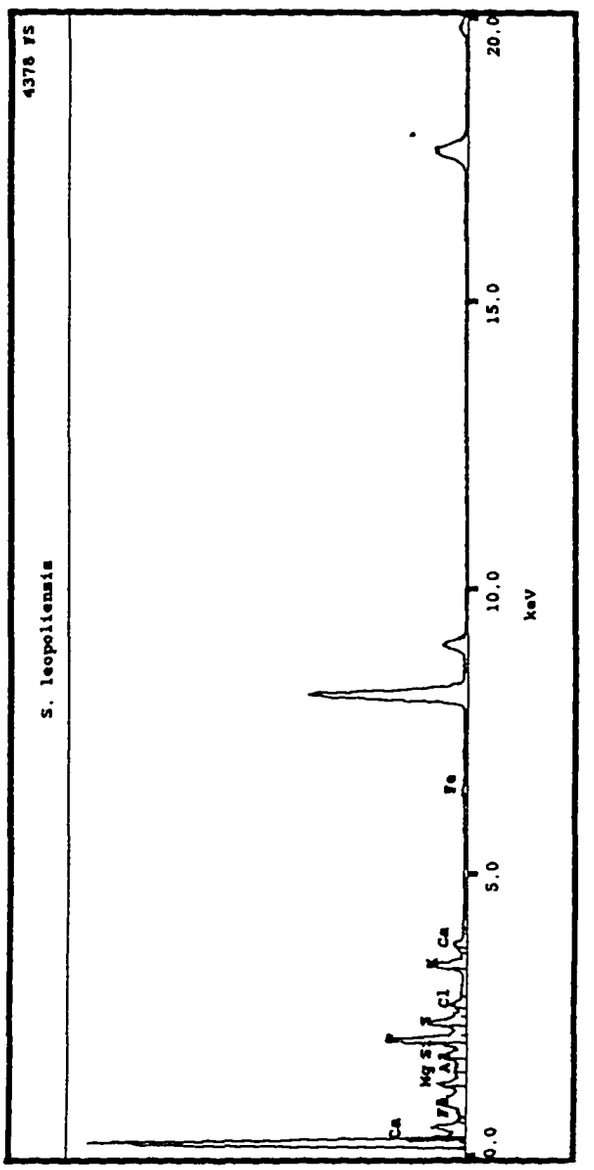

Figure 43. Spectrum of a polyphosphate body of an air dried control cell of *S. leopoliensis*. The distinguishable peaks are Mg, P, S, Cl, K, and Ca.

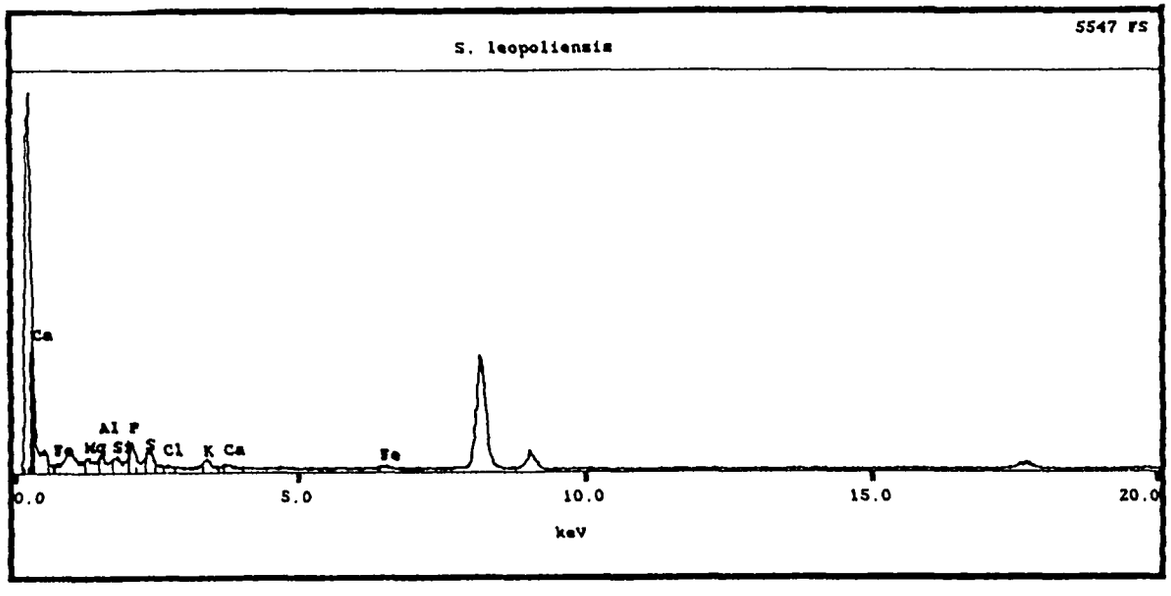

Figure 44. Spectrum of the cytoplasm of an air dried *S. leopoliensis* cell that was exposed to 0.037 mM Al. Only minor peaks for Mg, P, S, Cl, K, and Ca appear. No Al is detectable at this concentration.

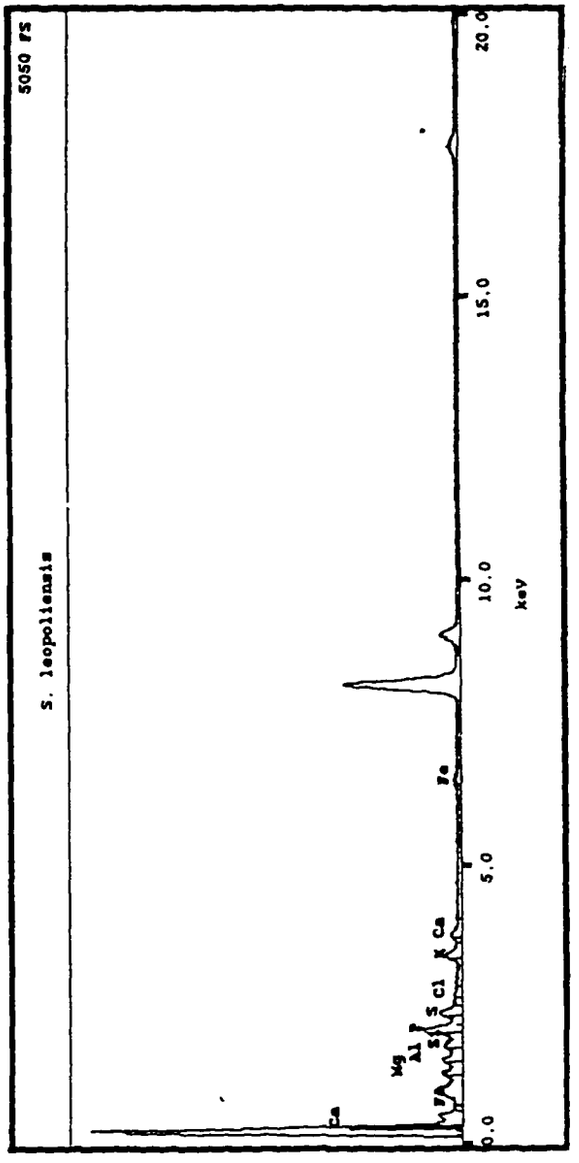

Figure 45. Spectrum of the cell wall of an air dried *S. leopoliensis* cell that was exposed to 0.037 mM Al. P, S, and K are most notable.

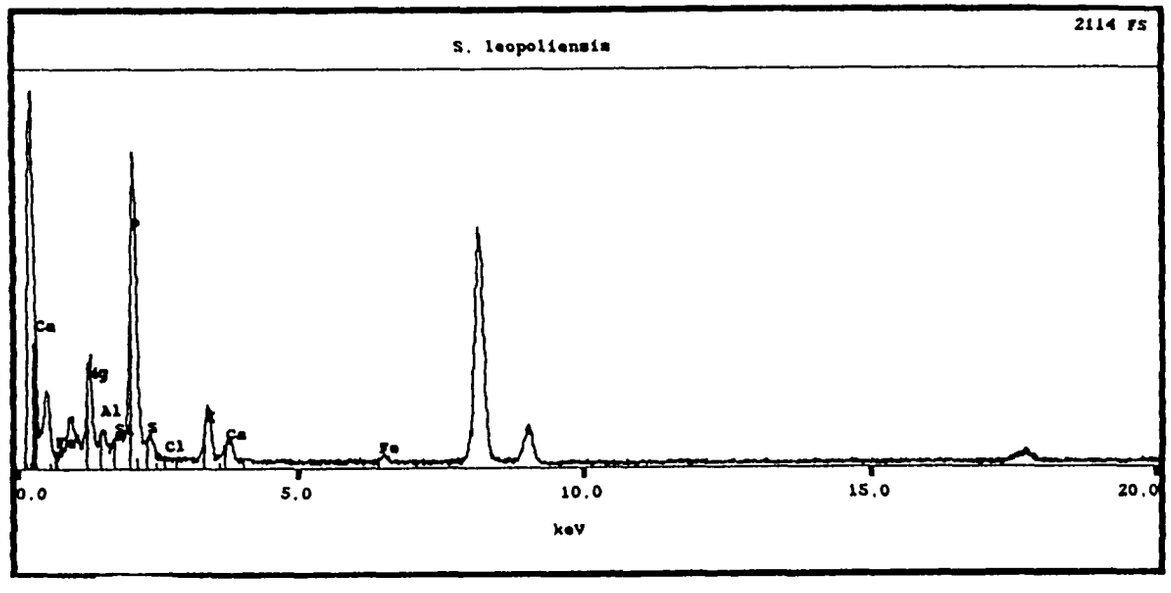

Figure 46. Spectrum of a polyphosphate body of an air dried *S. leopoliensis* cell that was exposed to 0.037 mM Al. Note the P peak. Compare with Figure 43, a spectrum from a control cell.

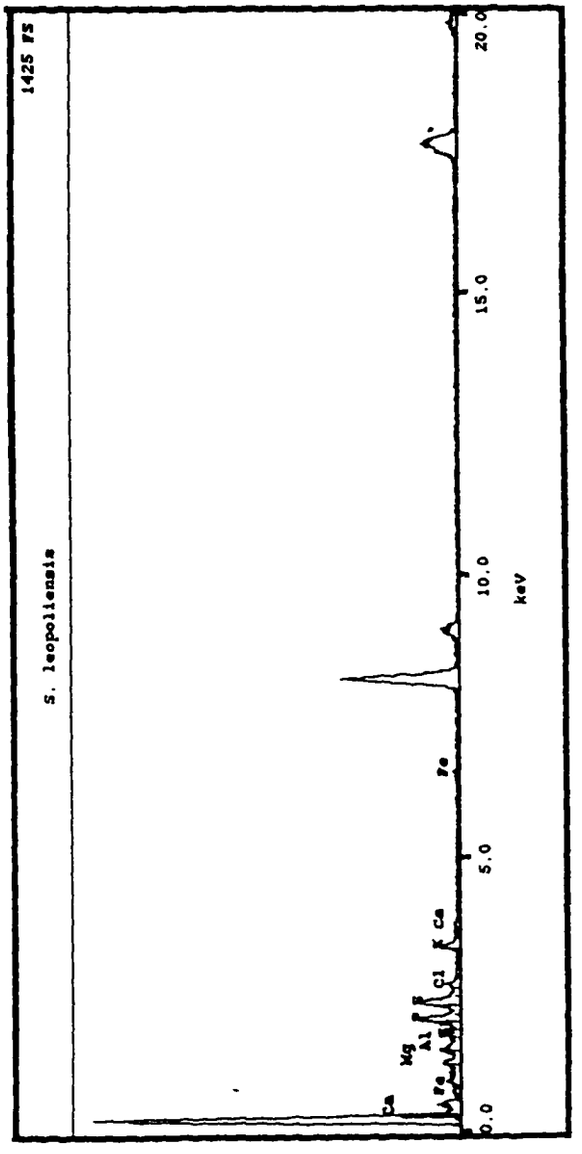

Figure 47. Spectrum of the cytoplasm of an air dried *S. leopoliensis* cell that was exposed to 0.185 mM Al. The P, S, and K peaks are *distinguishable but not prominent*.

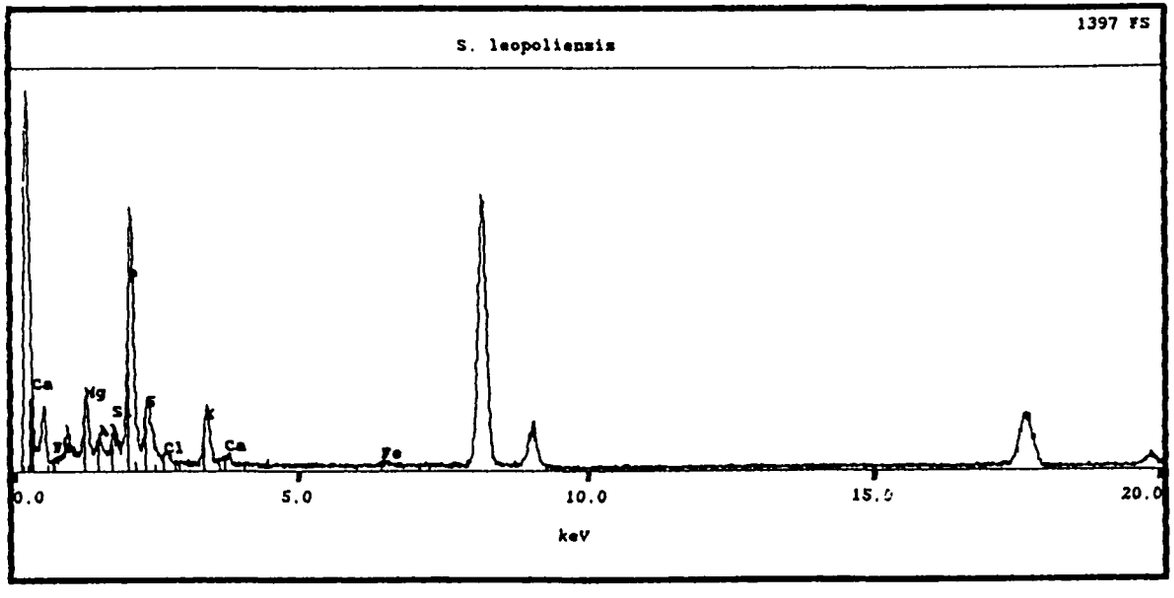

Figure 48. Spectrum of a polyphosphate body of an air dried *S. leopoliensis* cell that was exposed to 0.185 mM Al. Note the prominent P peak as well as those for Mg, S, and K. The Al peak is more distinct than in previous treatments.

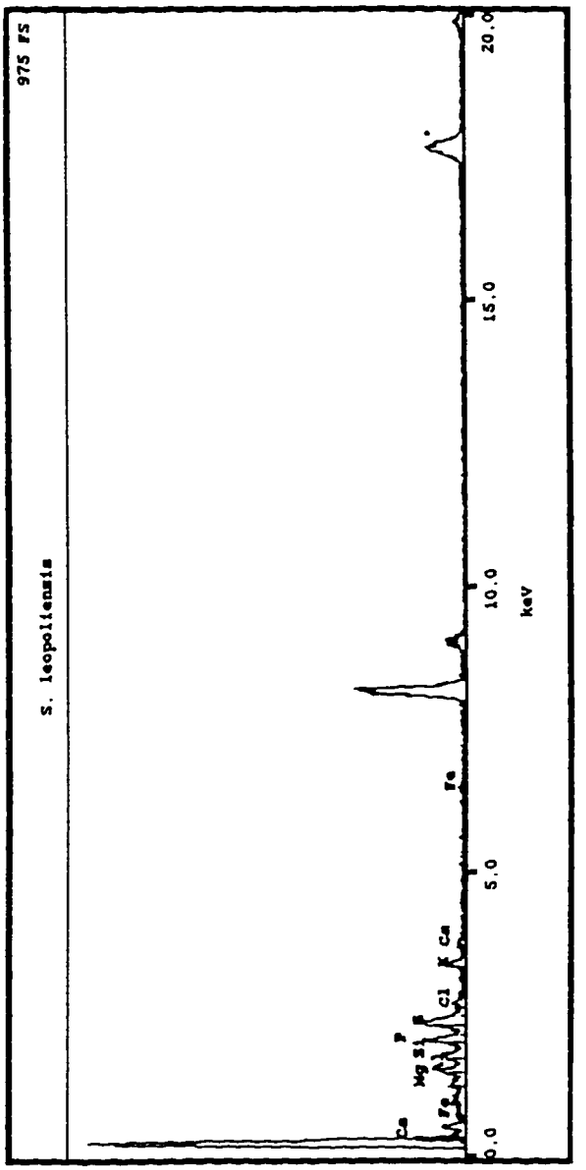

Figure 49. Spectrum of the cell wall of an air dried *S. leopoliensis* cell that was exposed to 0.185 mM Al.

spectra showed not only a steep phosphorus peak, but also clearly distinguishable peaks for magnesium, sulfur, and potassium (Figure 50). Of these, the magnesium peak was strongest. No aluminum peak is evident.

Upon exposure to 0.370 mM aluminum, both the cytoplasm (Figure 51) and the cell wall (Figure 52) produced spectra with peaks for most of the ions that barely projected above the baseline. The chlorine peak of the cytoplasm is negligible, as are the phosphorus and magnesium peaks of the cell wall. The slight increase in the aluminum peak of the cytoplasm (51) is accompanied by an increase in silicon. This combination was observed in other sectors (not reported here). The pattern produced by 0.185 mM aluminum is repeated in cells exposed to 0.370 mM (Figure 53). A prominent phosphorus peak was recorded along with increases in the peaks for magnesium, sulfur, and potassium. Again, no chlorine peak is recorded, nor is there strong indication of the presence of aluminum.

Phytochelatins

Phytochelatins were not detected in *S. leopoliensis*.

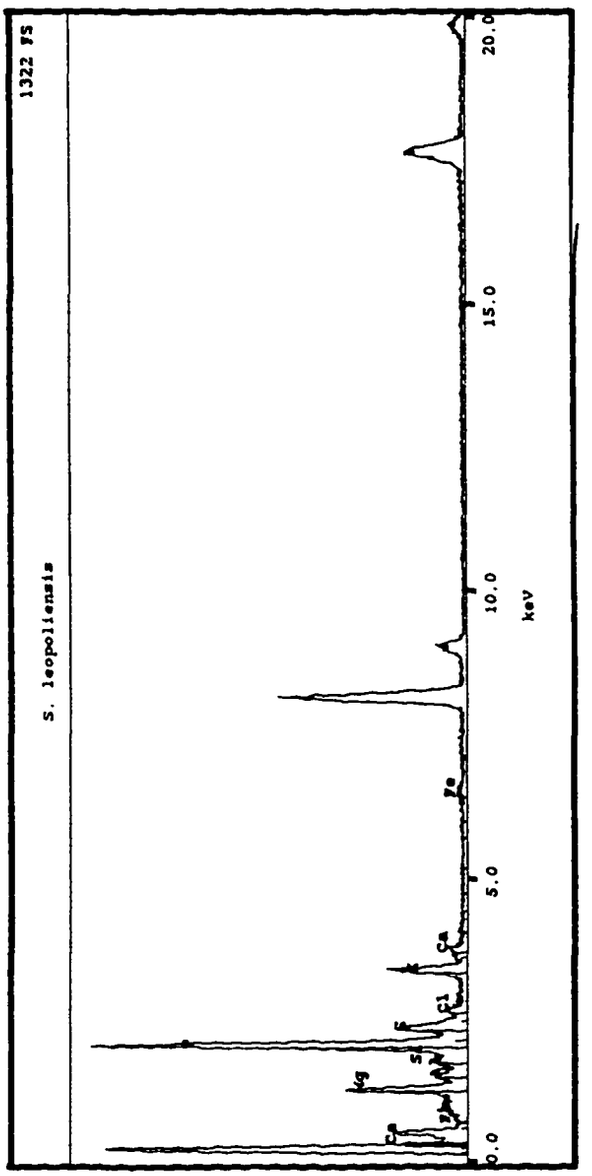

Figure 50. Spectrum of a polyphosphate body of an air dried *S. leopoliensis* cell that was exposed to 0.185 mM Al. Note the increase in the strength of the peaks for Mg, P, S, and K over those in Figure 48.

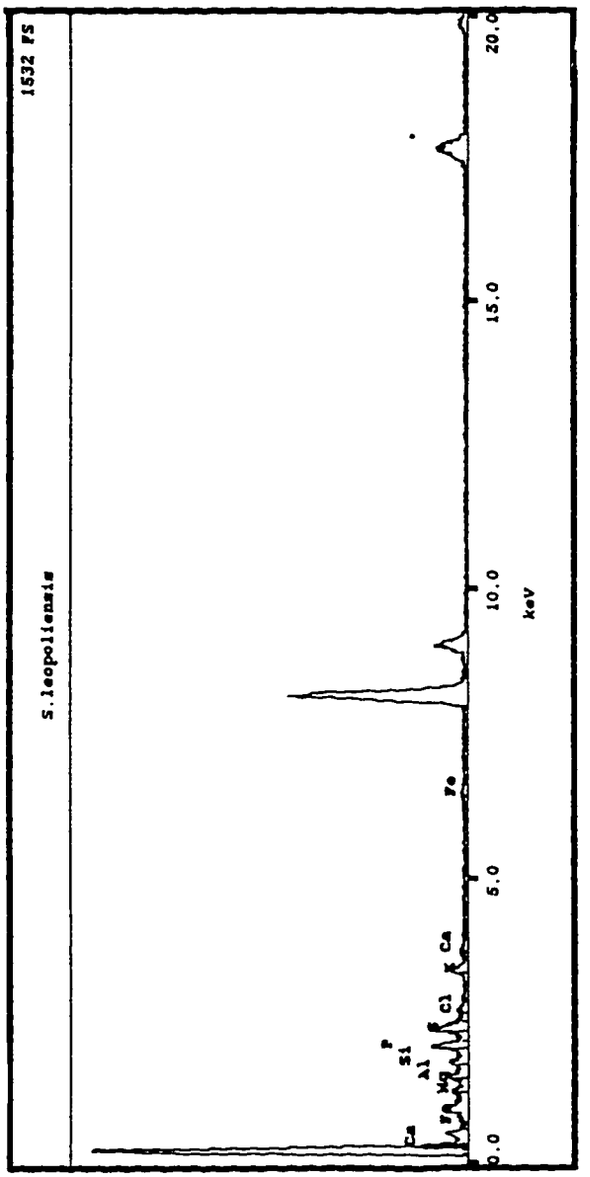

Figure 51. Spectrum of the cytoplasm of an air dried *S. leopoliensis* cell that was exposed to 0.370 mM Al.

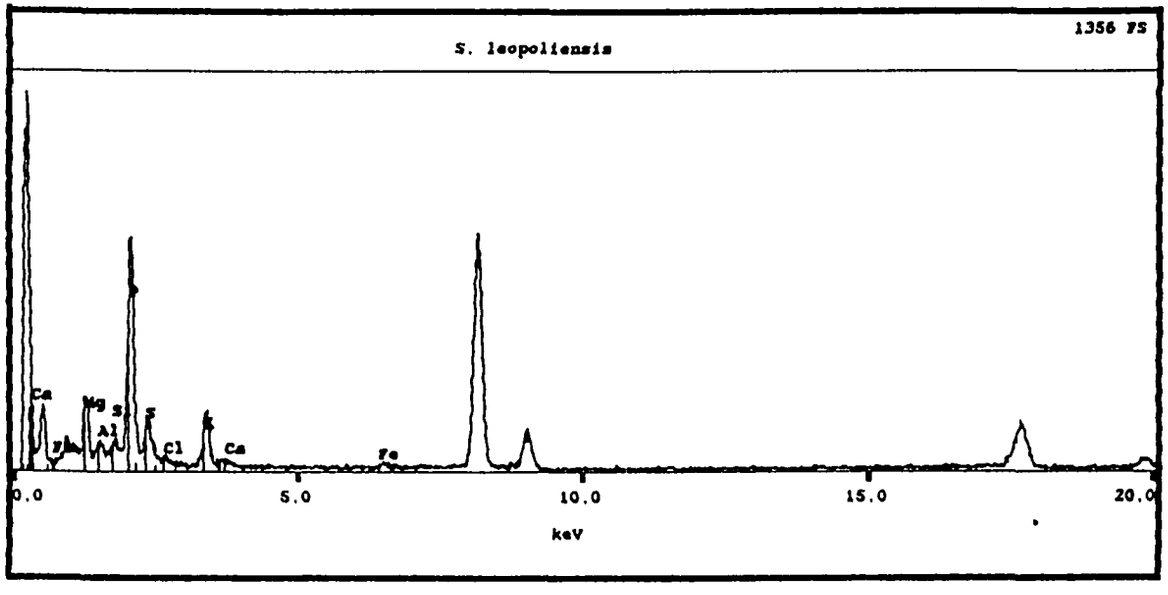

Figure 53. Spectrum of a polyphosphate body of an air dried *S. leopoliensis* cell that was exposed tom 0.370 mM Al. The peaks for Mg, P, S, and K are strong. Compare with Figure 50.

DISCUSSION

Quantification of protein and turbidity as a determinant of growth proved to be consistently reliable. The difficulty of counting cells in a filamentous species such as *A. doliolum* resulted largely from limitation of the light microscope in providing sharp focus throughout the length of a filament. Even up-and-down focusing did not allow for accurate cell counting. Dry weight determination gave varying results as well but no reason for the difficulty became apparent. Cell counting with a cell as small as *S. leopoliensis* is tedious and laborious.

The drastic decrease in protein production with increases in metal concentration indicates aluminum's capacity to inhibit growth. Upon exposure to the two highest concentrations, 0.23 mM and 0.37 mM, no viable cells of either species remained, consequently no protein could be detected. This is a notable comparison with control cells in which 58 μg were detected in *A. doliolum* and 124 μg in *S. leopoliensis*. The effective concentration (EC_{50}) of the metal on *A. doliolum*, 0.197 mM, compares favorably to reported data on aluminum and aquatic plants (Gostomski, 1990) as well as to a related species, *A. cylindrica* (Pettersson *et al.*, 1985). The Environmental Protection Agency (EPA) uses 750 μg/L as the acute criterion and 87 μg/L as the chronic criterion for protection of aquatic organisms against toxic effects of aluminum (Gostomski, 1990). The effective concentrations (EC_{50}) for each of the species reported here are well below these values, but compare favorably to EC_{50} values for other aquatic plants (Gostomski, 1990). *A. doliolum* with a value of 0.197 mM is very close to a related species, *A. cylindrica*, which is reported to have an EC_{50}

of 0.185 mM (Pettersson *et al.*, 1985) The growth response of *S. leopoliensis* resulted in an EC_{50} of 0.145 mM which is an indication that it is a more sensitive species.

That pH is inextricably linked to metal toxicity is strongly reflected in the current literature (Rai *et al.*, 1981; Lindemann *et al.*, 1990; Smith, 1990; Andersson, 1992; Husaini and Rai, 1992). The effect is reported to result from reduced toxicity in alkaline environments because ions precipitate into complexes which are rendered biologically inactive. These same ions at low pH levels, particularly below pH 6.5, are released and compete with hydrogen ions for adsorption at the cell's surface (Ramelow *et al.*, 1991) or they may complex with anionic ligands (Beveridge, 1989; Remacle, 1990).

Aluminum behaves as do many metals when introduced to a range of pH levels. It is a strongly hydrolyzing metal, and as such, is relatively insoluble in the neutral pH range, 6.0 to 8.0. In the acidic range, pH <6.0 as well as the alkaline, pH >8.0, the metal becomes more soluble making more ions available for chemical and biological activity (Driscoll and Schecher, 1990). This behavior of aluminum is clearly demonstrated by three regions in southern Norway, the site of a study to compare acid deposition and aluminum mobilization (Christopherson *et al.*, 1990). Two of these headwater catchments have received heavy deposition resulting in pH readings of 4.2 and 5.0 with corresponding aluminum levels of 20 μM and 3 μM, respectively. The third catchment, a pristine area which has been spared heavy deposition, has a pH of 7.5 with less than 1 μM aluminum. Tests of the soils of each region showed them all to be acidic. This is an indication that the aluminum in the third region is tightly bound in the soil.

Despite the prevalence of conditions similar to that described above, Cronan *et al.* (1986) report that some environments with complexing organic ligands have low aluminum levels at pH readings as low as 4.2.

Cyanobacteria as well as many algae experience optimal physiology in the neutral pH range so it is not uncommon to see notable variations when cells are grown in either acid or alkaline environments and even greater variations with exposure to heavy metals. *Scenedesmus sp.* and *Chlorella sp.* were collected from two headwater streams and exposed to various concentrations of aluminum at pH 5.0 (Lindemann *et al.*, 1990). When cells were exposed abruptly, a decrease in growth could be detected within 20 to 40 hours. This response is reported to be the result of rapid and gross adsorption as well as cell aggregations induced by changes in cell wall properties. This rapid reaction at the cell surface is followed by slow but widespread uptake into the cell proper. Conversely, when the aluminum was gradually added to the cultures, the decrease in growth rate was delayed some 70 to 240 hours. In reporting the results of tests to determine the influence of culture density, pH, organic acids and divalent cations on the ability of immobilized cells of *A. doliolum* and *Chlorella vulgaris* to take up nutrients and metals from their environment, Mallick and Rai (1993) determined that the cells' efficiency at nutrient and metal uptake decreased progressively as the pH varied in either direction (pH 4.0 to pH 10.0) from pH 7.0. It should be noted that this test involving removal of metal ions from the environment demonstrates that the cells' efficiency decreases as they approach the very pH range in which more metal contaminants would be found.

Effects of the Interaction of Aluminum and pH on the Ultrastructure of *A. doliolum* and *S. leopoliensis*

Any factor which affects cell growth would reasonably affect cell volume. The change in *A. doliolum's* cell volume might be explained as a result of aluminum's binding to essential constituents of the cell (Mallick and Rai, 1993). The observation of localization of aluminum in the nucleus where it increased DNA synthesis (Johnson and Wood, 1990), coupled with the recording of an increase in cell size with exposure to aluminum (Greger *et al.*, 1992) addresses the significant increase in cell volume of *A. doliolum* at the neutral pH. The increase in cell size observed by Greger *et al.* (1992) was attributed to incomplete cell division.

The poor correlation between detection of aluminum in the cell wall and the dramatic change in cell wall volume does not diminish the significance of the change. Eleftheriou *et al.* (1993) observed thickening of the cell wall in aluminum-treated *Thinopyrum* (roots) despite in the presence of electron-opaque deposits and malformations at acidic pH. They hypothesized that the increase was manifestation that a defense mechanism had been activated and that the mechanism for producing cell wall materials had not been seriously affected by the metal. Such mechanisms could account for the increase in cell wall volume in *A. doliolum.* Conversely, the decrease in cell wall volume observed in *S. leopoliensis* agrees with the response of *Chara* to aluminum at pH 4.4 (Reid *et al.*, 1995). Reid *et al.* (1995) report that aluminum appears to disrupt normal cell wall growth in two ways: (i)

by reducing the calcium concentration to a level below which cross-linking of pectic residues cannot occur and (ii) by cross-linking with cell wall components and altering the structure.

The nature of the cyanobacterial polyhedral body is clearly defined. This very prominent and angular body, first described by Jensen and Bowen (1961), has as its main constituent 5 to 15 polypeptides of ribulose 1,5-biphosphate carboxylase/oxygenase (RuBisCo). This highly abundant protein is documented as the enzyme which catalyzes carbon fixation. Jensen (1990, 1994), however, questions that function within polyhedral bodies since they tend not to be located near thylakoids. He more readily accepts a storage function because they have been observed in non-photosynthetic spores. If there is no photosynthetic role, the significant decrease in the volume as well as the number of polyhedral bodies in *A. doliolum* at the acidic and alkaline pH levels could be related to the capacity of aluminum to bind to essential cellular constituents (Mallick and Ria, 1993), in this case the stored RuBisCo. If, on the other hand, significant photosynthesis is conducted within the polyhedral body, the structural and number changes might indicate displacement of enough magnesium or iron to affect chlorophyll content and, in doing so, alter photosynthetic productivity. Quite the opposite observation has been reported for *Scenedesmus obtusiusculus* in which the iron and magnesium concentrations increased upon exposure to aluminum at acidic pH (Greger *et al.*, 1992). This increase in the number of polyhedral bodies was observed in *S. leopoliensis*. The difference is that *Scenedesmus obtusiusculus* cells were phosphorus enhanced.

Aluminum's affinity for phosphorus is reported to result in phosphorus deficiency (Tornqvist, 1988; Greger *et al.*, 1992). In the current study, however, *A. doliolum* showed significant increases in volume and number of polyphosphate bodies at the acidic pH and an increase in the number of these inclusions at the alkaline pH. This response might indicate either some level of aluminum tolerance or the intervention of an ameliorating agent. Exley *et al.* (1993) found that aluminum toxicity in the freshwater diatom, *Navicula pelliculosa*, was ameliorated by silicon which reduced the metal's competition with phosphorus.

Baxter and Jensen (1986) observed that when placed in phosphorus-rich medium, phosphorus-starved cells of *Plectonema boryanum* accumulated in 1 minute the amount of phosphorus recorded in control cells after 14 days of growth. This affinity for phosphorus is further demonstrated by *A. cylindrica* which recovered when placed in phosphorus-rich medium after experiencing phosphorus depletion induced by aluminum (Pettersson, 1989). The uptake of sufficient phosphhorus to overcome aluminum is thought to result from facilitation by a carrier which is activated by calcium and magnesium. These ions are known to bind metal cations. The structural translation of this mechanism is the polyphosphate body, a cellular inclusion which was first described by Bringmann (1952) and thoroughly investigated by Jensen (1968, 1969). The presence of phosphorus, calcium and magnesium within these bodies along with their increase in number and mass upon the cell's exposure to heavy metals strongly support a role in metal detoxification. Jensen *et al.* (1982) reached this conclusion after determining that metals were

sequestered in greater concentrations in polyphosphate bodies than in cell sectors which did not contain these electron-dense bodies. *A. doliolum* in this study appears to have overcome the phosphate-binding or phosphate-depleting capacity of aluminum. The drastic reduction in polyphosphate body volume observed in treated cells of *S. leopoliensis* at the acidic pH implies failure of these cells to increase their phosphorus uptake.

The response of cyanophycin granules of *A. doliolum* to aluminum at the acidic pH agrees strongly with the inverse of the observation that cyanophycin granule polypeptide (CGP) accumulates with phosphorus deficiency (Pettersson, 1989) and nitrogen excess (Allen, 1984). The immense increase in the number and volume of polyphosphate bodies is reportedly the result of accelerated phosphorus uptake (see above); this condition, quite reasonably, should then limit the accumulation of cyanophycin granule polypeptide into the characteristic structured granules. These somewhat spherical bodies, thought to be unique to cyanobacteria (Allen, 1984) are described as co-polymers of L-arginyl and L-aspartic acid in a 50:50 ratio and as nitrogen storage units (Jensen, 1993). If we consider the suggestion by Stevens *et al.* (1981) that a) cyanophycin is synthesized from internal conversion of cellular protein, and b) that this conversion is accompanied by a decrease in cellular nitrogen with phosphorus reduction, the second requirement (excess nitrogen) for synthesis of cyanophycin granules is satisfied. In the case of *A. doliolum*, apparently the preponderance of phosphorus was accompanied by limited nitrogen accumulation and the conversion of cellular proteins into cyanophycin.

The lipid inclusions of the cyanobacteria are reported to be of the same nature as those of the eukaryotic chloroplast (Jensen, 1993). The metal binding capacity of algal lipids (Rai *et al.*, 1981; Rai and Raizada, 1987) could account for the notable decrease in these inclusions among *A. doliolum* cells exposed to the metal at the acidic pH. The response of the cells in the alkaline range, however, points to a defense or detoxification mechanism. According to Wolk (1973) sulfoquinivosdyl diglyceride, a predominant ccyanobacterial lipid, demonstrates an affinity for sulfur-binding cations. If such a condition prevails with *A. doliolum* and aluminum, the increase in the number of lipid inclusions at pH 9.0 may indicate yet another means through which prokaryotic and eukaryotic cells cope with environmental flux.

Significant changes in the thylakoidal surface area must be analyzed with respect to changes in membrane integrity as well as (cellular) environmental affects on the efficiency of photosynthesis. The degradation of thylakoidal surface area in *S. leopoliensis* at the acidic pH indicates not only aluminum uptake, but also intrathylakoidal deposition. The change in thylakoidal membrane integrity can be attributed to lipid phase changes induced by aluminum (Pettersson, 1989), while the latter contention is supported by cellular reduction or depletion of carbon dioxide which deprives cells of chlorophyll a; the absence of chlorophyll a results in the loss of thylakoids (Jensen, 1993). One of the few significant changes to occur at the neutral pH is the increase in intrathylakoidal space observed in *S. leopoliensis*. This observation makes the increase in thylakoidal surface area at pH 9.0 appear to be a compensation to accommodate the combination of aluminum and the alkaline environment. The significant increase in

intrathylakoidal space among *A. doliolum* cells exposed to the metal at the acidic pH does not agree with the absence of variation in thylakoidal surface area in the same cells.

Cellular Incorporation of Aluminum

The many changes observed in total cell volume and cellular inclusions among *A. doliolum* and *S. leopoliensis* confirm the entry of aluminum into the cells at all pH levels tested. Energy dispersive x-ray microanalysis (EDX) in the current study was used to determine uptake and localization of aluminum in air-dried cells to determine if certain cellular sectors, particularly cell wall, cytoplasm and polyphosphate bodies, were targeted. The concentrations of aluminum were low (0.037 mM, 0.185 mM and 0.370 mM), but even low concentrations are significant if they provide some insight into threshold concentrations and when they allow some comparison between treated and untreated cells.

Analysis of spectra for cell wall, cytoplasm, and polyphosphate bodies in untreated cells of *A. doliolum* and *S. leopoliensis* compare favorably with respect to magnesium (Mg), sulfur (S), phosphorus (P), chlorine (Cl), potassium (K), and calcium (Ca). This elemental composition along with enhancement of the phosphorus peak in electron dense bodies (visible even within air-dried cells) identifies them as the polyphosphate bodies found among cyanobacteria (Jensen *et al.*, 1977; Jensen *et al.*, 1982; Rachlin *et al.*, 1984; Jensen, 1993; Jensen and Corpe, 1994).

Cells exposed to the lowest concentration of aluminum, 0.037 mM, did not generate an aluminum peak. However, the intensification of polyphosphate body spectra, particularly the phsophorus peak, of both species indicates a change in the composition of this inclusion. The polyphosphate bodies of *A. doliolum* and *S. leopoliensis* thus appear to localize metals as do those reported for other species (Jensen *et al.*, 1982; 1986; Baxter and Jensen, 1980; Pettersson *et al.*, 1985a). The change in cell wall and cytoplasmic elemental composition induced by aluminum in *S. leopoliensis* was negligible, but the slight change within the cytoplasm of *A. doliolum* could indicate sequestration of aluminum within some cellular sector which was not visible in cells prepared as these were. Absence of the metal from the cell wall does not agree with the response of *A. cylindrica* (Pettersson, 1985b) to a comparable concentration.

The spectra of polyphosphate bodies exposed to 0.185 aluminum repeat the prominent phosphorus peak of cells at the middle concentration. *S. leopoliensis* occasionally produced a strong magnesium peak which might indicate that the integrity of the polyphosphate body is retained at this concentration.

A. doliolum exposed to 0.370 mM aluminum showed a loss of magnesium from the cytoplasm and polyphosphate bodies and of potassium from the cell wall. This behavior is documented to result from a metal-induced change in membrane permeability allowing the loss of certain ions from the cell (Baxter and Jensen, 1980; Rai *et al.*, 1990; Jensen *et al.*, 1982). Both species retained the strong phosphorus peak for polyphosphate bodies at this concentration, a response that supports the detoxification role proposed for this

inclusion. The presence of aluminum could not be confirmed in either species.

The Effect of pH on Ultrastructure

The influence of pH appears to be greater on *S. leopoliensis* where the number of cellular inclusions that was significantly affected was the same as those changed by the interaction of aluminum and pH. The influence was less pronounced in *A. doliolum*. It should be noted that *S. leopoliensis* was exposed to a lower concentration of aluminum. These results, then, could indicate that this species is more sensitive to the interaction of pH and aluminum.

APPENDICES

Appendix A

Composition of Culture Medium-Modified Fitzgerald (Fitzgerald *et al.*, 1952)

	mg/liter
$NaNO_3$	124
$K_2HPO_4 \cdot 3H_2O$	13
$MgSO_4 \cdot 7H_2O$	25
$CaCl_2 \cdot 2H_2O$	36
Na_2CO_3	20
$Na_2SiO_3 \cdot 9H_2O$	58
Ferric Citrate	3
Citric Acid	3
Gaffron's minor element solution-	0.04 ml.

pH is adjusted to 7.2 by adding 1 N HCL or 1 N NaOH

Gaffron's solution:

	g/liter
H_3BO_3	3.10
$MgSO_4 \cdot 4H_2O$	2.23
$ZnSO_4 \cdot 7H_2O$	0.287
$(NH_4)_6Mo7O_{24} \cdot 4H_2O$	0.088
$CuSO_4 \cdot 5H_2O$	0.125
$Co(NO_3)_2 \cdot 6H_2O$	0.146
$Al2(SO_4)_3KSO_4 \cdot 24H_2O$	0.474
$NiSO_4(NH_4)SO_4 \cdot 24H_2O$	0.474

$Cd(NO_3)_2 \cdot 4H_2O$	0.154
$Cr(NO_3)_3 \cdot 7H_2O$	0.037
$V_2O_4(SO_4)_3 \cdot 16H_2O$	0.035
$Na_2WO_4 \cdot 24H_2O$	0.033
KBr	0.119
KI	0.083

Appendix B

Fixation of experimental samples.

A: Preparation of solutions:

1. Stock Michaelis Buffer:

   a. Weight: 1.94 g sodium acetate ($NaC_2H_3O2 \cdot 3H_2O$)
   2.94 g sodium barbital
   3.40 g sodium chloride

   b. Add water to (a) to reach 100 ml and dissolve the chemicals, then store the buffer in a refrigerator.

2. Osmium Tetraoxide:

   A stock solution of 2% OsO4 was made by dissolving 1 g of osmium tetraoxide in 50 ml of distilled water. The solution was stored in a Pyrex glass stoppered bottle, which was then placed in a tin can, and stored in a refrigerator.

3. 1% Bacto-tryptone:

   1 g of Difco bacto-tryptone and 0.5 g NaCl were dissolved in 100 ml distilled water. The solution was placed in small closed vials and stored frozen. The contents of the vials were thawed and mixed thoroughly before use.

B. Fixing Procedure of Modified Kellenberger *et al.*, 1958; Pankratz and Bowen, 1963):

1. Preparation of solutions:

a. 5 ml Stock Michaelis Buffer,

7 ml 0.1 N HCl,

13 ml distilled water,

0.25 ml 1.0 M $CaCl_2$;

then adjust the solution to pH 6.1 or 6.2.

b. Dilute solution (a) with an equal amount (1:1) of 2% OsO_4 then add 0.1 ml 1% bacto-tryptone per ml.

2. Fix samples in solution (b) for 3 hours at room temperature.

Appendix C

Embedding with Epon 812 (Luft, 1961)

A: preparation of Epon:

1. Mixture A:

62 ml Epon 812,

100 ml DDSA (Dodenyl Succinic Anhydride).

2. Mixture B:

100 ml Epon 812,

89 ml NMA (Nadic Methyl Anhydride).

Mixture A and mixture B were refrigerated, and brought to room temperature before opening to avoid water condensation.

3. Epon mixture for embedment: 1 part mixture A, and 2 parts mixture B were mixed in a disposable plastic beaker, and 0.2 ml of DMP-30 was added per 10 ml of the Epon mixture. It is important that all

components are thoroughly mixed so that no "streaks" are visible. Epon mixture should be freshly made before embedding.

B: Dehydration and embedding of fixed samples:

50% ethanol	5 -10 min
75% ethanol	5 -10 min
95% ethanol	5 -10 min
100% ethanol	5 -10 min
100% ethanol	5 -10 min
propylene oxide	5 -10 min
propylene oxide	5 -10 min
propylene oxide	5 -10 min
3 propylene oxide : 1 Epon	15 min
1 propylene oxide : 1 Epon	30 min
1 propylene oxide : 3 Epon	60 min

Transfer the sample into capsules then fill with pure Epon.

Polymerize the Epon by leaving capsules at:

room temperature	overnight
35° C oven	8-24 hours
45° C oven	8-24 hours
60° C oven	8-24 hours

Appendix D

Uranyl acetate-methanol stain (Stempak and Ward, 1964).

A: Preparation of the solution: 15 g hydrated uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2H_2O$) were dissolved in 50 ml of acetone-free absolute methanol with a magnetic stirrer. The solution was then

stored in a Pyrex glass-stoppered bottle at 4°C.

B: Staining: Grids were immersed in wells of a porcelain staining dish, section side up, for 10 minutes at room temperature. The wells were covered during this period to prevent evaporation of the methanol.

C: Washing: The grids were then rinsed with absolute methanol, ethanol, and water as listed below:

1. Absolute methanol	2 - 3 dips
2. Absolute methanol	10 - 20 dips
3. 100% ethanol	25 dips
4. 80% ethanol	50 dips
5. 50% ethanol	50 dips
6. distilled H_2O	50 dips

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