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A BIOSENSOR FOR CADMIUM BASED ON BIOCONVECTIVE PATTERNS

By David A. Noever and Helen C. Matsos

Space Science Laboratory
Science and Engineering Directorate

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16. Abstract

An *in vitro* method for monitoring cadmium, one of the most lethal bivalent heavy metals, can detect biologically active levels. The effects of cadmium tend to concentrate in protozoa far above natural levels and therein begin transferring through freshwater food chains to animals and humans. In a small sample volume (~5 ml) the method uses the toxic response to the protozoa, *Tetrahymena pyriformis*, to cadmium. The assay relies on macroscopic bioconvective patterns to measure the toxic response, giving a sensitivity better than 1 µg/l and a toxicity threshold to 7 µg/l for Cd^{+2}. Cadmium hinders pattern formation in a dose-dependent manner. Arrested organism growth arises from slowed division and mutation to non-dividing classes. Unlike previous efforts, this method can be performed in a shallow flow device and does not require electronic or chemical analyses to monitor toxicity.

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TECHNICAL MEMORANDUM

A BIOSENSOR FOR CADMIUM BASED ON BIOCONVECTIVE PATTERNS

INTRODUCTION

Protozoa such as *Tetrahymena pyriformis* take up heavy metals from water, producing internal concentrations greater than surrounding levels. [1] As a consequence, these freshwater organisms serve as successful model systems for studying aquatic contamination, as well as for detecting toxicity. Compared to direct chemical analysis, toxicity assays using protozoa have potential advantages: (1) high levels of accumulation make for sensitive detection; (2) *in vivo* monitoring gives an integrated picture of pollution, thus not masking intermittent exposure; (3) as a more direct indicator, it can describe biological levels likely to enter into higher food chains; and (4) practical removal systems can be based on regrowth or early isolation of contaminated organisms.

Previous work has used *Tetrahymena* as an indicator for cadmium toxicity. [1] As a quantitative monitor, the basis for its assessment has been either specific growth rate, biomass or ultrastructure. [2] For example when added to 2 day old *Tetrahymena* cultures, cadmium ions inhibit further growth in a dose-dependent manner. About 30% inhibition was detected in the presence of 10 μM cadmium concentration, with virtually 100% inhibition at 30 μM. No significant changes were detected in 1 μM cultures. These assessments have been confirmed in various studies using different media, in each case requiring either cell counting procedures, x-ray microanalysis, or electron microscopy. For practical assays using a large statistical sample, these laboratory techniques can be both laborious and prohibitively expensive.

The present work uses macroscopic bioconvective patterns as a monitor to assess cadmium toxicity. Biconvection leads to dynamic patterns appearing at critical concentrations above 10^5-10^6 negatively geotaxic organisms ml^{-1}. They arise from the density inversion of organisms heavier than their suspending media. [3] On the scale of many millions of organisms, these patterns reflect a variety of cellular changes including motility, number, density, etc.

Like previous monitors, pattern formation shows cadmium inhibition in a dose-dependent manner. The advantages of such a monitor are: (1) its greater sensitivity to detect 1 μM cadmium levels; (2) relative freedom from complex laboratory procedures or equipment; and (3) ease in training operators and

portability. The principal disadvantage of such a monitor is its relatively larger sample size (~200 ml). This volume is required for rapid assay within a few days and some techniques to avoid the latter difficulty are discussed.

MATERIALS AND METHODS

Stock cultures of the protozoa, *Tetrahymena pyriformis* (ATCC), were grown axenically in autoclaved proteose peptone/ yeast media. [1] Fresh 100 ml growth medium was innoculated with 1 ml of cells harvested from stock at a stationary growth phase. After 2 days, when these cultures attained early logarithmic growth, they were divided into equal 100 ml portions and supplemented with cadmium chloride ($CdCl_2$ * 2.5 H_2O) in a fresh 100 mls of medium. Final cadmium concentration varied between 1 and 100 µg/l. Cultures were incubated at 28.0 C constant temperature during a 24-hr photoperiod (20 lux over 400-700 nm, supplied by cool white fluorescent tubes). The growth of *Tetrahymena* was monitored by cell counting using a hemacytometer.

Within 2-3 days following final seeding, biconvective patterns were induced by first concentrating the cultures. This was accomplished in 200 ml portions, wherein the cells were harvested by drip filtration (either with 25 psia vacuum pressure or without pumping) through a 0.2 µm mesh. Over several hours, the unattended culture volume was allowed to fall to 10 mls, thus yielding a maximum organism density of approximately 10^6 ml^{-1}.

The assay was carried out in Petri dishes, 4.8 cm in diameter and 0.8 mm in depth. The 10 ml lots of concentrated cultures (media plus organisms) was transferred from filters to dishes using 5 ml plastic pippettes. Protozoan growth was assessed quantitatively by measuring the formation times of macroscopic patterns and the number of nodal points in the pattern. These measures have been used previously to monitor organism activity within a pattern. [4] In a similar vein, qualitative observations of pattern clarity also reflect both organism viability and population across a narrow range of cadmium concentrations.

RESULTS AND DISCUSSION

The assay method was tested by examining the effects of cadmium concentrations between 1-100 µg/l Cd^{+2}. Each dilution inhibited growth sharply over a narrow range and the median inhibitory concentration was estimated to be between 5-7 µg/l Cd^{+2}. The dose dependence of macroscopic pattern response paralleled previous growth studies, [1] but with slightly greater sensititivity in the 1-5 µg/l Cd^{+2} range. Motility or size changes, each of which individually could

account for this improved sensitivity (compared to cell counting alone), was not examined, although qualitatively cadmium exposure tended to result in irregular size changes [1] and slowed motility.

For different cadmium levels, comparison of polygonal patterns can be aided by a statistical set characterized by average pattern size, the probability distribution of polygons according to the number of sides, and the correlation between the number of polygonal sides and the average number of sides for its neighbors. Thus by photographing patterns, then digitizing the images, bioconvective patterns can be compared not only to detect cadmium, but also to match with other two-dimensional networks such as foam layers and crystal grain boundaries. These have recently been reviewed. [5] Many of these quantitative relations are universal, such as a linear dependence of average cell perimeter on the number of sides. These laws continue to hold for different cadmium levels, but with varying coefficients which offer additional techniques to detect the presence of heavy metals.

Culture age was examined, since it is known that with increasing age *Tetrahymena* respiratory capacity decreases and pH increases. Because of small diluent volumes, the effect of cadmium addition on pH, osmolarity, gas tensions, etc. are negligible. Systematic variation of these variables, nevertheless, has been reported to have no measurable effect on their viability as chemical indicators. [6] There was good agreement between the current growth curves (figs. 1 and 2), previously published results [1] for *Tetrahymena,* and the bioconvective indicator employed macroscopically.

Tetrahymena is a well-characterized organism, long used as a biological indicator. [7] The biological action of cadmium has been described previously. [1] As seen after 2 days' exposure to 10 μM Cd^{+2}, *Tetrahymena* undergoes changes in cytoplasm and nucleus. In macronucleus, the nucleoli show irregular shape and consist of mainly fibrillar material; these effects have been attributed to the inhibition of RNA synthesis. In cytoplasm, irregularly-shaped autophagic vacuoles appear with increased lipid drops and dense granules. In cells exposed to 100 μM cadmium, mitochondrial disintegration occurs within 1 hour of exposure. In addition to ultrastructure changes, chemical content is disturbed--both an accumulation of 2.4 mmoles of cadmium per kg of wet cells (after 2 days exposure) and enhanced sulfur content. It is known that cells synthesize metal- chelating, sulfur-rich proteins (e.g., metallothioneins) in the presence of heavy metals, notably cadmium.

The use of microorganisms to detect toxicity has been reviewed. [8] The heavy metal cadmium was used here to illustrate the potential usefulness of bioconvective patterns as a monitor for assays. By culturing for only 3 days, the *Tetrahymena* assay was able to detect to 1 μg/l Cd^{+2}. The assay is simple to perform and requires no elaborate apparatus or procedure. *Tetrahymena* grows rapidly and reliably in axenic media and following growth, no special precautions are

required to maintain sterility during the assay.

While for the present test the method described is adequate, some improvements can increase sensitivity and convenience. For example, other media choices (such as defined Rosenbaum's medium) show a protective effect for cadmium with an approximately 50% survival rate at cadmium dosages which prove lethal in proteose-peptone. [1] It is possible that a shorter growth time (<3 days) is obtainable using larger culture volumes, which then can be filtered to the same 10 ml for final testing. However, if larger volumes are inconvenient, small vials (~10-20 ml) can be used with an extended growth time. Other workers have reported pattern formation spontaneously and without any filtration or other concentration scheme. [3] Hence in principle a successful assay outcome should prove possible without any transfer of cells, but rather in the smaller test dishes themselves.

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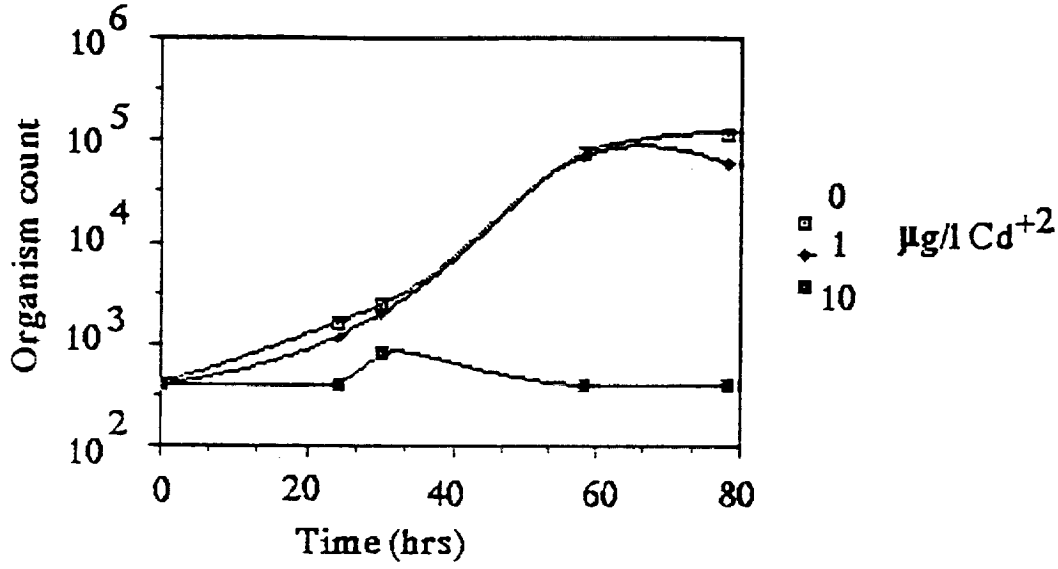

Figure 1. Effect of cadmium on the growth of *Tetrahymena pyriformis* population. The cultures were supplemented with cadmium at time T=0 hours. The concentration of cadmium is shown at right between 0-10 µg/1 Cd^{+2}.

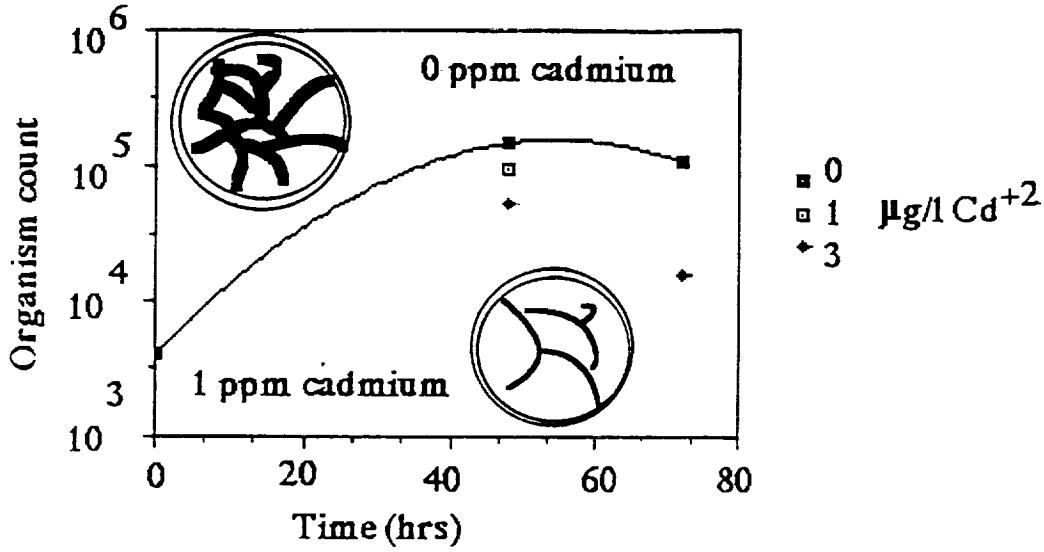

Figure 2. Effect of low doses of cadmium on the growth of *Tetrahymena pyriformis* population. The cultures were supplemented with cadmium at time T=0 hours. The concentration of cadmium is shown at right between 0-3 µg/1 Cd^{+2}.

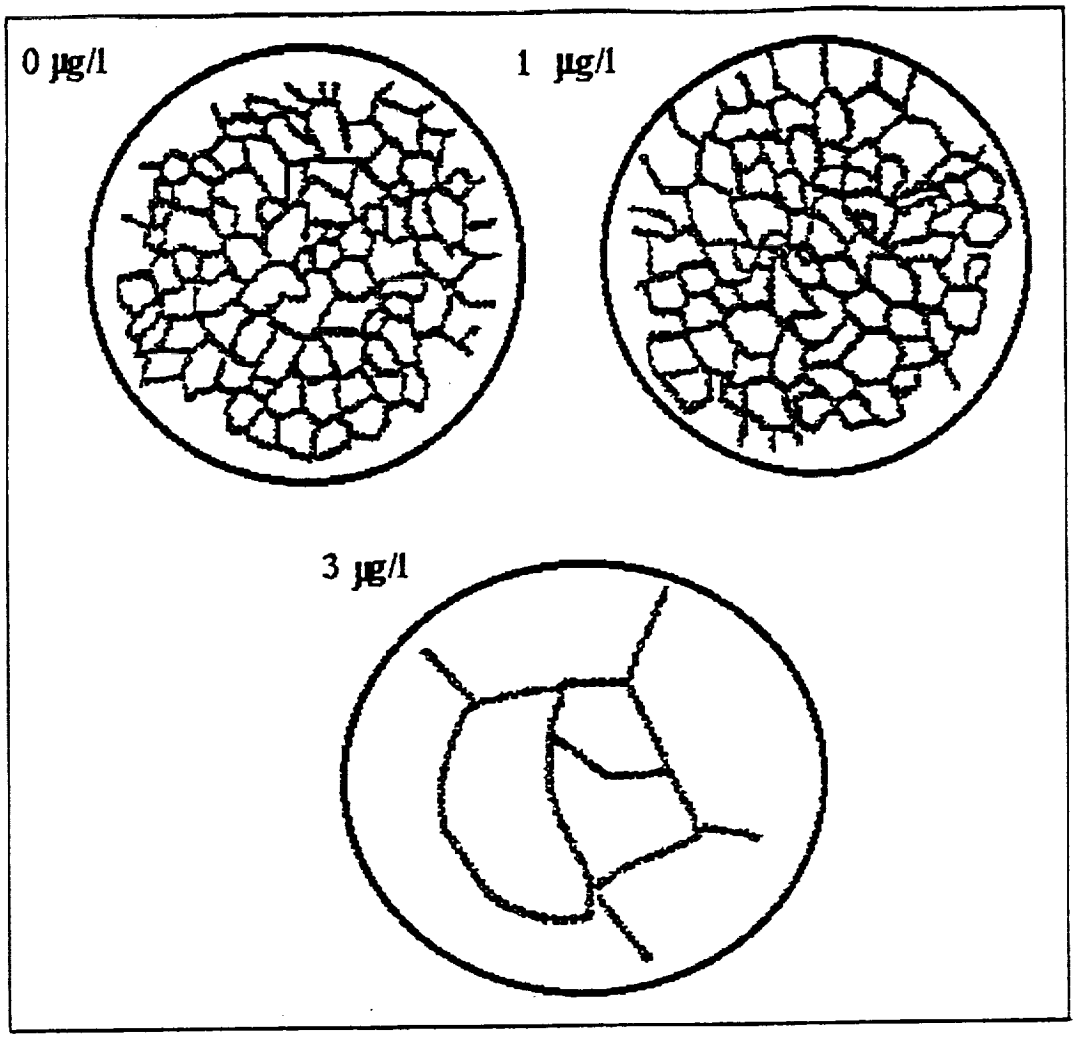

Figure 3. Characteristic *Tetrahymena pyriformis* bioconvective patterns as a function of cadmium concentration between 0-3 µg/1 Cd^{+2}.

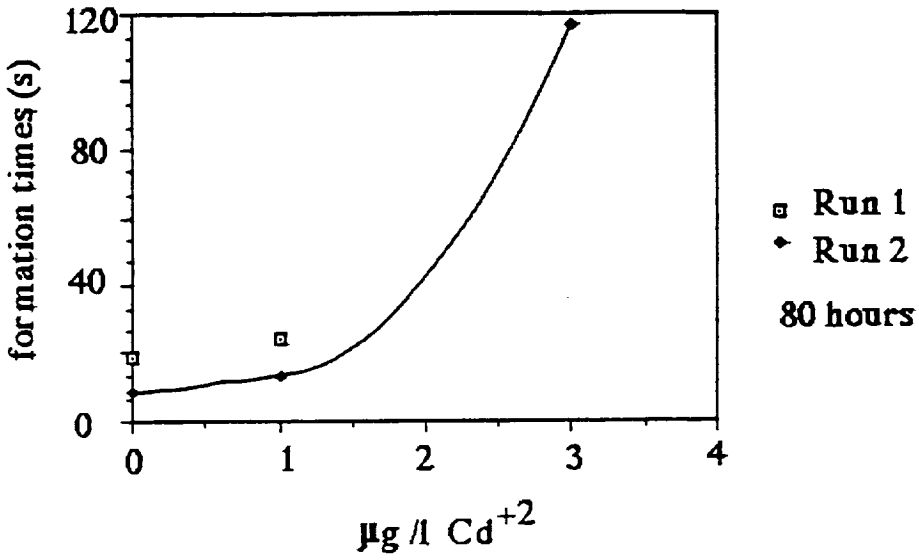

Figure 4. Formation times for *Tetrahymena pyriformis* bioconvective patterns as a function of cadmium concentration. Runs 1 and 2 were for different cultures and organism counts, but both detected the difference between 0-1 µg/1 Cd^{+2}.

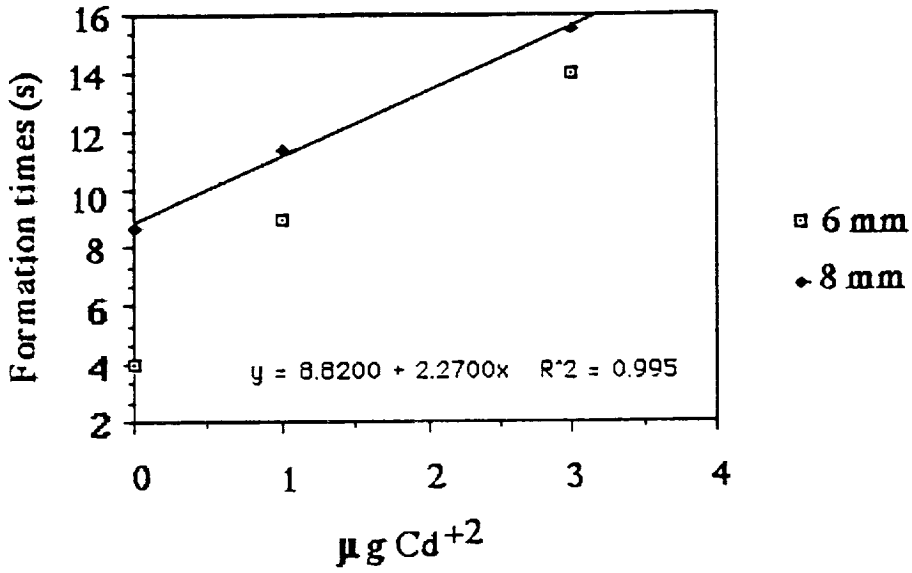

Figure 5. Depth dependence for *Tetrahymena pyriformis* bioconvective patterns as a function of cadmium concentrations. Runs 1 and 2 were for different depths but for the same organism counts, but both detected the difference between 0-1 µg/1 Cd^{+2}.

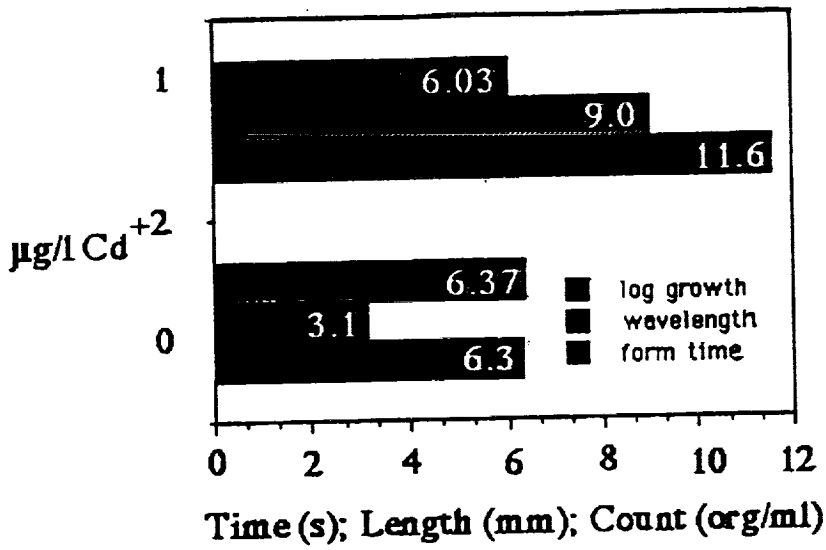

Figure 6. Summary comparison between *Tetrahymena pyriformis* bioconvective patterns as a function of cadmium concentration between 0-1 µg/1 Cd^{+2}. Bar coloring is indicated in bottom right insert for log growth, typical pattern dimension, and formation time.

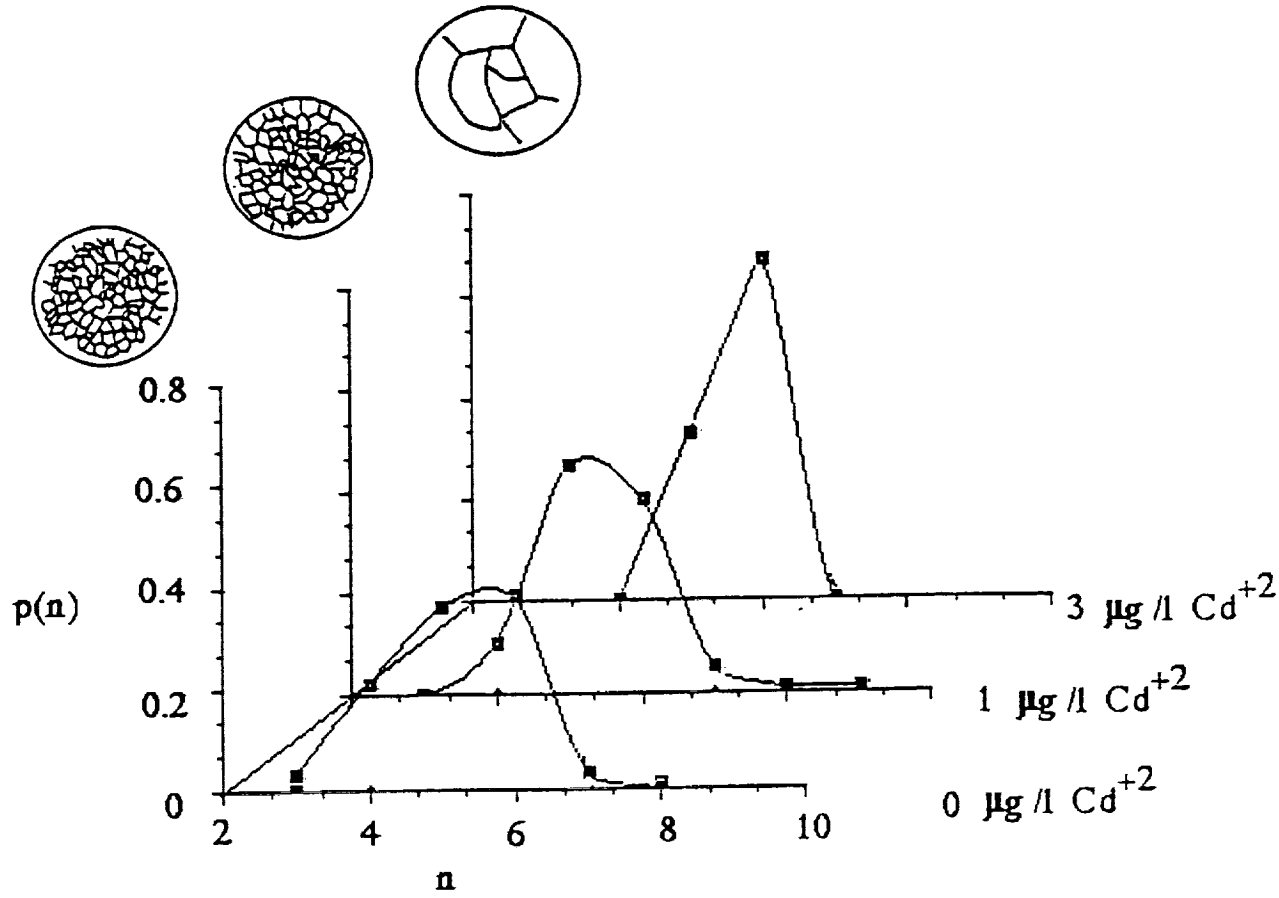

Figure 7. *Tetrahymena pyriformis* bioconvective pattern cell-side distribution for varying cadmium concentrations. Inserts above the y-axis show digitized pictures of actual pattern changes, with p(n) expressing the fraction of n-sided cells. The sharp peak for 3 µg/1 Cd^{+2} is the result of three patterns only as shown.

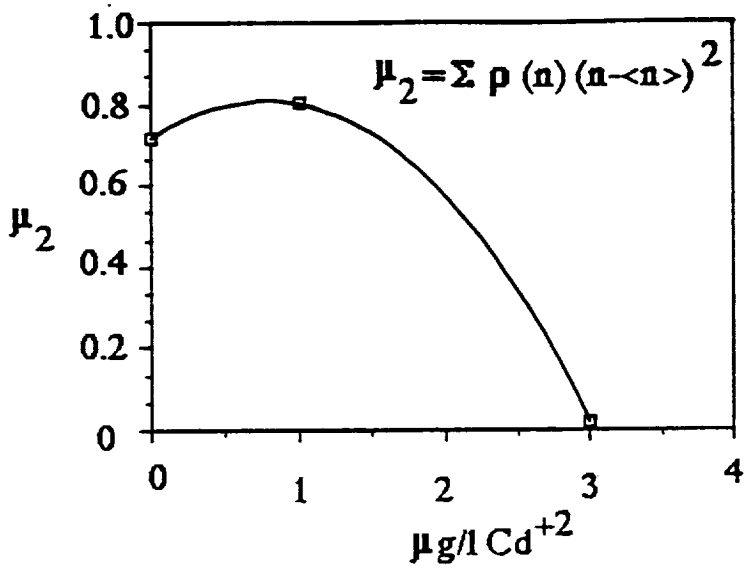

Figure 8. The second moment μ_2 of the *Tetrahymena pyriformis* bioconvective pattern cell-side distribution for varying cadmium concentrations. The upper insert defines the second moment in terms of the probability p(n) of an n-sided pattern found within a general network with average <n>. Results show an abrupt change between 1-3 $\mu g/l\ Cd^{+2}$.

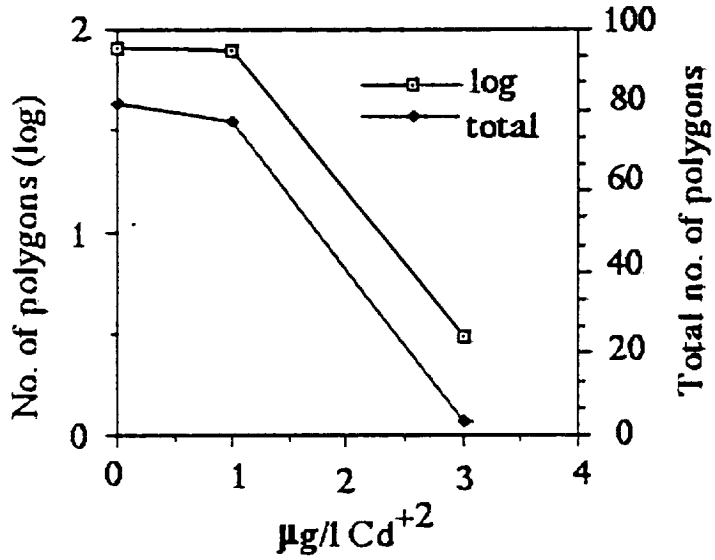

Figure 9. Number of bioconvective polygons for *Tetrahymena pyriformis* patterns as a function of cadmium concentration between 0-3 $\mu g/l\ Cd^{+2}$. Results show an abrupt change between 1-3 $\mu g/l\ Cd^{+2}$.

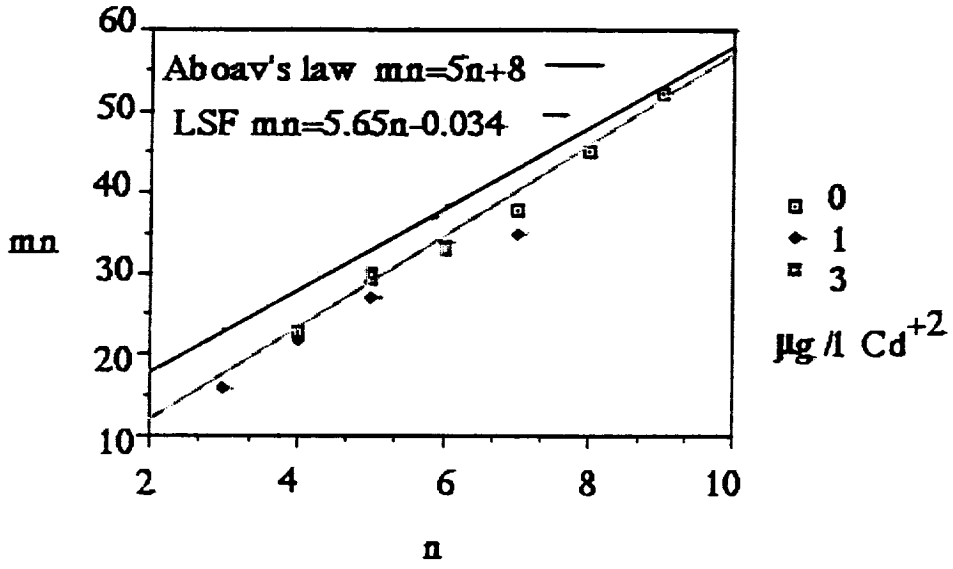

Figure 10. Test of Aboav's law for bioconvective polygons of *Tetrahymena pyriformis* patterns as a function of cadmium concentration between 0-3 µg/l Cd^{+2}. All three cadmium levels indicate a correlation between the number of sides of a polygon, n, and the average number of sides of its neighbors, m(n). Aboav's law predicts the relation plotted as the solid line, whereas the broken line is a least squares fit (LSF) of the data for 0 µg/l Cd^{+2}.

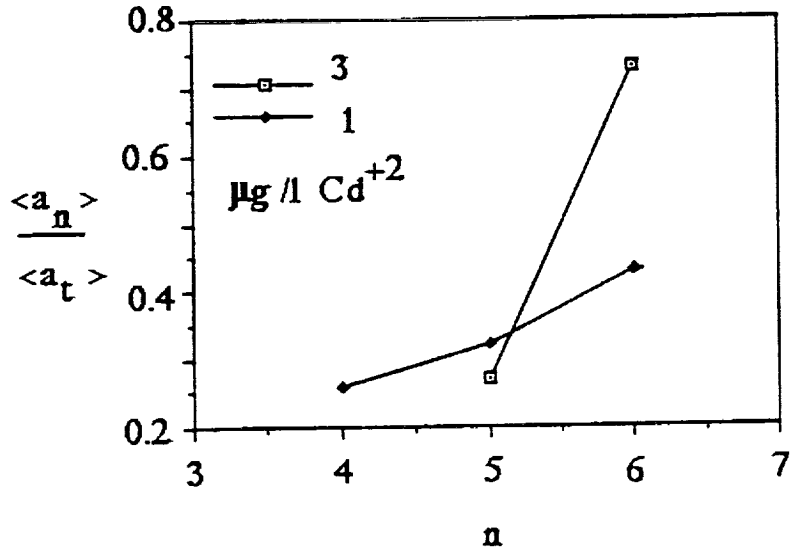

Figure 11. Average polygonal area $\langle a_n \rangle / \langle a_t \rangle$ as a function of n sides for bioconvective polygons of *Tetrahymena pyriformis* patterns as a function of cadmium concentration between 1-3 µg/l Cd^{+2}. Data for 1 µg/l Cd^{+2} indicate a linear fit would be appropriate. Results show an abrupt change between 1-3 µg/l Cd^{+2}.

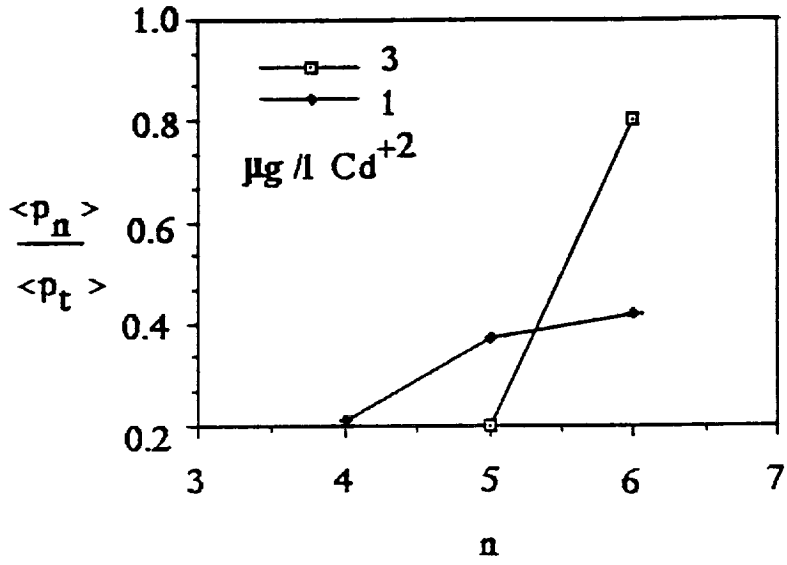

Figure 12. Average polygonal perimeter $\langle p_n \rangle / \langle p_t \rangle$ as a function of n sides for bioconvective polygons of *Tetrahymena pyriformis* patterns as a function of cadmium concentration between 1-3 µg/1 Cd^{+2}. Data for 1 µg/1 Cd^{+2} indicate a linear fit would be appropriate. Results shown an abrupt change between 1-3 µg/1 Cd^{+2}.

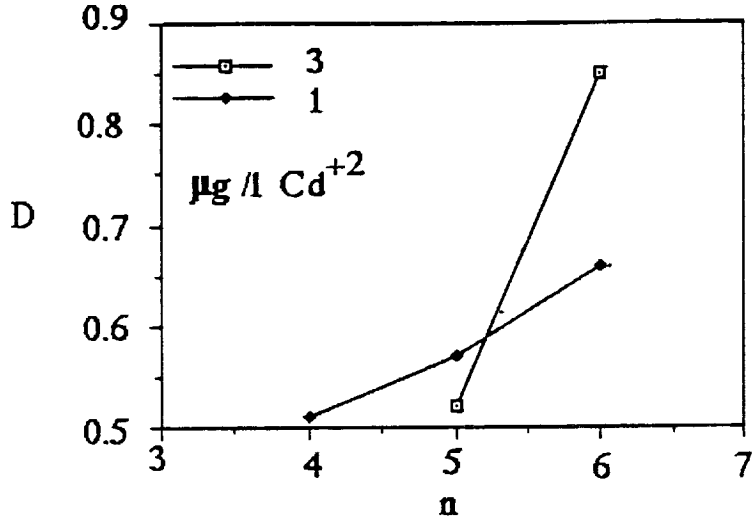

Figure 13. Average maximum polygonal dimension D as a function of n sides for bioconvective polygons of *Tetrahymena pyriformis* patterns as a function of cadmium concentration between 1-3 µg/1 Cd^{+2}. Data for 1 µg/1 Cd^{+2} indicate a linear fit would be appropriate. The units of D represent a relative length for comparison, as calculated from $D=(4A)^{1/2}/\pi$ and Fig. 11. Results show an abrupt change between 1-3 µg/1 Cd^{+2}.

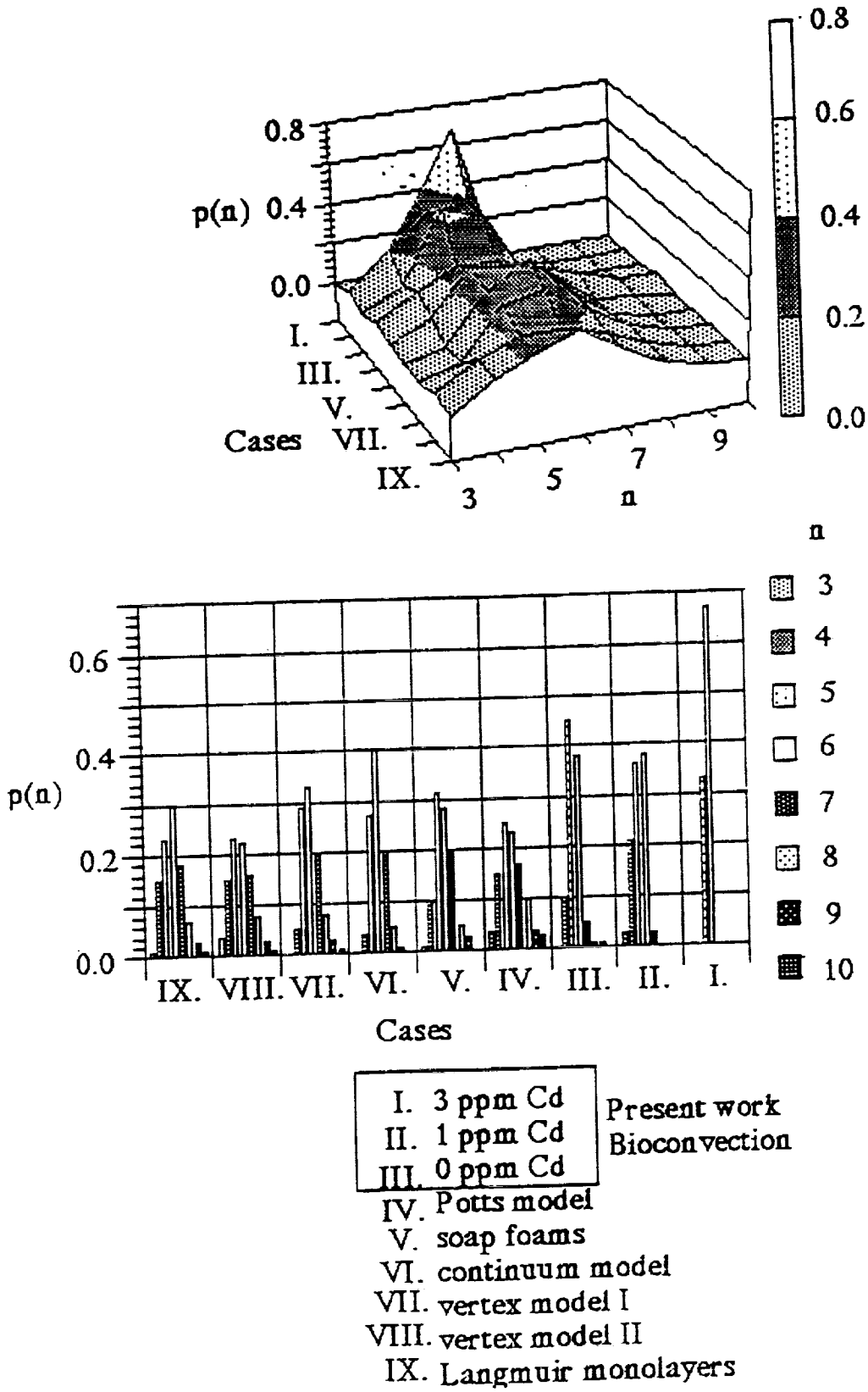

Figure 14. Comparison of polygon-side distributions from the present bioconvective experiment, from various work on foams, and numerical simulations. Some universal characteristics appear as seen in: Cases I-III, this bioconvective work on cadmium; case IV, Potts model, Srolovitz et al. [9]; case V, soap foams, Stavans and Glazier [10]; case VI, continuum model, Beenekker [11]; case VII, vertex model I, Kawasaki et al. [12]; case VIII, vertex model II, Kawasaki et al. [13]; case IX, Langmuir monolayers of foam [5].

APPROVAL

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The information in this report has been reviewed for technical content. Review of any information concerning Department of Defense or nuclear energy activities or programs has been made by the MSFC Security Classification Officer. This report, in its entirety, has been determined to be unclassified.

E. Tandberg-Hanssen

E. TANDBERG-HANSSEN
Director
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