ENGINEERING-PDH.com
ONLINE CONTINUING EDUCATION

BIOLOGICAL PHOSPHOROUS REMOVAL FROM IMPACTED WATER

Main Category:	Environmental Engineering
Sub Category:	-
Course #:	ENV-127
Course Content:	20 pgs
PDH/CE Hours:	2

OFFICIAL COURSE/EXAM

(SEE INSTRUCTIONS ON NEXT PAGE)

WWW.ENGINEERING-PDH.COM
TOLL FREE (US & CA): 1-833-ENGR-PDH (1-833-364-7734)
SUPPORT@ENGINEERING-PDH.COM

ENV-127 EXAM PREVIEW

- TAKE EXAM! -

Instructions:

- At your convenience and own pace, review the course material below. When ready, click "Take Exam!" above to complete the live graded exam. (Note it may take a few seconds for the link to pull up the exam.) You will be able to re-take the exam as many times as needed to pass.
- Upon a satisfactory completion of the course exam, which is a score of 70% or better, you will be provided with your course completion certificate. Be sure to download and print your certificates to keep for your records.

Exam Preview:

1. Nutrients such as nitrogen, phosphate, and organic carbon are typically growth-promoting substrates that, when present in high concentrations, allow for unchecked growth of photosynthesizers.
    a. True
    b. False
2. One potential method of immobilizing cells is using silica sol-gel. Sol-gel is a process by which siliceous molecules in a solution (sol) agglomerate to form a network (gel). Studies have shown that E. coli encapsulated in sol-gel can remain metabolically active for over ____ months.
    a. 2
    b. 4
    c. 6
    d. 8
3. According to the reference material, current approaches to the algal bloom problem have been reactionary, treating the symptoms of entrenched blooms.
    a. True
    b. False
4. According to the reference material, how many different bacterial strains were tested for their ability to accumulate phosphate?
    a. 2
    b. 3
    c. 4
    d. 5

5. According to the reference material, wash tests were performed with methanol, bleach, and sterile water. Methanol was found to kill the encapsulated bacteria, even at short exposure times.
    a. True
    b. False
6. According to the reference material, which of the following phosphate accumulating organisms proved to be the most effective at phosphorous uptake?
    a. Nocardioides luteus BAFB
    b. Rhodococcus jostii RHA1
    c. Gordonia sp. KTR9
    d. Microlunatus phosphovorus
7. Ultimately, it was determined the best method with maximum cell activity was tandem washes in sterile water for ten min each. However, on average, __% of the replicates had cells escape/media contamination.
    a. 15
    b. 30
    c. 35
    d. 45
8. According to the reference material, the number of freshwater sources in the U.S. with documented hypoxia has increased dramatically.
    a. True
    b. False
9. According to the reference material, N. luteus had a faster rate of uptake at 4.6 ppm PO4 /h (1.59 mmol P/h), it only reduced phosphate concentration by ___% before releasing some (40 ppm) back into the media at 116 h.
    a. 5
    b. 10
    c. 15
    d. 20
10. According to the reference material, this concept has potential for very large scale up, moderate cost, high profile, environmental stewardship as an ecologically friendly, non-invasive solution for phosphate removal with the value-added benefit of producing commercially available phosphorous.
    a. True
    b. False

Abstract

Nutrient input into waterways from agriculture runoff, sewage, and other sources is a major cause of water pollution and cultural eutrophication. Nitrogen and phosphate are growth-limiting nutrients, that when present in high concentrations, allow for unchecked growth of cyanobacteria, resulting in reduced water quality and the potential for proliferation of harmful algal blooms (HABs). Physical removal of these nutrients from waterways is needed. Immobilized phosphorous accumulating bacteria could be used as a phosphate biofilter to absorb and accumulate phosphate from impacted water. The research presented herein demonstrates the immobilization or encapsulation of phosphate accumulating bacteria in a silica sol-gel matrix that can be suspended in a high phosphate water system and then retrieved to collect excess phosphate (PO_4). Growth and PO_4 uptake was monitored in free-living and sol gel immobilized, pure cultures of the phosphate hyperaccumulating bacterium *Microlunatus phosphovorus*. Phosphate was accumulated at a rate of 3.3 parts-per-million (ppm) PO_4 /h (1.11 mmol P/h) and 80 mmol P/g cell dry wt/h, respectively. Application of immobilized cells to phosphate impacted waters for uptake and removal of phosphate would allow for large-scale removal of phosphate, thus reducing the eutrophication effect and limiting the potential for HABs.

Contents

Figures and Tables

Figures

Tables

Unit Conversion Factors

Multiply	By	To Obtain
degrees Fahrenheit	(F-32)/1.8	degrees Celsius
feet	0.3048	meters
inches	0.0254	meters
inch-pounds (force)	0.1129848	newton meters
microinches	0.0254	micrometers
ounces (U.S. fluid)	2.957353 E-05	cubic meters
pints (U.S. liquid)	0.473176	liters
quarts (U.S. liquid)	9.463529 E-04	cubic meters
square inches	6.4516 E-04	square meters

Acronyms and Abbreviations

APCRP	Aquatic Plant Control Research Program
ATP	adenosine triphosphate
DoD	Department of Defense
EL	Environmental Laboratory
EPP	Environmental Processes Branch
EPED	Environmental Processes and Engineering Division
ERDC	Engineer Research Development Center
HABs	Harmful Algal Blooms
HCl	hydrochloric acid
h	hours
LB	Luria Broth
MTES	methyltriethoxysilane
ml	Mililiter
ML	Microlunatus
min	minutes
OD	Optical Density
PAOs	Phosphate Accumulating Organisms
PHA	polyhydroxyalkanoates
PO_4	Phosphate
ppm	parts-per-million
SGM	sol-gel matrix
TEOS	tetraethylorthosilicate
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency
WWTPs	Wastewater Treatment Plants

1 Introduction

1.1 Background

Nutrient input into waterways from agriculture runoff, sewage, and other sources is a major cause of water pollution. This process, known as cultural eutrophication, impairs many recreational and commercially important waterways (Chislock et al. 2013). Nutrients such as nitrogen, phosphate, and organic carbon are typically growth-limiting substrates that, when present in high concentrations, allow for unchecked growth of photosynthesizers. Affected sites are not always known, reported, or fully characterized. The availability of these nutrients allows organisms such as cyanobacteria (blue-green algae) to voraciously consume these abundant nutrients, "crowding out" aquatic plants and beneficial green algae, which are important food sources for littoral grazers. Significant cyanobacterial growth may result in reduction in water clarity and light penetration, leading to plant die off, hypoxia, or anoxia.

Another significant and potentially dangerous issue is the proliferation of harmful algal blooms (HABs). Numerous species of cyanobacteria and some true algae (i.e., golden algae) are known to produce toxins that can affect morbidity and mortality of aquatic and terrestrial wildlife and that of humans and domestic animals. Freshwater algal blooms are ubiquitous in the U.S., and without a common reporting mechanism the number of incidences per year is unknown. However, large-profile blooms resulting in toxin release are generally reported and are compiled by various governmental agencies as well as interest groups.

Current approaches to the algal bloom problem have been reactionary, treating the symptoms of entrenched blooms. Unfortunately, algal blooms are a symptom, and the underlying cause is the high nutrient levels which allow the algal blooms to recur. In most cases agricultural or municipal runoff of phosphorous and nitrogen are the primary nutrients released into these waterways, with phosphorous being the predominant growth limiting nutrient (Kortsee et al. 1994). Numerous regulations have been introduced to curb this nutrient input, including reductions in phosphorous-containing fertilizers for municipal and commercial use (summarized at https://www.epa.gov/nutrient-policy-data/what-epa-doing-reduce-nutrient-pollution). Public education also has been important in reducing nutrient

input. For example, the U.S. Environmental Protection Agency (USEPA) has compiled a list of useful educational resources for the individual including webinars, videos, pamphlets, and links to other entities with site specific information (https://www.epa.gov/nutrientpollution/harmful-algal-blooms#act). Other means for combating eutrophication have included algaecides and herbicides, and decreasing the photosynthetic ability of aquatic life through shading or additive obscurants like dyes (Chislock et al. 2013). However, in order to prevent recurrence, physical removal of these nutrients from the waterway or watershed is needed.

One approach for phosphate removal is by using phosphate accumulating organisms (PAOs). Some municipal wastewater treatment plants (WWTPs) utilize PAOs as a biological method for removing excess nutrients from their system. Numerous strains of phosphorous hyperaccumulating bacteria have been employed in municipal wastewater treatment plants for nutrient removal. These PAO bacteria accumulate phosphate intracellularly during aerobic respiration as a mechanism for storing phosphate for adenosine triphosphate (ATP) production during anaerobic conditions (Keasling et al. 2000). As the cells proliferate in the aerobic phase of wastewater treatment, they take up phosphate before entering the settling phase, where they are added to the solid sludge material and separated from the treated water (Keasling et al. 2000; Martin et al. 2006). While this is an effective approach in a contained system such as a waste water treatment plant, introduction of such cells to a water body would result in temporary sequestration of phosphate into cells, but not permanent removal from the system. In order to effectively remove phosphate from water bodies where flocculating the cells is not possible, some other method is needed to immobilize cells for removal.

1.2 Approach

One potential method of immobilizing cells is using silica sol-gel. Sol-gel is a process by which siliceous molecules in a solution (sol) agglomerate to form a network (gel). The specifics of the sol-gel system used herein are presented in the Methods and Materials Section. Sol-gel encapsulation can be performed at physiologically relevant temperatures and pH thus making it ideal for preserving biological activity (Gill and Ballesteros 2000). In addition, silica is biologically inert and resistant to many biological degradation mechanisms and the low cost makes it attractive for industrial applications (Yu et al. 2005). Encapsulation in sol-gel is also known to maintain activity of microbial cells much longer than in liquid

culture. Studies have shown that *E. coli* encapsulated in sol-gel can remain metabolically active for over two months (Mutlu et al. 2015). Previous work has also demonstrated that encapsulation of cells within a sol-gel matrix effectively inhibits cell proliferation (Nassif et al. 2002; Premkumar et al. 2002).

1.3 Objective

The goal of this study was to find a way to encapsulate phosphate accumulating bacteria, introduce them into open water systems, allow them to take up phosphate, then remove the cells and phosphate entirely from the water. The results indicate that encapsulated cells are an effective means of sequestering phosphate, however a more robust encapsulation matrix is needed to inhibit cell escape.

1.4 Scope

The team hypothesized that sol-gel encapsulation of PAOs would enable efficient removal of phosphate from water through recovery of the introduced cells. To our knowledge this work is the first attempt to immobilize PAOs in an environmentally inert silica matrix (sol-gel) for use as a phosphate biofilter. Repeated application of immobilized cells could allow for large-scale removal of phosphorous, thus reducing the eutrophication effect and limiting the potential for harmful algal blooms.

2 Methods and Materials

2.1 Growth of pure cultures

Four bacterial strains were tested for their ability to accumulate phosphate. *Microlunatus phosphovorus* (ATCC 700054), which is a known phosphate accumulator, *Gordonia* sp. KTR9 (Indest et al. 2013; Indest et al. 2010), *Rhodococcus jostii* RHA1 (Indest et al. 2015; Indest et al. 2016), and *Nocardioides luteus* BAFB (Jung et al. 2002), which are actinomycetes known to produce high concentrations of polyhydroxyalkanoates (PHA) in a process often linked to phosphate accumulation (Seviour et al. 2003). Cells were grown in 50 ml *Microlunatus* (ML) medium (Nakamura et al. 1995) cultured in 250 ml flasks at 30° C, shaking for 48 hours (h), pelleted, washed twice in sterile saline, then inoculated into 50 ml fresh ML medium in 250 ml flasks at an optical density (OD) 0.04 at 600 nm, in triplicate, at 30° C, shaking for 116 h.

2.2 Monitoring of phosphate uptake

Phosphate concentrations were measured colorimetrically with a LaMotte Series 1200 colorimeter (LaMotte Company, Chesterton, MD.) as O-phosphate via the ascorbic acid method which utilizes ammonium molybdate and antimony potassium tartrate. Reagents and methods were provided with the LaMotte Series 1200 instrument. Limits of detection were between 0.01–3 ppm. Samples with concentrations above 3 ppm were diluted appropriately in phosphate-free water.

2.3 Sol-gel encapsulation

Bacterial cells were encapsulated in a sol-gel matrix (SGM) following the method of Dickson and Ely, 2011 (Dickson and Ely 2011) as described in Table 1. The ratio of tetraethylorthosilicate (TEOS) and methyltriethoxysilane (MTES) was varied to empirically determine the best formulation for highest phosphate uptake and least bacterial cell escape. The acidified precursor consisting of TEOS, MTES, water, and HCl was stirred for 12 h prior to encapsulating cells. First trials began by incorporating cells at~108 cells/ml into plastic 15 ml test tubes or 1/8 in. sterile silicon tubing, expulsing the material once solidified, and cutting the SGM into ¼–½ in. plugs.

Table 1. Cell encapsulation formulation as determined empirically to preserve physiological activity and reduce escape of cells from the matrix (modified from Dickson and Ely 2011).

Formulation component	Volume (ml)	Notes
Sol-gel precursor		
Tetraethylorthosilicate (TEOS)	6.4	Can be scaled up or down proportionally. Final pH is ≈ 1.5. Add to sterile flask containing stir bar and mix for >12 h.
Methyltriethoxysilane (MTES)	1.6	
Water	14.85	
Concentrated hydrochloric acid (HCl)	0.75	
Total precursor volume	23.6	
Encapsulated cells		Can be scaled up or down proportionally. Final pH is ≈ 7. Add to sterile container, mix well, and distribute quickly before solidifying (≈ 30 min).
Cells in medium or buffer (10^8 cells/ml)	1.0	
0.2M Tris HCl pH7.5	12.5	
sol-gel precursor	4.5	
Total cell mix volume	18.0	

2.4 Sol-gel washing trials

Cells embedded in SGM plugs were placed in sterile saline, methanol, or bleach solution to remove cells from the surface. Methanol was used at 100% strength for washes of 1, 5, or 10 minutes (min), followed by a five minute sterile water wash. Bleach washes were conducted for one minute each at 8, 0.8, 0.008, and 0.0008% followed by three, one minute water rinses. Sterile water rinses were also performed for five and ten min, twice each. Outgrowth tests were performed in 0.5× Luria Broth (LB) with a 0.5 mg/ml tetrazolium violet indicator. This allowed for the direct observation of metabolism in the pellets by the formation of purple color formation (Figure 1) indicative of substrate reduction via metabolic activity, and escape of cells into the media by turbidity and purple color formation. It is important to note that 0% escape was never reliably achieved, one of three replicates usually showed outgrowth of cells, consistent with other reports (Nassif et al. 2002).

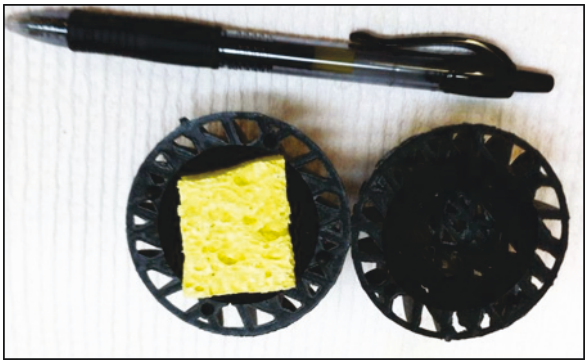
Figure 1. Outgrowth test to determine survival and escape of cells from the SGM in which they were embedded. Purple color indicates cell activity.

2.5 Phosphate uptake by cells in SGM

The first tests were done in 96-well format (250 µl wells) to determine the physiological activity and survival of the encapsulated cells over time. *M. phosphovorus* cells were added to a SGM and 100 µl poured into multiple wells of a 96-well plate. Activity over time (53 days) was assessed by addition of 0.5× Luria Broth (LB) with 0.5 mg/ml tetrazolium violet to selected wells which were then monitored for purple color in the SGM. This activity was generally evident within 1–6 h.

Sterile cellulose sponges (autoclaved, 1 in.× 0.75 in. cylinders) were then used as a support matrix for the SGM, as it became evident that the SGM plugs were too fragile to be placed in a water system without support. The sponges were saturated with 8 ml liquid SGM and cells which solidified within the pore spaces of the sponge. The sponge-SGM was then encased in a 3 in. hard-shelled, flow-through sphere (pond or aquarium bioball, purchased from Como, Inc. on Amazon.com(Figure 2), this was not only protective against innumerable physical insults that would be encountered in a waterway, but also allows for easy collection. One bioball was added to each of the 1 L beakers filled with 600 ml 0.5×LB and phosphate uptake was monitored in the same fashion as the free-living pure culture experiments. Sterile control SGM was also prepared using glycerol in place of cells.

Figure 2. Protective plastic flow-through encasement for sol-gel embedded cells.



3 Results and Discussion

There are many phosphate accumulating organisms that could be used in this study. To date, *Microlunatus phosphovorus* (ATCC 700054), a bacterial strain that was isolated from activated sludge of a municipal wastewater treatment plant and was determined to hyperaccumulate phosphate (PO4) (Nakamura et al. 1995), and three actinomycete strains, *Gordonia sp*. KTR9, *Rhodococcus jostii* RHA1, and *Nocardioides luteus* BAFB have been tested. Growth (Figure 3) and PO4 uptake (Figure 4) was monitored in free-living pure cultures of these four strains. *M. phosphovorus* was most effective at phosphorous uptake (44% reduction in the phosphate concentration) with a calculated rate of 3.3 ppm PO4 /h (1.11 mmol P/h) which is consistent with published values (Santos et al. 1999). Although N. luteus had a faster rate of uptake at 4.6 ppm PO4 /h (1.59 mmol P/h), it only reduced phosphate concentration by 20% before releasing some (40 ppm) back into the media at 116 h. Both of these rates, however, are substantial since environmentally relevant conditions approaching 1 ppm are considered eutrophic. Upon switching the cells to an anaerobic environment, the PO4 was released at a rate of 1.75 ppm PO4/h (0.6 mmol P/h) (data not shown), thus allowing control of the uptake and release of PO4 by the bacteria from the water.

Figure 3. Comparative growth of potential phosphate accumulating organisms over 116 h.

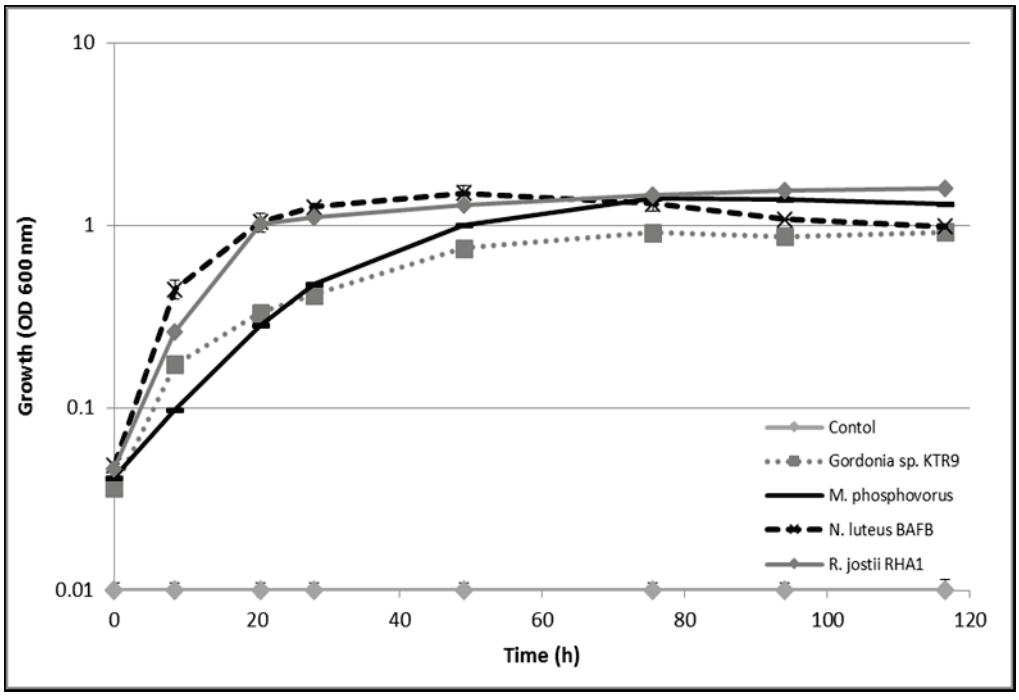
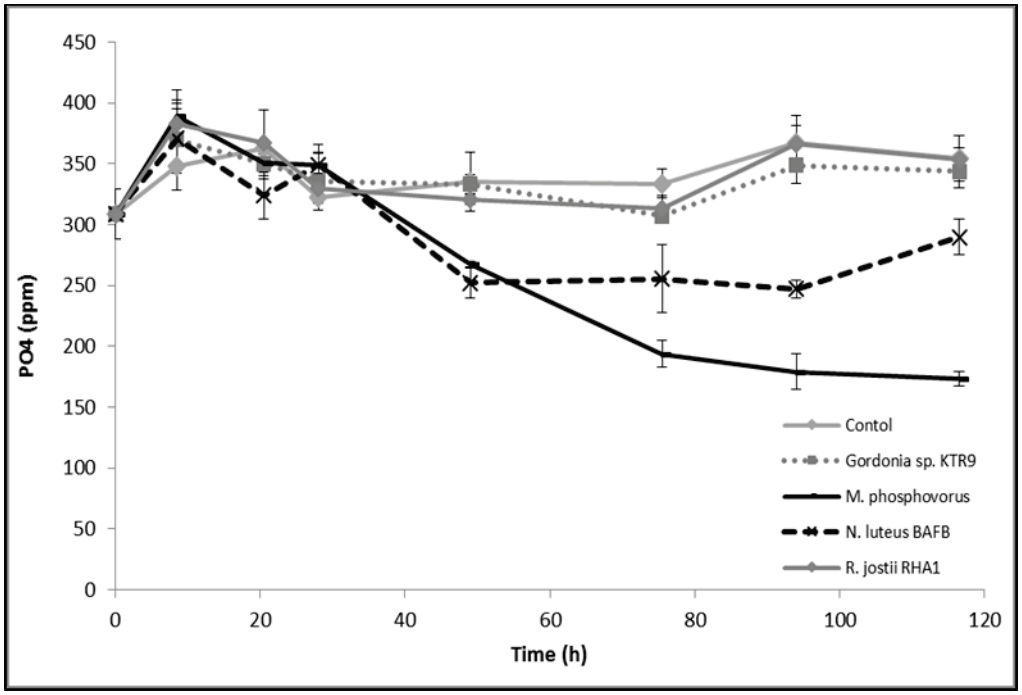
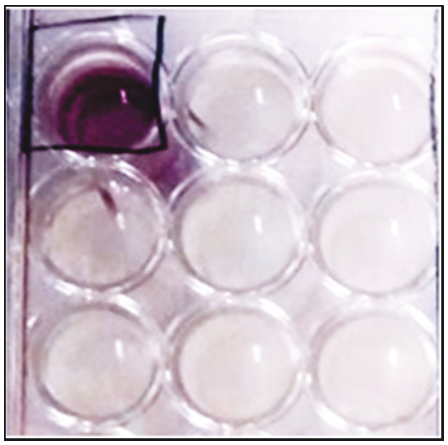


Figure 4. Phosphate uptake from growth medium of putative phosphate accumulating organisms over 116 h.



Encapsulation of viable cells within SGM has been demonstrated to improve the long-term stability and metabolic activity of cells when compared to liquid cultures (Saez et al. 2012; Yu et al. 2005). Silica sol-gels can be formulated under biologically compatible temperature and pH and are highly porous, allowing diffusion of substrates while retaining the encapsulated cells. Briefly, cells were encapsulated in a silica SGM (Table 1) to enable physiological functionality of *M. phosphovorus* over 53 days post encapsulation. *M. phosphovorus* was still capable of converting the tetrazolium violet indicator to violet, indicating the cells were still active (Figure 5).

Figure 5. Sol-gel encapsulated *M. phosphovorus* cells in microplate wells. Purple color indicates metabolic activity.

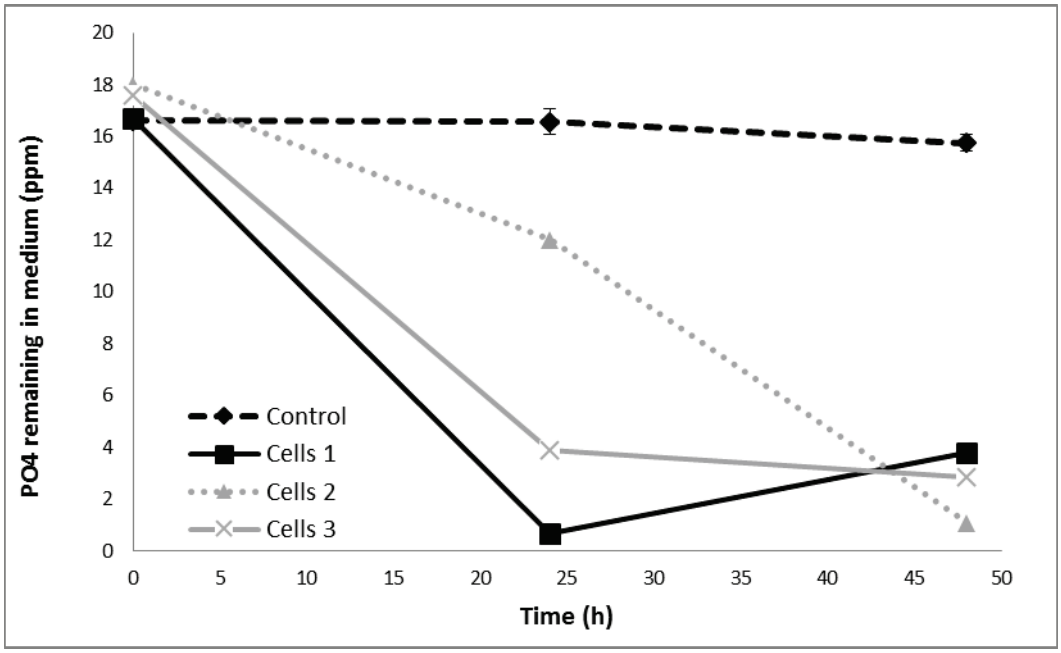


Cell escape was assessed with various methods as simple water washes were found to be unpredictable and limitedly successful. On average, one replicate from each triplicate experimental set exhibited outgrowth of the encapsulated cells into the media. Wash tests were performed with methanol, bleach, and sterile water. Methanol was found to kill the encapsulated bacteria, even at short exposure times. Diluted bleach was found to be somewhat effective at 0.8–0.08% with a 30-second exposure for preventing outgrowth, but the embedded cells along the periphery were physiologically inactive (clear margin around SGM pellets with purple throughout the interior) (Figure 1), affecting rates of phosphate uptake significantly (data not shown). Ultimately, it was determined the best method with maximum cell activity was tandem washes in sterile water for ten min each. However, on average, 30% of the replicates had cells escape/media contamination. The cell escape assays were unpredictable as some triplicate samples were all contaminated, while others had one or no contamination events. No-cell controls were always run and found to be void of contamination, indicating these events were due to cell escape. The frequency of contamination of cells “leaking” into the medium after numerous washes is indicative of the SGM being compromised and allowing cells to be liberated from the encasement. A method to ensure the cells will not “leak” into the environment will be necessary before this technology can be taken to field scale.

Once cells were encapsulated in the sol-gel and sponge matrix, phosphate uptake was monitored. As seen in Figure 6, the replicates were variable in terms of cell escape which resulted in media contamination easily

visualized by turbidity. However, the encapsulated cells were capable of accumulating phosphate from the medium. One cause of cell escape was thought to be physical breakage of the SGM, even when cast in a sponge.

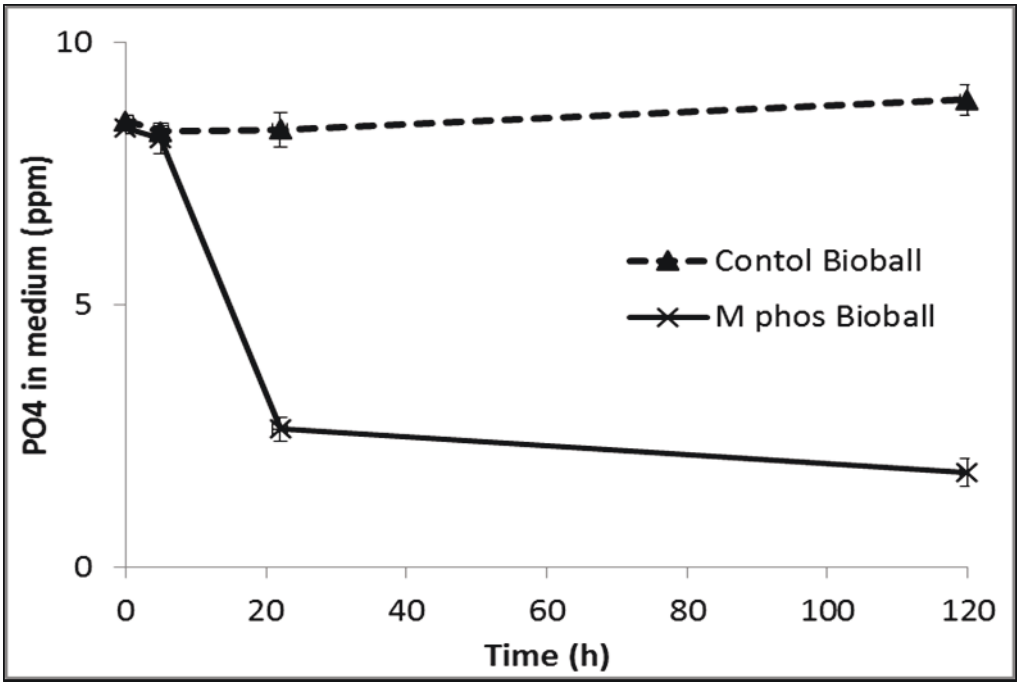
Figure 6. Phosphorous uptake from ML medium with by sol-gel encapsulated *M. phosphovorus* cells in a sponge encasement. Individual replicates shown due to contamination of the medium with escaped cells. No-cell control SGM in sponge remained sterile (black, dashed line).



In order to fortify the structural integrity of the SGM, the sponge-SGM-cells complex was placed inside a porous, 3 in., hard plastic bioball. Phosphate uptake was again monitored and these trials were more successful, albeit at slower rates than free living cells. This is likely due to the ability of free living cells to replicate while the encapsulated cells did not have enough space within the SGM pores to divide (Nassif et al. 2002; Premkumar et al. 2002). In comparing the total cell density of the free living cells to the SGM cells, the approximate cell number/ml treated volume of the free living cells (8.6×10^7) was 150 times greater than the SGM cells (5.8×10^5). However, a total of 78% of the PO_4 was removed over 120 h, and although the uptake of phosphate from encased cells was about 1/10 the rate of free-living cells, 0.3 ppm PO_4 was removed per hour. (Figure 7). This rate translates to 80 mmol P/g cell dry wt/h, this is much higher than seen in many biological phosphate removal batch reactors. For example, researchers observed rates of 8 mmol P/g suspended solids/h (Nittami et al. 2011), and 0.645 mmol P/g suspended solids/h (Ong et al. 2013), in sequencing batch reactors with WWTP inoculum under optimal

conditions, and 3.34 mmol P/g cells/h with *M. phosphovorus* inoculum (Santos et al. 1999). This difference in rate is partially explained by the fact that in the WWTP inoculated reactors P uptake was compared to total suspended solids rather than cell dry weight, underrepresenting the active PAO community (Nittami et al. 2011; Ong et al. 2013). Furthermore, the sequencing batch reactor study with *M. phosphovorus* was conducted in minimal salts medium (Santos et al. 1999), while this study was conducted in half-strength rich medium for one culturing episode or batch.

Figure 7. Phosphorous uptake from ML medium with no PO_4 added by sol-gel encapsulated *M. phosphovorus* cells in a sponge encasement inside bioballs. No contamination was observed in the encapsulated cells (solid line) or the no-cell control (dashed line).



4 Conclusion

The number of freshwater sources in the U.S. with documented hypoxia has increased dramatically. This research demonstrates the feasibility of phosphate sequestration by encapsulated cells. In order to use this technology in natural systems numerous hurdles must be overcome. The conceptual design of this effort is a novel method for reduction and removal of phosphorous from freshwater systems in an effort to curb harmful algal blooms and hypoxia events. The goal was to find a way to encapsulate phosphate accumulating bacteria, introduce them into open water systems, allow them to take up phosphate, then remove the cells and phosphate entirely from the water. The results indicate that encapsulated cells are an effective means of sequestering phosphate, however a more robust encapsulation matrix is needed to inhibit cell escape.

The results presented herein represent a starting point for this concept, but the ability to keep the bacteria trapped within the SGM is a major issue that needs critical attention. This concept has potential for very large scale up, low cost, high profile, environmental stewardship as an ecologically friendly, non-invasive solution for phosphate removal with the value added benefit of producing commercially available phosphorous.

References

Chislock, M. F., E. Doster, R. A. Zitomer, and A. E. Wilson. 2013. Eutrophication: causes, consequences, and controls in aquatic ecosystems. *National Education Knowledge* 4(4):10.

Dickson, D.J., and R. L. Ely. 2011. Evaluation of encapsulation stress and the effect of additives on viability and photosynthetic activity of *Synechocystis* sp. PCC 6803 encapsulated in silica gel. *Applied Microbiology and Biotechnology* 91:1633–1646.

Gill, I., and A. Ballesteros. 2000. Bioencapsulation within synthetic polymers (Part 1): Sol-gel encapsulated biologicals. *Trends in Biotechnology* 18(7):282–296. https://doi.org/10.1016/S0167-7799(00)01457-8.

Indest, K. J., J. O. Eberly, and D. E. Hancock. 2015. Expression and characterization of an *N*-oxygenase from *Rhodococcus jostii* RHAI. *The Journal of General and Applied Microbiology* 61:217–223. https://doi.org/10.2323/jgam.61.217.

Indest, K. J., J. O. Eberly, D. E. Hancock, C. M. Jung, M. R. Carr, and G. A. Blakeney. 2016. *Rhodococcus jostii* RHA1 TadA-homolog deletion mutants accumulate less polyhydroxyalkanoates (PHAs) than the parental strain. *The Journal of General and Applied Microbiology* 62:213-316. https://doi.org/10.2323/jgam.2016.03.002.

Indest, K. J., D. Hancock, C. M. Jung, J. O. Eberly, W. W. Mohn, L. D. Eltis, and F. H. Crocker. 2013. Role of nitrogen limitation in transformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by *Gordonia* sp. strain KTR9. *Applied and Environmental Microbiology* 79:1746–1750.

Indest, K. J., C. M. Jung, H. P. Chen, D. Hancock, C. Florizone, L. D. Eltis, and F. H. Crocker. 2010. Functional characterization of pGKT2, a 182 kb plasmid containing the *xplAB* genes involved in the degradation of RDX by *Gordonia* sp. KTR9. *Applied and Environmental Microbiology* 76:6329–6337.

Jung, C. M., C. Broberg, J. Giuliani, L. L. Kirk, and L. F. Hanne. 2002. Characterization of JP-7 jet fuel degradation by the bacterium *Nocardioides luteus* strain BAFB. *Journal of Basic Microbiology* 42:127–131. https://doi.org/10.1002/1521-4028(200205)42:2<127::AID-JOBM127>3.0.CO;2-C.

Keasling, J., S. Van Dien, P. Trelstad, N. Renninger, and K. McMahon. 2000. Application of polyphosphate metabolism to environmental and biotechnological problems. *Biochemistry* 65:324–331.

Kortsee, G. J. J., K. J. Appledoorn, C. F. C. Bonting, E. W. J. vanNiel, and H. W. vanVeen. 1994. Biology of phosphate-accumulating bacteria involved in enhanced biological phosphorous removal FEMS. *Microbiology Reviews* 15(2-3):137–153. https://doi.org/10.1111/j.1574-6976.1994.tb00131.x

Martin, H. G., N. Ivanova,V. Kunin, F. Warnecke, C. W. Barry, A. C. McHardy, C. Yeates, S. He, A. A. Salamov, E. Szeto, E. Dalin, N. H. Putnam, H. J. Shapiro, J. L. Pangilinan, I. Rigoutsos, N. C. Kyrpides, L. L. Blackall, K. D. NcMahon, and P. Hugenholtz. 2006. Metagenomic analysis of two enhanced biological phosphorous removal (EBPR) sludge communities. *Nature Biotechnology* 24:1263–1269.

Mutlu, B. R., S. Yeom, L. P. Wackett, and A. Aksan. 2015. Modelling and optimization of a bioremediation system utilizing silica gel encapsulated whole-cell biocatalyst *Chemical Engineering Journal* 259:574–580. http://doi.org/10.1016/j.cej.2014.07.130.

Nakamura, K, A. Hiraishi, Y. Yoshimi, M. Kawaharasaki, K. Masuda, and Y. Kamagata. 1995. *Microlunatus phosphovorus* gen nov., sp. nov., a new gram-positive polyphosphate-accumulating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* 45:17–22.

Nassif, N., O. Bouvet, M. Noelle Rager, C. Roux, T. Coradin, and J. Livage. 2002 Living bacteria in silica gels. *Nature Materials* 1:42–44.

Nittami, T., H. Oi, K. Matsumoto, and R. J. Seviour. 2011. Influence of temperature, pH and dissolved oxygen concentration on enhanced biological phosphorus removal under strictly aerobic conditions. *New Biotechnology* 29:2–8. http://doi.org/10.1016/j.nbt.2011.06.012.

Ong, Y. H., A. S. M. Chua, B. P. Lee, and G. C. Ngoh. 2013 Long-term performance evaluation of EBPR process in tropical climate: start-up, process stability, and the effect of operational pH and influent C:P ratio. *Water Science Technology* 67(2):340–346.

Premkumar, J. R., E. Sagi, R. Rozen, S. Belkin, A. D. Modestov, and O. Lev. 2002 Fluorescent bacteria encapsulated in sol–gel derived silicate films. *Chemistry of Materials* 14(6):2676–2686. doi:10.1021/cm020020v.

Saez, J. M., C. S. Benimeli, and M. J. Amoroso. 2012. Lindane removal by pure and mixed cultures of immobilized actinobacteria. *Chemosphere* 89(8):982–987. https://doi.org/10.1016/j.chemosphere.2012.06.057.

Santos, M. M., P. C. Lemos, M. A. M. Reis, and H. Santos. 1999. Glucose metabolism and kinetics of phosphorous removal by the fermentative bacterium *Microlunatus phosphovorus*. *Applied and Environmental Microbiology* 65:3920–3928.

Seviour, R. J., T. Mino, and M. Onuki. 2003 The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiology Reviews* 27(1):99–127. https://doi.org/10.1016/S0168-6445(03)00021-4.

Yu, D., J. Volponi, S. Chhabra, C. J. Brinker, A. Mulchandani, and S. K. Singh. 2005. Aqueous sol-gel encapsulation of genetically engineered *Moraxella* spp. cells for the detection of organophosphates. *Biosensors and Bioelectronics* 20(7):1433–1437. https://doi.org/10.1016/j.bios.2004.04.022.