

**Construction of an Anaerobic Gassing Station to Assist in Isolation
and Characterization of Members of a Hydrogen-Utilizing, Chromate-
Reducing Consortium**

Faculty Research Grant Proposal

Submitted to the Faculty Development Committee by:

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I. Project Summary

Chromium is a unique element, functioning as both an essential micronutrient and a carcinogen depending on the valence state. Hexavalent chromium [Cr(VI)] is carcinogenic, mutagenic, and readily soluble in water, allowing it to move easily in aquatic environments. Many industrial processes are known to generate large quantities of Cr(VI)-laden wastewater which must be treated before release. On the other hand, trivalent chromium is necessary for functioning of animal cells, 100-fold less toxic than the hexavalent form, and is nearly insoluble in water. Thus, conversion of the hexavalent form to the trivalent is a satisfactory treatment strategy. Current treatments bring about a chemical reduction but are costly and create other forms of waste. Finding a biological system to catalyze hexavalent chromium reduction presents an economical alternative to current regimes. This requires a deeper knowledge of the biochemistry of the organisms capable of these reactions, a knowledge which is currently unavailable.

Organisms of importance in contaminated ecosystems would likely be categorized as strictly anaerobic, growing only in the absence of molecular oxygen. Cultivation of such organisms would require the use of an anaerobic gassing station to remove all oxygen molecules from gases and materials which might come into contact with microbial cultures. After construction of the gassing station is completed it will be essential in techniques used to study anaerobic organisms such as Cr(VI)-reducing or methanogenic. This funding will allow the purchase of parts and equipment to construct such a system making it possible to begin a research program in anaerobic microbiology at Elmhurst College.

II. Narrative

1. Current Situation

Industrial practices including electroplating, leather tanning, pigment manufacture, corrosion inhibition, and fungicide production generate large quantities of chromium-laden wastewater, which must be treated before discharge. About 170,000 tons of chromium wastes are released into the environment annually. The widespread use of chromium as well as the improper disposal of chromium-laden wastes has led to areas of serious environmental contamination. The prevalence of chromium contamination has resulted in the classification of chromium as one of the seventeen most important environmental toxicants by the U.S. Environmental Protection Agency.

Chromium exists in oxidation states ranging from 0 to +6, although, only two states, Cr(III) and Cr(VI), are commonly observed in environmental samples. The valence state and relative solubility of chromium are dependent on environmental conditions (pH, temperature, redox potential) and the presence of other organic and inorganic molecules. Trivalent chromium, or Cr(III), an essential trace nutrient in the human diet, has relatively low toxicity and is nearly insoluble at neutral pH, and thus, nearly immobile in the environment. Conversely, hexavalent chromium, or Cr(VI) is acutely

toxic, mutagenic, teratogenic, and carcinogenic. In addition, Cr(VI) is highly mobile in the environment, mainly due to its soluble nature.

Common treatment technologies for removal of chromium from industrial waste include ion exchange, electrodepositing, and chemical reduction with iron and sulfur-containing solutions (FeSO_4 , Na_2SO_3 , NaHSO_3 , and $\text{Na}_2\text{S}_2\text{O}_5$) followed by precipitation. These methods, while effective, can be quite costly, requiring high energy input or large quantities of chemical reagents, and can create other forms of waste with unique environmental concerns. The biological reduction of Cr(VI) to Cr(III) may provide a less costly approach to soil and aquifer remediation.

Although many bacterial strains have been shown to mediate reduction of Cr(VI) to Cr(III), few studies have examined the in situ potential of microbial Cr(VI) reduction in aquifers. The stimulation of existing microbial populations may result in microbial chromium reduction, potentially reducing the migration of Cr(VI) in aquifers. Thermodynamic predictions suggest that Cr(VI) reduction will occur after oxygen and nitrate depletion but prior to ferric iron reduction, sulfate reduction, or methanogenesis. Previous work on this project has shown this to be the case. While many bacteria are known to reduce chromium under aerobic conditions, other facultative organisms reduce Cr(VI) only under anaerobic conditions, while strictly anaerobic iron-reducing and sulfate-reducing bacteria require, by nature, highly reducing conditions.

These highly reducing conditions demand the complete absence of oxygen, as oxygen can be toxic to some organisms upon exposure. For example, exposure of methanogenic organisms to oxygen can be lethal. Conducting research on strictly anaerobic bacteria requires special techniques for cultivation in the absence of oxygen. A functional anaerobic laboratory must have an anaerobic chamber, a flexible chamber filled with nitrogen and hydrogen (95:5) in which anaerobic organisms could be handled. In addition, a gassing station, where oxygen would be flushed out of bacterial cultures or any other equipment necessary for handling these organisms, would be essential. Establishing a research project in anaerobic microbiology without both of these components is impossible. Anaerobic chambers are very costly and other funding sources have been sought to obtain this equipment. However, a gassing station is something that is built with gas-tight connections, copper tubing, and a high-powered vacuum pump (for pulling oxygen out of sealed vessels). Building a gassing station would allow a continuation of studies on chromium-reducing bacteria, both in sediments as well as in pure culture.

2. The Project Plan

At the beginning of summer, a gassing station will be constructed in a permanent location out of Swagelok fittings, copper tubing, a heavy-duty vacuum pump, and a tube furnace. The basic theory behind an anaerobic gassing station is that gas (a mix of either nitrogen or hydrogen with carbon dioxide) flowing out of the cylinder would enter a copper column. This column, filled with copper shavings, and heated

to 150° C, would remove any oxygen molecules from the flow of gas by causing a chemical reaction between the copper and oxygen to give cuprous oxide. Gas exiting the column would be free of oxygen and safe for use in the cultivation of strictly anaerobic bacteria. This oxygen-free gas would move through copper tubing to exit at either a manifold or cannula port. In a manifold system, a series of 6-8 outlets would allow an exchange of headspace gases in sealed vessels when combined with the action of the vacuum pump. By this system, existing gas in a sealed bottle could be vacuumed out and replaced with oxygen-free gas. Use of the cannula would allow oxygen to be flushed out of syringes or other equipment necessary to handle anaerobic organisms. Removing all oxygen from syringes allows safe passage of the organisms from one vessel to another without loss of biomass due to oxygen exposure. I will be building the gassing station myself with supplies purchased from this grant. As I am not teaching this summer, I can focus on construction of this equipment.

Upon completion, the gassing station will be used to establish an anaerobic microbiology research program at Elmhurst College. One avenue that this research would take would be to study anaerobic transformation of heavy metals. I have studied this subject for some time, determining the physiochemical conditions necessary for hexavalent chromium reduction to occur in a contaminated aquifer. At the conclusion of my graduate career, I was working with a mixture of bacteria which was able to use hydrogen as an energy source to reduce hexavalent chromium to a non-toxic form. This consortium grew and carried out the reduction in only 4-5 days, making it the fastest anaerobic chromate-reducing consortium reported in the literature. Working to continue isolation and characterization of individual chromate-reducing organisms from this mixture will be the major research focus of this project.

I also wish to expand my studies specifically to methanogenic bacteria, which gain energy by metabolizing hydrogen (H₂) and carbon dioxide (CO₂) to form methane (CH₄). These organisms are among the most fastidious of the strict anaerobes and grow at very low redox potentials. Methanogens have never been shown to transform metals but molecular sequencing has shown that they possess heavy-metal reductases and heavy metal ATPases (enzymes that reduce heavy metals and form adenosine triphosphate [ATP], respectively). It would be logical for these organisms to have metal transforming abilities since they are among the most primitive organisms on Earth, having existed in the "primordial soup," an environment high in heavy metals and devoid of oxygen. This research project would take time because most of the anaerobic bacteria grow more slowly than aerobic organisms (one month compared to 24 hours).

In addition to allowing the start-up of anaerobic microbiology research at Elmhurst College, it is possible that in the future the anaerobic gassing station could be used in an advanced microbiology course to study microbial ecology of complex biological systems. Similarly, this project would be excellent for giving undergraduate students direct research experience. The most competitive students leaving college are those that participated in an independent research project or internship. These

experiences give students an advantage when moving into either professional school (graduate or medical) or into the job market (industry).

3. Faculty Expertise

I have worked with anaerobic bacteria for nearly 10 years. My research focused on the physical/chemical conditions necessary for stimulation of metal transformation by naturally occurring bacteria in a contaminated aquifer. I also spent one year as a post-doctoral associate studying inhibition of methanogenic metabolism by pure culture organisms. In both cases, the research centered on use of an anaerobic gassing station. Finally, before joining the Elmhurst College faculty I was employed by the U.S. EPA as a post-doc where I built a gassing station for use in studying organic chemical transformations by anaerobic bacteria.

4. Plans for Evaluation and Dissemination

During the course of this research, progress will be presented at national meetings for the American Society of Microbiology. When a significant body of acceptable results has been collected a paper will be submitted to a peer-reviewed journal. Regional and local opportunities to share this research progress will also be considered, especially at research symposia held on campus.

III. Time Line

Construction of the anaerobic gassing station will be complete one month after receiving all necessary equipment and connections. After completion of the gassing station, my time will be split for the remainder of the summer (80% research; 20% teaching preparation). During the coming semesters research will proceed and interested undergraduates will be involved, trained, and given a specific aspect of the project with which to work.

IV. Budget

Please see Excel budget spreadsheet (Grant Budget) below. All prices were gathered from on-line catalogs of Fisher Scientific, Swagelok, Inc., and Cole-Parmer and are the most current prices for the items listed.

Necessary Parts	Part Number	Supplier	Price	Quantity	Total Co
Vacuum Pump	01-183-52	Fisher	\$2,002.00	1	\$2,002.00
Heating Tape	11-463-51B	Fisher	\$85.00	1	\$85.00
Temperature Controller for heating tape	11-463-50	Fisher	\$162.00	1	\$162.00
Temperature Regulator	11-463-49	Fisher	\$114.00	1	\$114.00
Nonabsorbant cotton	07-895	Fisher	\$24.70	1	\$24.70

Copper tubing (1/8")	22308302	Fisher	\$32.45	1	\$32.
Copper tubing (1/4")	22308303	Fisher	\$38.11	1	\$38.
Copper turnings	AC317912500	Fisher	\$27.40	2	\$54.
Gas regulator (Hydrogen)	10-572-1K	Fisher	\$172.20	1	\$172.
Gas regulator (Nitrogen)	10-572-1E	Fisher	\$171.00	1	\$171.
Vacutainer needles (22 gauge)	02-665-17	Fisher	\$19.53	1	\$19.
Butyl Rubber tubing	14-168B	Fisher	\$38.91	1	\$38.
Glass syringes (2cc), Becton Dickinson 2440	14-823-10A	Fisher	\$34.30	2	\$68.
Blunt end needles (19 gauge X 4")	14-825-16G	Fisher	\$77.43	1	\$77.
Polyethylene tubing	1417016	Fisher	\$67.32	1	\$67.
1/8 inch union cross	B-200-4	Swagelok	\$9.40	6	\$56.
Three-way ball valves	B-41XS2	Swagelok	\$31.10	3	\$93.
Union tee (1/8")	B-200-3	Swagelok	\$6.20	1	\$6.
Metering valve	B-2MG	Swagelok	\$37.20	2	\$74.
Teflon ferrules	TFE-204-1	Swagelok	\$27.20	1	\$27.
Teflon ferrules	TFE-203-1	Swagelok	\$27.20	1	\$27.
Compound Gauge	G-68007-01	Cole-Parmer	\$87.25	1	<u>\$87.</u>
TOTAL					\$3,500.

V. Current and Previous grants

None

VI. Publications

Marsh, T.L., and M.J. McInerney. 2001. Hydrogen bioavailability as a measure of potential for chromate reduction. *Appl. Env. Microbiol.* 67(4):1517-1521.

Marsh, T.L., Leon, N.M., and M.J. McInerney. 2000. Physiochemical factors affecting chromate reduction by aquifer materials. *Geomicrobiol. J.* 17(4):291-303.

Marsh, T.L. 2000. Anaerobic reduction of hexavalent chromium by subsurface microorganisms. Invited oral presentation at ASM General Meeting, May 21-25, 2000.

Marsh, T.L., and M.J. McInerney. 1999. Factors affecting reduction of hexavalent chromium by aquifer materials. Oral presentation at International Symposium on Subsurface Microbiology, August 22-27, 1999.

Marsh, T.L., and M.J. McInerney. 1999. Hydrogen bioavailability as a measure of chromate reduction potential. Presented at ASM General Meeting, May 30-June 3, 1999.

Marsh, T.L., and M.J. McInerney. 1998. Microbially-mediated reduction of hexavalent chromium. Oral presentation at U.S. Geological Survey Annual Meeting for Norman Landfill, February 24-25, 1998.

Marsh, T.L., and M.J. McInerney. 1997. Biological reduction of hexavalent chromium by aquifer sediments. Presented at ASM General Meeting, May 4-8, 1997.

Marsh, T.L., V.E. Worrell, N. Leon, and M.J. McInerney. 1996. Biological reduction of hexavalent chromium by aquifer sediments. Presented at The Art of Anaerobes Conference, August 16-17, 1996. Accepted for publication in Volume 6, Number 1 of BioFactors.

McInerney, M.J., and V.E. Worrell, N. Leon, and T.L. Marsh. 1996. Development of techniques for *in situ* bioremediation of chromium contaminated soil and groundwater: Phase 1. Laboratory evaluation. U.S. EPA Final Technical Program Report. Submitted August 1996.

Marsh, T.L., X. Xiang, R.M. Knapp, M.J. McInerney, P.K. Sharma, and B.E. Jackson. 1995. Mechanisms of microbial oil recovery by *Clostridium acetobutylicum* and *Bacillus* Strain JF-2. In Conference proceedings, The Fifth International conference on Microbially Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems.

Marsh, T.L. and M.J. McInerney. 1995. Physiology of a halotolerant, thermotolerant, exopolysaccharide-producing *Bacillus* species. Oral presentation at ASM Missouri Valley Branch Annual Meeting, March 31- April 1, 1995.