# ELUCIDATING THE ROLE OF TOXIN-INDUCED MICROBIAL STRESS RESPONSES IN BIOLOGICAL WASTEWATER TREATMENT PROCESS UPSET

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# ELUCIDATING THE ROLE OF TOXIN-INDUCED MICROBIAL STRESS RESPONSES IN BIOLOGICAL WASTEWATER TREATMENT PROCESS UPSET

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## (ABSTRACT)

The overall hypothesis of this work is that the physiological microbial stress response could serve as a rapid, sensitive, and mechanistically-based indicator of process upset in biological wastewater treatment systems that receive sporadic shock loads of toxic chemicals. The microbial stress response is a set of conserved and unique biochemical mechanisms that an organism activates or induces under adverse conditions, specifically for the protection of cellular components or the repair of damaged macromolecules. Using traditional immunochemical analysis techniques, the heat shock protein, GroEL, was found to be induced in activated sludge cultures exposed to perturbations of chemicals at all concentrations tested (cadmium, pentachlorophenol, and acetone) or heat stress. As total cadmium concentrations increased above 5 mg/L, there was a significant and consistent increase in effluent volatile suspended solids concentrations from activated sludge sequencing batch reactors relative to unstressed controls, but there was no additional increase in GroEL levels.

Stress proteins may serve as sensitive and rapid indicators of mixed liquor toxicity which can adversely impact treatment process performance, but GroEL may not be a good candidate protein for this purpose due to the lack of a dose/response relationship. Additionally, production of stress proteins did not explain the significant deflocculation upsets that were characteristic of many of the industrially-relevant chemicals tested, including pentachlorophenol and cadmium. Although the purpose of stress response mechanisms is protective at the cellular level, the effect may be disruptive at the macroscopic level in engineered bioreactor systems.

The goal of the second research phase was to determine whether the bacterial glutathione-gated, electrophile-induced potassium efflux system is responsible for deflocculation observed due to shock loads of toxic electrophilic (thiol reactive) chemicals. The results indicate significant $K^+$ efflux from the activated sludge floc structure to the bulk liquid in response to shock loads of 1-chloro-2,4-dinitrobenzene (CDNB), N-ethylmaleimide (NEM), 2,4-dinitrotoluene (DNT), 1,4-benzoquinone (BQ), and $Cd^{2+}$ to a bench-scale sequencing batch reactor (SBR) system. In most cases, the stressor chemicals caused significant deflocculation, as measured by an increase in effluent volatile suspended solids (VSS), at concentrations much less than that required to reduce the maximum specific oxygen uptake rate by 50% ($IC_{50}$). This suggests that electrophile-induced activated sludge deflocculation is caused by a protective bacterial stress mechanism (as hypothesized) and that the upset event may not be detectable by aerobic respirometry. More importantly, the amount of $K^+$ efflux appeared to correlate well with the degree of deflocculation.

The transport of other cations including sodium, calcium, magnesium, iron, and aluminum, either to or from the floc structure, was negligible as compared to $K^+$ efflux. In bench-scale SBRs, it was also determined that the $K^+$ efflux occurred immediately (within minutes) after toxin addition and then was followed by an increase in effluent turbidity. $K^+$ efflux and deflocculation

responses were similar for bench-scale SBRs and continuous-flow reactor systems, indicating that the periods of elevated exogenous substrate levels typical in SBR systems are not required to activate electrophile-induced $K^+$ efflux or deflocculation. This also suggests that the initial and rapid efflux of $K^+$ immediately following electrophile addition is the factor that leads to deflocculation, not the increase in bulk liquid $K^+$. *Sphingomonas capsulata*, a bacterium consistent with that found in biological wastewater treatment systems, *Escherichia coli* K-12, and activated sludge cultures exhibited very similar dynamic efflux/uptake/efflux responses due to the electrophilic stressors, NEM and CDNB, and the thiol reducing agent, dithiothreitol (DTT).

The polyether ionophore antibiotic, nigericin, was used to artificially stimulate $K^+$ efflux from *S. capsulata* and activated sludge cultures. Thus, glutathione-gated $K^+$ efflux (GGKE) activity may cause $K^+$ release from the cytoplasm of activated sludge bacteria into the floc structure and extracellular polymeric substances (EPS) and then diffusion-limited transport into the bulk liquid. It was not possible to resolve the effect of the GGKE system on changes in bulk liquid or floc-associated pH. However, calculations indicate that the localized $K^+$ concentration within the floc structure immediately after chemical stress is consistent with that known to induce floc disruption as a result of KCl addition. Using alkaline phosphatase as a periplasmic marker as well as fluorescent membrane-permeable and impermeable nucleic acid stains, it was determined that a negligible amount of the $K^+$ efflux response was due to lysis of activated sludge microorganisms. The current results are very promising and are the first to suggest that activated sludge upset (i.e. deflocculation) may be caused by a specific protective stress response in bacteria.

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## TABLE OF CONTENTS

**Introduction**……...1
Microbial Stress Responses……..…2
Research Hypothesis…….……...4
Experimental Objectives…….…….5
References……5

**Chapter 1. Literature Review…**……..…….……8
Deflocculation of Activated Sludge……..……8
The Prokaryotic Glutathione-Gated $K^+$ Efflux System…….……..12
Toxicity of Electrophilic Chemicals……25
Bioflocculation of Activated Sludge……27
References……34

**Chapter 2. The Immunochemical Detection of Stress Proteins in Activated Sludge Exposed to Toxic Chemicals** ..……..……45

**Chapter 3. A Physiological Mechanism for Activated Sludge Deflocculation Caused by Shock Loads of Toxic Electrophilic Chemicals.**……..…..……..…46
Abstract……..46
Introduction……47
Methodology……..52
Results and Discussion……..57
Conclusions……71
Acknowledgements……72
References……..72

**Chapter 4. Implicating the Glutathione-Gated Potassium Efflux System as a Cause of Activated Sludge Deflocculation in Response to Shock Loads of Toxic Electrophilic Chemicals** ……77
Abstract……77
Introduction……78
Materials and Methods……81
Results and Discussion……86
Conclusions……105
Acknowledgements……106
References……106

**Appendix A. Data for Chapter 2….**……A1

**Appendix B. Data for Chapter 3**……B1

**Appendix C. Data for Chapter 4**……C1

**Vita - Charles B. Bott**

## LIST OF TABLES

**Chapter 3. A Physiological Mechanism for Activated Sludge Deflocculation Caused by Shock Loads of Toxic Electrophilic Chemicals**

Table 1. $IC_{25}$, $IC_{50}$, and $IC_{75}$ Concentrations……..…59

Table 2. Effluent Particle and Mixed Liquor Cation Analysis……..68

**Chapter 4. Implicating the Glutathione-Gated Potassium Efflux System as a Cause of Activated Sludge Deflocculation in Response to Shock Loads of Toxic Electrophilic Chemicals**

Table 1. Soluble NEM and Effluent VSS Concentrations…….……93

## LIST OF FIGURES

**Chapter 1. Literature Review**

Figure 1. Recognized $K^+$ Transport Systems in *E. coli*……13
Figure 2. Glutathione-Gated Electrophile Induced $K^+$ Efflux System……...21

**Chapter 3. A Physiological Mechanism for Activated Sludge Deflocculation Caused by Shock Loads of Toxic Electrophilic Chemicals**

Figure 1. Reactions of Glutathione……50
Figure 2. Cation Measurement Procedure……..56
Figure 3. Deflocculation Screening Results……...59
Figure 4. NEM- and CDNB-Induced $K^+$ Efflux and Deflocculation……62
Figure 5. $K^+$ Efflux and Deflocculation Versus NEM and CDNB……..65
Figure 6. $K^+$ Efflux and Deflocculation Over Time……69

**Chapter 4. Implicating the Glutathione-Gated Potassium Efflux System as a Cause of Activated Sludge Deflocculation in Response to Shock Loads of Toxic Electrophilic Chemicals**

Figure 1. Batch *S. capsulata* $K^+$ Efflux Stress Experiment……89
Figure 2. Activated Sludge SBR NEM/DTT/NEM Experiment……91
Figure 3. Activated Sludge Dynamic NEM/DTT/NEM Experiment……..94
Figure 4. Activated Sludge Dynamic CDNB/DTT/CDNB Experiment…..95
Figure 5. *S. capsulata* NEM and Nigericin Addition……..98
Figure 6. Activated Sludge $K^+$ Efflux for NEM and Nigericin Addition…..100
Figure 7. Effluent NTU for Activated Sludge NEM and Nigericin……101

# INTRODUCTION

Biological wastewater treatment plants are dynamic systems which are often exposed to changing environmental conditions, such as variations in biochemical oxygen demand (BOD) loading, wet weather inflows, and sporadic toxic discharges. Changes in wastewater composition can impose a range of process responses. Those responses, which are most often reported by operators after a toxic upset event, were determined through a survey conducted by the Water Environment Research Foundation and include poor BOD removal, disrupted nitrification, and deflocculation of activated sludges (17). It is generally necessary or desirable to alter operational conditions in response to these environmental changes if acceptable or optimal effluent quality is to be maintained. Unfortunately, operators are often forced to function in a reactive rather than proactive mode as a result of changing influent quality. Few studies have been done under well-controlled experimental conditions with indigenous microbial communities to show how certain wastewater conditions (**source**) impact activated sludge treatment processes (**effect**) (17). In the absence of established correlations between various sources and their effect(s), operators often cannot determine which control actions to implement in response to wastewater shock loads to most effectively mitigate process impact. To develop a better understanding of how source and effect are related, we propose identifying the predominant biochemical, chemical and/or physical **causes** that link source and effect.

Molecular biology tools have provided process engineers, scientists, and operators with methods for determining how operational and design decisions influence activated sludge community structure and function. To date, genomic-based tools and fatty acid methyl ester content/distribution have been used predominantly for studying how activated sludge populations adapt structurally to deviations in conditions. However, in order to fully comprehend how activated sludge communities respond to variations in their environment, the physiological changes brought about by short-term adaptive responses need to be considered as well. It was hypothesized that rapid microbial stress mechanisms are detectable physiological responses which serve as a first line of defense against environmental perturbations. Although their primary role is to protect cells, the net effect of these mechanisms in biological wastewater treatment cultures may be to disrupt process performance at the macroscale. Therefore, we propose studying these mechanisms to determine their role in source-cause-effect relationships in

chemically perturbed activated sludge communities. Once these relationships are established for indigenous microbiological communities, it should be possible to identify appropriate mitigation or prevention strategies that will allow treatment plants to be operated satisfactorily once exposed to dynamically changing wastewaters. Additionally, understanding the mechanisms invoked under these conditions will lead to targeted monitoring or sensing strategies. These upset early warning devices can be integrated with computerized decision/support systems, which will help operators determine how to respond most appropriately so that treatment plant efficiency and effluent quality are optimized.

**Microbial Stress Responses**

It has been well established that bacterial cells can activate stress responses that either protect cells against damage (protective stress responses) or repair cells after damage has occurred (remedial stress responses) (15). Protective and remedial stress responses are comprised of both unique and conserved adaptive physiological responses that are induced or activated in bacteria to survive in adverse environmental conditions. Many of these responses are quite rapid (constitutively expressed, or activated in seconds), as would be necessary to prevent lethal effects from highly toxic stressors. Despite the clear importance of these mechanisms to ecological development in complex microbial communities, the environmental microbiology and engineering communities have focused little on this topic. In contrast, catabolic mechanisms for a range of chemical toxins (stressors) have been studied extensively over the past few decades. Activation of genes for biodegrading potentially toxic chemicals can take minutes to hours for a cell that is genetically capable, depending upon the presence of other substrates, the concentration of the stressor, and the history of catabolic enzyme induction. Consequently, the relative importance of stress responses to sudden influxes of stressors on process performance remains largely unstudied.

*Remedial Stress Responses.* Stress proteins are rapidly and highly inducible in all cells in response to sudden environmental changes; this response has, in part, refueled the recent resurgence in proteomics (7). Stress proteins are believed to help cells recover from damage upon exposure to a wide variety of stressors, such as heat (19,20), starvation (20), anaerobiosis

(19), and oxidative conditions (20). For example, the response to sudden increases in temperature at the molecular level involves the synthesis of well characterized heat shock proteins that repair damaged proteins through refolding or reassembly, or degrade nonessential proteins (14). Whereas the term "stress protein" has traditionally been equated to heat shock proteins and other similar chaperones that repair (or remediate) damaged proteins, it is appropriate to consider stress proteins within a broader context, including those that promote a protective function. For example, glutathione S-transferases (GSTs) may be involved in both the glutathione response and selected catabolic activities, in addition to remedial chaperonin activities (13,16); despite this broad stress-related functionality, they remain largely unstudied in bacterial systems (21). In addition, studies have shown that many proteins are rapidly generated in pure cultures of *Escherichia coli* (3,8) and *Pseudomonas putida* (18) after exposure to sublethal concentrations of toxic organic or inorganic compounds. However, most of those proteins were of unknown function, and it appeared that some unique proteins were induced in response to different classes of chemical stressors (3).

*Protective stress responses.* Once a chemical stressor enters the cytoplasm, it may damage critical macromolecules. Bacteria can respond by activating efflux pumps that remove undesirable chemicals from the cytoplasm (not discussed here) or influence cytoplasmic chemistry by transporting cations and/or changing pH. A broad range of functions exists for efflux systems; however, this work focuses on those that respond to electrophilic chemicals (both organic chemicals and toxic heavy metals). A cation efflux mechanism ubiquitous in Gram-negative bacteria that protects cytoplasmic macromolecules from electrophilic (oxidative) stressors involves a cysteine-containing tripeptide called glutathione. The sulfhydryl substituent of reduced glutathione (GSH) is susceptible to spontaneous (nonenzymatic) electrophilic attack by many oxidants (1,2). It appears that GSH acts in a sacrificial manner, scavenging harmful electrophiles to form glutathione-S-conjugates in order to prevent macromolecular oxidations and electrophilic additions. The glutathione-S-conjugate activates the glutathione-gated $K^+$ efflux (GGKE) system, thereby allowing significant release of intracellular $K^+$ from the cell (4,5,10). Cytoplasmic acidification has been measured concurrently with $K^+$ efflux, and it was suggested that acidification may be the mechanism that affords the cell protection from electrophilic stress (9,11,12).

**Research Hypothesis**

*Stress protein Hypothesis.* It may be possible to identify a range of indicator proteins (both conserved, such as heat shock proteins, and unique) that are rapidly generated in response to xenobiotic stress. Collectively, these proteins may serve as "fingerprints" to indicate the chemical properties of stressors which impose toxicity to bacterial cells by different mechanisms. As such, these stress protein fingerprints may provide valuable information about the physiological state or health of a biological treatment system. It follows that exposure to chemical stressors can also cause treatment performance deterioration. Therefore, it is important to establish relationships between stress protein generation patterns and biological treatment process performance problems in response to pulse inputs of chemical stressors. It is particularly meaningful if these relationships are established using microorganisms indigenous to the treatment process being studied.

*Deflocculation Hypothesis.* It was an unexpected observation that the most significant mode of process upset in response to shock loads of two industrially relevant electrophiles (pentachlorophenol and cadmium) was deflocculation of the mixed liquor, producing a cloudy suspension of small particles that did not settle in the reactor effluent. Although increased levels of a specific heat shock protein were measured in the mixed liquor as a result of chemical stress, the deflocculation phenomena could not be explained by the physiological induction of heat shock proteins (6). In addition, there appeared to be no legitimate physicochemical basis for electrophile-induced deflocculation. For example, based on the polyvalent cation bridging model of bioflocculation (see Chapter 1), the addition of cadmium, a divalent cation, should not result in a significant weakening of floc structure. These preliminary observations led to a comprehensive literature review of electrophile-induced stress responses in bacteria. As a result, it was hypothesized that a specific protective microbial stress response, the GGKE system as discussed above, is responsible for activated sludge deflocculation in response to shock loads of electrophilic chemicals. Furthermore, it has been suggested that, in general, microbial stress responses are involved with the upset of activated sludge systems receiving influent perturbations of chemical toxins. Identifying source-cause-effect relationships, such as that

proposed here, will provide a basis for development of new monitoring technologies and operational strategies for systems under the influence of influent chemical perturbations.

**Experimental Objectives**

The overall objectives of this research project are presented as follows:

- Evaluate the relationship between the induction of a specific heat shock protein, GroEL, in mixed liquor and process performance deterioration in a bench-scale activated sludge system receiving shock loads of toxic chemicals that are known to cause upset problems in biological treatment systems.
- Evaluate a source-cause-effect relationship in which the **source** represents shock loads of industrially relevant electrophilic toxins, the **cause** is physiological with respect to GGKE activity and physicochemical due to floc structure deterioration with excess monovalent cation in the EPS, and the **effect** is deflocculation of the mixed liquor.

Specific tasks that address these objectives will be discussed within the individual chapters of this dissertation.

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# Chapter 1. LITERATURE REVIEW

## Deflocculation of Activated Sludge

In a recent survey of industrial and domestic wastewater treatment plant operators, it was determined that 70% of those responding had experienced some form of process upset due to an influent disturbance (68). The most common modes of biotreatment upset were ineffective COD/BOD removal, ineffective nitrification, deflocculation, and non-filamentous foaming, while non-filamentous bulking was less common (68). Transient deflocculation in activated sludge systems is a critical issue for many municipal and industrial wastewater treatment facilities, but it is a problem for which "cause and effect" is usually not correlated (68). For example, deflocculation is not as well defined as filamentous bulking. Using microscopic examination, an operator can observe the proliferation of filamentous bacteria and then unambiguously attribute the excess filaments to sludge bulking (59). In addition, when filamentous bulking is first observed, the operator can immediately initiate mixed liquor chlorination as a temporary control of filamentous bacteria to improve settling before surface water contamination occurs.

In the case of deflocculation, the only available indication of upset conditions is usually a rapid increase in effluent suspended solids (turbidity) and a deterioration in sludge dewatering properties. If the deflocculation event is severe and prolonged, significant activated sludge system washout can occur without ever recognizing the cause of the problem or the time of initiation (49,68,70,71,87). Xenobiotic-induced deflocculation results in severe environmental problems beyond the obvious loss of biomass and the associated discharge of biochemical oxygen demand (BOD). In addition to the effluent toxicity associated with the soluble fraction, it is likely that deflocculating xenobiotics are also sorbed to the deflocculated particles and thus washed out of the system, thereby resulting in even higher effluent toxicity. In treatment plants where biological nutrient removal is employed, total phosphorous (TP) removal will be severely reduced if polyphosphate containing bacteria are among the deflocculated particles. Deflocculation also results in possible release of pathogens to receiving streams and increased $Cl_2$ demand in disinfection processes. If tertiary filtration is used, deflocculation will result in an obvious increase in backwash requirements.

Before discussing the known causes of transient activated sludge deflocculation, an important distinction must be made between two specific mechanisms of deflocculation. This review will exclusively address deflocculation caused by shock loading of a specific group of inhibitory chemicals. Sustained dispersed growth (pinpoint floc), which is induced by steady-state operation at solids retention times (SRT) greater than about 10 days or at low organic loading rates (51,59,97), will not be considered, even though it is realized that the physiological age of the floc may make it more or less vulnerable to transient deflocculation by inhibitory discharges.

*Heavy Metals.* It has been reported that shock loading of several toxic heavy metals, including divalent cadmium, mercury, and zinc, causes rapid and significant deflocculation of activated sludge. Neufeld (87) investigated this phenomena using laboratory sequencing batch reactors (SBR), and demonstrated that $Cd^{2+}$, $Hg^{2+}$, and $Zn^{2+}$ all resulted in significant deflocculation. As expected, a decrease in the measured SVI was generally observed with a concurrent increase in effluent suspended solids (87). However, because of some very significant omissions concerning the reactor feed composition as well as extremely high background suspended solids concentrations ($Zn^{2+}$ and $Cd^{2+}$ experiments), it is difficult to draw further conclusions from the presented data (87). It is important to note that deflocculation was observed with $Hg^{2+}$ addition within hours of shock loading, while in the $Cd^{2+}$ and $Zn^{2+}$ experiments, significant deflocculation was not observed until 3-4 days after shock loading (87). This was most likely caused by the extremely high background turbidity shown for these experiments.

There have been some conflicting reports as to the affects of shock loading or batch addition of copper ($Cu^{2+}$). Bruus et al. (19) reported that added cupric ion (~725 mg/L $Cu^{2+}$) caused $Ca^{2+}$ release (~10 mM $Ca^{2+}$) from the floc by ion-exchange mechanisms, but that sludge dewatering properties (SRF) actually improved with added $Cu^{2+}$. The author suggested this was caused by stronger complexing of polysaccharide carboxylate substituents by $Cu^{2+}$ (i.e. copper is able to bridge EPS better than $Ca^{2+}$) (19). In parallel activated sludge pilot systems, McDermott et al. (71) indicated that, with shock loads of $Cu^{2+}$ up to 100 mg/L, COD removal was somewhat affected for approximately 36 hours, but effluent turbidity did not increase (71). At a shock load of 210 mg/L $Cu^{2+}$, both effluent suspended solids and COD increased (71). For a 410 mg/L

$Cu^{2+}$ shock load, the effluent COD significantly increased, while the effluent suspended solids unexpectedly improved to below that shown for the 210 mg/L shock (71). McDermott et al. (71) also demonstrated that activated sludge, which was acclimated to 25 mg/L $Cu^{2+}$, had a significantly higher steady-state effluent TSS than an unacclimated control. Unfortunately, $Ca^{2+}$ concentrations were not measured, and therefore ion-exchange mechanisms could not be addressed.

The insufficiency of information with respect to deflocculation caused by toxic heavy metals is not likely due to the insignificance of the problem. It is believed that the lack of full-scale system verification, supporting the notion of heavy metals induced deflocculation, is due to the fact that treatment plant operators can rarely link "cause and effect" well enough to conclude that a specific influent component was the cause of a deflocculation event. A transient occurrence of deflocculation may be observed as an increase in effluent turbidity, but if no samples were analyzed at that specific time for heavy metals, there would be no evidence of a "cause" for deflocculation.

*$Cl_2$ Control of Filaments.* Excessive filamentous growth in activated sludge systems results in poorly settling sludge, characterized by a high SVI (bulking) but very low supernatant turbidity (59). Temporary control of filamentous bacteria is typically achieved by adding low levels of $Cl_2$ directly into an activated sludge basin (or into a well-mixed RAS line). This is done in hopes of selectively killing or inhibiting the filamentous bacteria, which tend to be more exposed to the bulk liquid than the floc microorganisms (59). Overdosing of $Cl_2$ has been shown to result in rapid deflocculation, both in full-scale and in laboratory systems (21,59). It is important to note that the mode of deflocculation caused by $Cl_2$ overdosing results in the same form of deflocculated particles and polymers as that typically observed for shock loads of heavy metals and selected organics (59). Specifically, deflocculation results in a turbid suspension of stabilized pinpoint flocs characterized by a somewhat cloudy appearance concurrent with a rapid decrease in the SVI (18,59). The current theory of the mechanism for $Cl_2$ deflocculation is that excess hypochlorous acid (HOCl) oxidizes floc EPS and bacterial cells resulting in floc disintegration (59). The important aspect for this review is concerned with the strong electrophilic properties of HOCl (110). That is, HOCl is a powerful oxidant which is quite

effective in the inactivation of bacterial cells as promoted by recognized electrophilic reactions with cell proteins and nucleic acids (53).

*Uncharacterized Organic Compounds.* In many cases, activated sludge treatment systems, especially those treating variable and poorly characterized industrial waste streams, undergo periods of sludge deflocculation in which the cause of the problem and the time of initiation are never known. It is expected that some of these occurrences may be related to transient deflocculation caused by various inhibitory and industrially derived organic compounds. There has been very little work done in this area and no references were found in the technical literature. Considering the review of the potassium efflux system presented below, organic compounds, which are expected to be potent deflocculants, will most likely contain electrophilic substituents such as nitro groups, halogenated organics, organic sulfonic acids, and carbonyl groups (110). Examples of potentially relevant electrophiles include nitrobenzenes, halogenated aliphatics and aromatics, cyclic carbonyls, and aliphatic aldehydes.

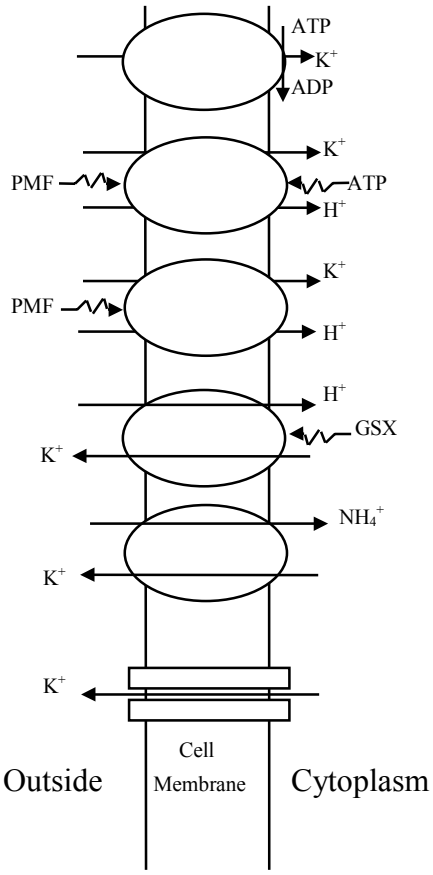
*Turbulent Flow Considerations.* In order to elucidate a mechanism for deflocculation, the effects of turbulent flow on an activated sludge floc must be addressed in conjunction with the mechanisms of floc strength reduction. Floc breakup mechanisms in turbulent shear flow systems are strongly influenced by the physical floc structure and the bonding orientation of primary particle clusters. As opposed to commonly studied metal hydroxide flocs, which are composed of a conglomeration of loosely bound primary particles, activated sludge flocs generally contain an intricate network of filamentous organisms that appear to function as the "backbone" of the floc structure (51,59). The primary particle clusters in an activated sludge floc represent a diverse range of bacterial species and EPS and are of a size range from approximately 2.5 to 12 μm (60).

Experimental analysis as well as intuitive observations have determined that there are two primary mechanisms of activated sludge floc degradation. Floc breakup as a result of primary particle erosion is caused by surface shearing (turbulent drag) and occurs when the shear strength of the bonds holding the primary particle clusters on the surface is exceeded (94,95). The result is the production of stable and dispersed "pin-point" flocs (deflocculation) that are poorly

removed from solution by gravity clarification. Filament fracture occurs when excessive tensile stresses are imposed on an AS floc. Physical rupture of the floc produces two smaller floc fragments rather than nonsettleable dispersed particles. Although the floc fracture breakup mechanism is not an important source of primary particles, it can explain the experimentally determined relationship between the peak floc size and the in-situ velocity gradient (95). Modeling of floc breakup will not be emphasized in this proposal, but it is important to note that Parker et al. (94,95) previously developed a useful hydrodynamic model, incorporating both aggregation and disintegration mechanisms, that can be applied to the unique physical characteristics of activated sludge flocs.

**The Prokaryotic Glutathione-Gated $K^+$ Efflux System**

The potassium uptake and efflux systems have been relatively well studied in *Escherichia coli* and have been shown to be quite analogous for many other Gram-negative bacteria (5,6,7,8,15,16,25,27,28,33,104). In *E. coli*, the monovalent cation regulation system is very complex with at least three $K^+$ uptake systems and at least two independent efflux systems (Figure 1). To add to this complexity, there are also organic substrate and ammonia transporters which have been suggested to involve symbiotic influx or efflux of $K^+$ (10). Potassium is the major cation regulating intracellular pH ($pH_i$) and osmotic turgor pressure in Gram-negative bacteria and serves as the counter ion for many negatively charged protein residues and negatively charged substrate molecules/intermediates within the cytoplasm (12,13,32,72,76,106). As a result, $K^+$ is usually highly concentrated in the cytoplasm up to 1.0 mole/L (5). It has been demonstrated that *E. coli* growing in media with only 0.1 to 1.0 μM $K^+$ (at normal ionic strength) can have a steady-state intracellular $K^+$ concentration up to 0.5 moles $K^+$/L of cytoplasmic fluid (5,108). The previous observation requires that these bacteria have effective uptake systems with a very high affinity for extracellular potassium as well as tightly regulated efflux systems that are induced only as a result of severely inhibitory conditions. Although the sodium ion may become important in severely $K^+$ limited conditions, its control of $pH_i$ and osmotic pressure is rarely as dramatic as that for the potassium system. As reviewed below, several researchers have demonstrated that chemical challenge by electrophilic compounds (both mild and strong oxidants) elicits a protective mechanism that prevents protein and DNA damage as a result of rapid efflux of intracellular $K^+$.




$K^+$ Tranport Systems

Kdp - High affinity inducible $K^+$ uptake

TrkGH - Low affinity constitutive $K^+$ uptake

Kup (TrkD) - Low affinity $K^+$ uptake

KefBC - GSX Activated $K^+$ Efflux

$K^+/NH_4^+$ Antiporter

Mechanosensitive - Stretch Activated Channel (KefA?)

Figure 1. Recognized $K^+$ transport systems in *E. coli*. Adapted from Bakker (5). ( ⁓▸ = Activation) (PMF = Proton Motive Force)

In addition to bacterial regulation systems, the potassium ion and its transport and cellular accumulation are also an important regulatory component of higher life forms (29,56,61). Cation uptake and efflux systems have been characterized in mammalian erythrocytes (103,118), and the importance of ion transport systems in general has been conserved in most eukaryotic cells. Potassium has been shown to be a critical factor in transpirational water loss from plants and is involved in turgor pressure regulation (67). Although the potassium flux mechanisms have not been well elucidated, transient activity of $Na^+,K^+$-ATPases, in conjunction with heat shock protein (Hsp70) induction, have been observed in fish gill epithelial cells in response to heavy metal, xenobiotic, and thermal stressors (105). It is important to note that only a few examples have been indicated from the many available references on ionic regulation and its effects on eukaryotic cells. Since considerable information was obtained with respect to potassium

transport in Gram-negative prokaryotes, there will be no further mention of eukaryotic mechanisms, other than the confirmation that ion control systems are a critical regulatory component of metabolism, intracellular pH, and internal pressure in many systems besides those evaluated below.

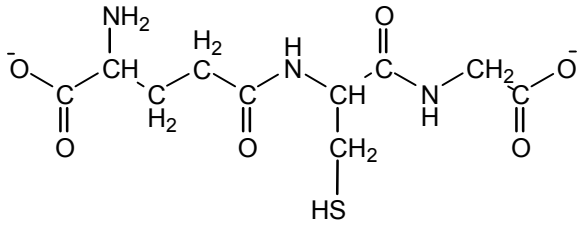
Most of the reported cation transport investigations have been performed using *E. coli* strain K-12 and derived mutants. Although some experimental work has been done on other Gram-negative organisms (e.g. *Vibrio alginolyticus*), this review and the genetic nomenclature refer to *E. coli* unless otherwise stated. However, it should be noted that homologous efflux systems (KefA and KefBC), which will be the focus of this review, have been found to exist in all Gram-negative organisms examined, but not in Gram-positives with the exception of *Staphylococus aureus* (see below) (15,28). In order to concentrate on the electrophile mediated control of the potassium efflux system, substantial information has been neglected from this proposal with respect to the recently elucidated characterization and regulation of the $K^+$ uptake systems (*kdp*, *trk*, and *kup*) as well as the specific cellular regulation of $pH_i$ and osmotic pressure. The reader is referred to the cited reviews of these related subjects for further information (5,6,12,13,14,17,26,32,52,101,108).

*Potassium Efflux System (KefBC).* It was originally thought that the KefB and KefC membrane transport proteins were associated with the low affinity *trk* potassium uptake system (see Figure 1) (33,100). In fact, TrkB and TrkC were reclassified as KefB and KefC when it was determined that these proteins represent specific glutathione gated potassium efflux/proton antiports (7,16,73). Before this distinction, Bakker and Mangerich (8), Meury et al. (74), and Booth et al. (16) demonstrated that E. coli, growing at either exponential- or stationary-phase, rapidly responded to 0.5 mM N-ethylmaleimide (NEM), a toxic sulfhydryl-reactive electrophile, with the efflux of nearly 90-95% of its intracellular $K^+$ pool within 20-30 minutes. In most cases, half of the $K^+$ efflux occurred during the first 2-3 minutes after NEM addition. The loss of intracellular $K^+$ was transient, and within a given period of time after NEM removal, almost all of the intracellular $K^+$ pool was recovered followed by a reinitiation of growth, provided a suitable substrate (74). Interestingly, it was determined that both the efflux and the recovery of intracellular $K^+$ were mediated without *de novo* protein synthesis (i.e. in the presence of

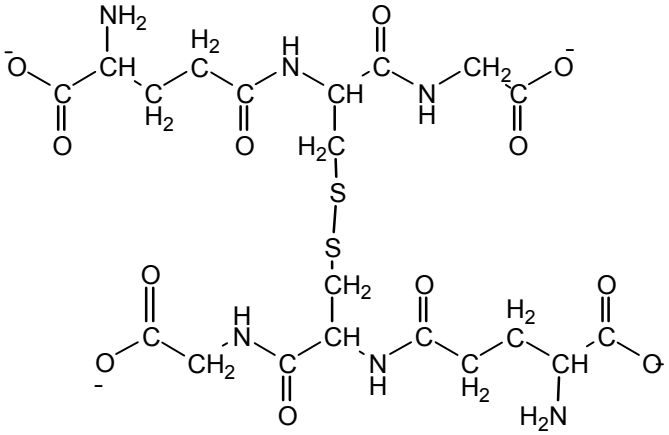
chloramphenicol) (31,74,75). Using mutagenesis and genetic analysis, Booth *et al.* (15,31,43,79) recently confirmed that KefB and KefC represent dual $K^+$ efflux systems, which are gated by reduced glutathione (GSH) and which are activated (to differing degrees) by glutathione adducts (GSX) and possibly also oxidized glutathione (GSSG). The more recent and relevant work that has been done with respect to this $K^+$ efflux system has been performed by Booth and coworkers within the last 10-12 years.

Glutathione is a tripeptide formally known as N-(N-L-γ-glutamyl-L-cysteinyl)glycine. GSH represents a significant fraction of the low molecular weight cytoplasmic thiol pool in both eukaryotic and prokaryotic cells (up to 95% in most Gram-negative bacteria) (3,30,36,115). The structure of GSH and GSSG at pH 7 is shown below (note the unusual γ peptide bond between the glutamate and cysteine residues). Fahey et al. (36) demonstrated that all Gram-negative bacteria tested had a significant amount of cytoplasmic GSH, and that for most of these organisms the GSH fraction comprised a majority of the non-protein thiol in the cell. However with the exception of *S. aureus*, all Gram-positive organisms tested had very low levels of GSH, and GSH was a much smaller fraction of the available cytoplasmic non-protein thiol pool (36). Interestingly, *S. aureus* was found to have GSH levels comparable to the lower range of Gram-negative bacteria (36). This exception may explain the observation by Douglas et al. (28) of KefBC homologue activity in only one Gram-positive organism, *S. aureus*. Fahey et al. (36) also indicated that the GSH content of E. coli increased significantly upon entry into stationary phase.

The thiol of GSH reacts <u>spontaneously</u> (non-enzymatic), especially at slightly elevated intracellular $pH_i$ (7.8-8.5), with many electrophilic oxidants that cause protein damage and DNA mutations (3,4). Although the mode of cell damage may be specific for each inhibitory electrophile, it appears that GSH acts in a sacrificial manner, scavenging harmful electrophiles so as to prevent macromolecular oxidations (22). In addition to inducing cellular protection mechanisms (e.g. $K^+$ efflux), it is also suspected that the GSX adducts activate putative detoxification pathways, and that GSX serves as the reactant for the first detoxification step rather than the electrophile itself (4,45,64,69). The reaction between GSH and several well known electrophilic sulfhydryl reagents is presented below:

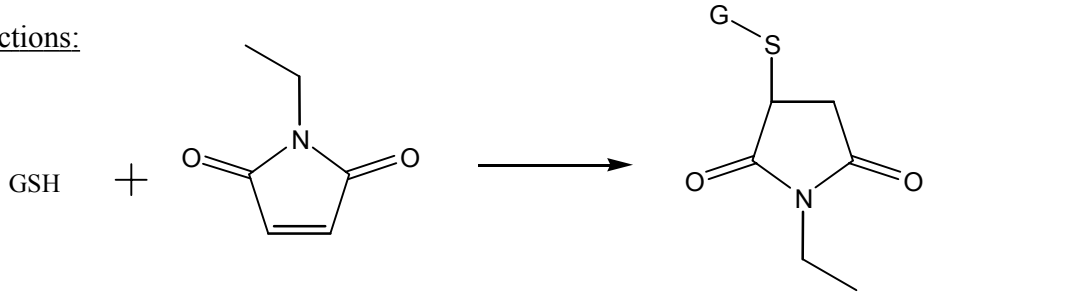


Reduced Glutathione - GSH

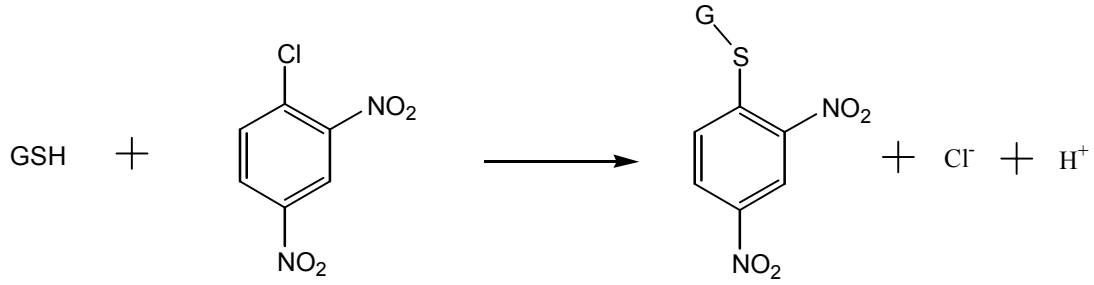


Oxidized Glutathione - GSSG

Reactions:



(N-ethylmaleimide)-NEM (N-ethyl-succinimido-S-glutathione)-ESG



(1-Chloro-2,4-dinitrobenzene)-CDNB (2,4-dinitrophenyl-S-glutathione)

The KefB and KefC potassium efflux system will be discussed, as it is relevant to this work, without significant discussion of the molecular biological methods and *E. coli* K-12 mutants used to characterize the biochemical mechanism. GSH analogues activate the KefB and KefC channels, while reduced GSH tightly inhibits efflux activity. As expected, before KefBC activity was even identified, Meury and Kepes (73) demonstrated that GSH-deficient mutants required very high concentrations of $K^+$ for growth, unless GSH was artificially supplemented. It was proposed that $K^+$ was "leaking" from the KefB and KefC channels and is consistent with GSH gating of KefB and KefC. KefB and KefC activity may also be caused by strong oxidants (e.g. t-butylhrdroperoxide and diamide), which oxidize GSH almost completely to GSSG and mixed protein disulfides (GSSP). These oxidants may actually induce KefB and KefC activity by removing reduced GSH from the KefB and KefC binding site. More recently, in contrast, Booth et al. (15) suggested that small amounts of strong oxidant GSX adducts were measured using HPLC.

Although the KefC protein has been much better characterized on a molecular and structural basis, it is known that these duplicate systems (KefB and KefC) function quite similarly (15,16,31,77). However, the relative activities of KefB and KefC differ depending on the functional group bound to (or complexed with) the GSH sulfhydryl substituent (43). KefB has also been demonstrated to be kinetically inferior to KefC with respect to $K^+$ efflux and was suggested to have broader GSX adduct specificity than KefC (16,86). Using limited selection of activators, Elmore *et al*. (31) suggested that smaller hydrophilic electrophiles (e.g. iodoacetate) elicit much less $K^+$ efflux for KefC than larger more hydrophobic GSH conjugates (e.g. CDNB). It was, however, eventually concluded that iodoacetate, duraquinone, and methylglyoxal (see below) activate KefB much more than KefC, while NEM, CDNB, p-chloromercuribenzoate, phenylmaleimide, diamide (diazenedicarboxylic acid bis-(N,N-dimethylamide)), and $HgCl_2$ all strongly activated both KefB and KefC (31,43,73,74,75,85,86).

Glutathione-S-conjugates (GSX) activate the potassium efflux channels, KefB and KefC, promoting rapid and significant efflux of $K^+$ from the cell. Booth et al. (16) demonstrated that KefB and KefC are actually $K^+$/proton antiports. Depending on the activation capability of a given electrophile, these researchers measured significant, though transient, acidification of the

cytoplasm synchronous with $K^+$ efflux (40,42,44). It is important to note that, for some electrophiles, the $pH_i$ measurements indicated that the discharged $K^+$ could not be completely accounted for by proton uptake (15). In fact, it was suggested that some influx of $Na^+$ was also associated with $K^+$ efflux (15). The slight amount of $Na^+$ uptake was attributed to the $pH_i$ regulated putative $Na^+/H^+$ antiport, which concurrently discharges a small number of the protons taken up by KefB and KefC system (15). It was also suggested that futile cycling of $Na^+$ and $NH_4^+$ may be involved with maintaining electroneutrality during $K^+$ efflux and cytoplasmic acidification. Averaging the results of $pH_i$ measurements from several experiments, Ferguson et al. (43,44,45,86) demonstrated a maximal $pH_i$ depression of 0.4-0.6, 0.6-1.0, 0.6-0.8, and 0.6-0.7 units for methylglyoxal (MG), NEM, CDNB, and iodoacetate stress, respectively. Even though this may not seem like a substantial decrease in $pH_i$, the regulation of intracellular pH is under very stringent control, and therefore any deliberate reduction in $pH_i$ should have a distinct function (45).

Ferguson and coworkers (15,37,38,40,42,44,86) eventually suggested that cytoplasmic acidification is the mechanism which actually affords the cell protection from electrophilic stress. Although the proof of the protective acidification mechanism is somewhat speculative, several pieces of circumstantial evidence, both from the research presented by Ferguson and coworkers (40,41,43,44,45,86) and from a variety of other unrelated observations, are presented. Firstly, since it is known that reduced sulfhydryl substituents of protein cysteine residues have a $pK_a$ of approximately 8.3, cytoplasmic acidification will result in a higher fraction of protonated thiols, thus making proteins with susceptible sulfhydryl substituents (e.g. active site thiols) less vulnerable to electrophilic attack. In addition, slight reductions in $pH_i$ have been associated with increases in DNA supercoiling (114), which in turn increases the binding capability of protective proteins (e.g. Dps). Cytoplasmic acidification also causes a reduction in the rate of transposition of insertion sequences (65) and suppresses the activity of mutagenic DNA base alkylating agents (e.g. N-methyl-N'-nitro-N-nitrosoguanidine) (93). It has been suggested that slight cytoplasmic acidification could also rapidly activate existing proteins which repair and protect DNA from oxidative stress, independent of changes in DNA supercoiling (42).

The most convincing evidence is that demonstrated by Ferguson *et al*. (40,41,43,44,45,86). Using culturable cell plate counting and $pH_i$ measurement, these researchers demonstrated that the actual value to which the $pH_i$ was depressed and not the magnitude of the decrease, was responsible for conferring resistance to cells stressed with MG and NEM (42,44,45). This response was further validated by using KefB$^-$ and KefC$^-$ mutants and by acidifying the cytoplasm with salts of weak, membrane permeable organic acids (e.g. sodium acetate) (42,44,45). It was found that the same degree of increased viability was obtained regardless of whether the intracellular acidity was provided by the KefBC system or external acidification (42,44,45). Protein synthesis was not required for increased short-term viability based on chloramphenicol addition, and therefore the damage that leads to cell non-viability must occur immediately upon electrophilic challenge (42,44,45). It was also determined that the time at which the external acidification was provided was critical to cell viability, thus indicating that a rapid rate of acidification, such as that provided by the KefB and KefC system, is necessary for immediate protection of cellular components (42,44,45). Although increased cell viability was obtained as a result of cytoplasmic acidification from NEM and MG addition, no increase in viability was observed for CDNB and iodoacetate challenge even though a comparable degree of $K^+$ efflux and internal acidification was measured (86). The authors suggest that this may be due to both a lesser degree of toxicity (electrophilic reactivity) and/or to a KefBC independent $K^+$ efflux and internal acidification that was observed at high CDNB and iodoacetate concentrations (86). Also of interest to this proposal, Summer and Goggelmann (111) demonstrated that the mutagenicity of both CDNB and 1-fluoro-2,4-dinitrobenzene, which is a more potent electrophile than CDNB, is inversely proportional to the concentration of GSH present in *Salmonella typhimurium* tester strains, thus indicating that GSH "scavenging" of electrophiles may also provide protection. As shown in Figure 2, it was recently concluded by Ferguson et al. (45) that cellular protection is provided by rapid acidification, which represses electrophile/macromolecule interactions and/or rapidly activates preexisting protection/repair pathways (i.e. without additional protein synthesis).

Meury et al. (74) and Bakker and Mangerich (8) conclusively demonstrated that NEM induced $K^+$ efflux can be reversed by addition of a strong reductant such as β-mercaptoethanol or dithiothreitol (8,74). These researchers added excess reductant to the growth medium after

nearly maximal $K^+$ efflux and observed a subsequent uptake of $K^+$ to a new steady-state level (8,74). After observing $K^+$ uptake, it was found that $K^+$ efflux could be re-induced by adding additional NEM (8,74). More importantly, these researchers determined that neither $K^+$ efflux nor reductant-induced recovery were inhibited by chloramphenicol addition, thus indicating that *de novo* protein synthesis was not required for either mechanism (8,74). Since the previous researchers did not evaluate the reaction of a specific reductant with GSX adducts, Elmore *et al*. (31), subsequently used thin layer chromatography (TLC) and radioactive NEM to demonstrate that $K^+$ efflux reversal was a result of GSX activator breakdown with dithiothreitol releasing reduced GSH. Finally, due to the stability of the thioether bond generated by CDNB reaction with GSH (as compared to the C-S-C bond formed by NEM), Booth et al. (15) indicated that none of the evaluated reducing agents were able to significantly react with 2,4-dinitrophenyl-S-glutathione, which would thereby terminate CDNB-induced $K^+$ efflux. The reader is referred to Chapter 4 for a more complete review of dynamic efflux/uptake/efflux and its importance for this research.

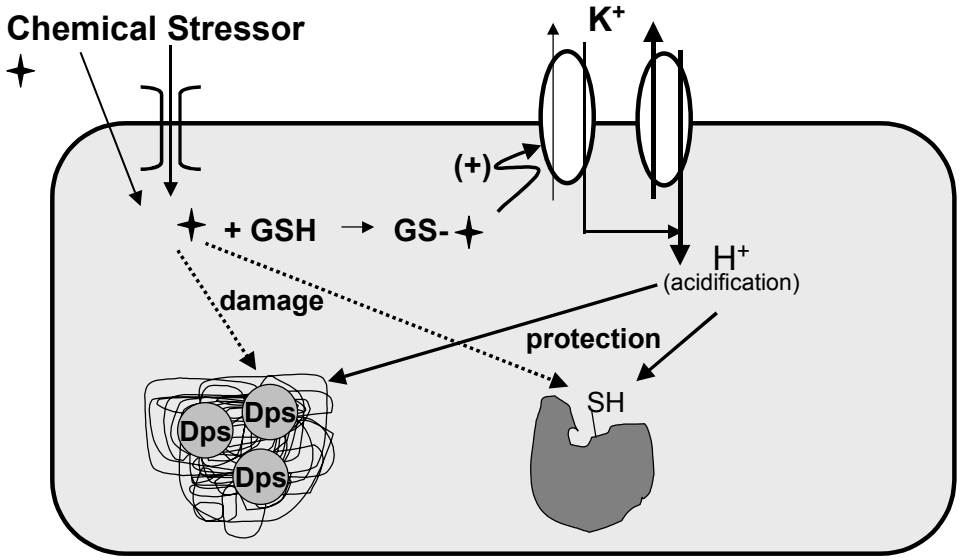



Figure 2. Suggested mechanism of Gram-negative bacterial protection (not detoxification) from electrophilic stress. Adapted from Ferguson et al. (42,45). An electrophilic chemical stressor enters a bacterial cell. Due to the high concentration of reduced glutathione (GSH) present in the cytoplasm, the electrophile immediately reacts with GSH (either spontaneously or through glutathione S-transferase) and activates the $K^+$ efflux channels in the cytoplasmic membrane. A counter flow of cations (typically protons) maintains a charge balance and cell turgor pressure. The influx of protons results in cytoplasm acidification. It is believed that acidification affords protection to macromolecules in the cell by making them less reactive with electrophiles, and activating other stress protection-related systems like Dps.

*Species diversity of Homologous KefB and KefC Activity:* In a survey of a 13 Gram-negative and Gram-positive organisms, Douglas et al. (28) demonstrated that all of the Gram-negatives tested had KefBC-type NEM-induced $K^+$ efflux activity. Although *Rhodobacter sphaeroides* and *Azospirillum brasilense* had slightly reduced activity as compared to *E. coli* 3000, *Salmonella typhimurium* LT2*, Enterobacter aerogenes*, *Erwinia carotovora*, and *Pseudomonas aeruginosa* exhibited $K^+$ efflux rates and magnitudes very similar to that observed for *E. coli* (28). Of the Gram-positives tested, including *Staphylococcus aureus, Staphylococcus caseolyticus, Bacillus cereus, Streptococcus faecalis, Streptococcus lactis,* and *Lactobacillus casei*, only *S. aureus* exhibited $K^+$ efflux activity, albeit somewhat slower than and to a lesser degree as that observed

with *E. coli* (28). Using both restriction endonuclease digested fractions and the entire cloned E. coli *kef*C gene sequence as probes for Southern blot hybridization, Douglas *et al.* (28) found no sequence homologies for the Gram-positives. Of the Gram-negative organisms tested, only *S. typhimurium* and *Ent. aerogenes* exhibited significant sequence homology to *E. coli kef*C, especially in the 5'-end of the gene (28). Although KefBC activity exists in most Gram-negative bacteria, the *kef*C sequence appears to have diverged significantly from that observed in E. coli.

Using a common Gram-negative marine bacterium, *Vibrio alginolyticus*, Nakamura *et al.* (84) demonstrated the presence of an NEM-induced (KefBC-like) $K^+/H^+$ antiporter, which acidified the cytoplasm to the same degree as that observed for E. coli, and which also could be reversed by the addition of β-mercaptoethanol. These researchers also found that, upon β-mercaptoethanol reversal, both the cytoplasmic $K^+$ concentration and the $pH_i$ were returned to approximately the same steady-state levels as before NEM addition (84).

*Mechanosensitive Channel Regulators.* The phenomena of mechanosensitive or stretch activated channels is currently being studied, and the structure and function of these systems is poorly understood (15). Stretch-activated channels are more relevant to the controlled release of $K^+$ on osmotic down-shock, but the presence of this mechanism should be addressed as another functional $K^+$ efflux system. Previous investigations by Cui *et al*. (24,25), using patch clamp recordings on giant *E. coli* protoplasts, revealed that the predominant mechanosensitive channels in the *E. coli* membrane discriminated poorly among the monovalent cations and were permeable to small amounts of both glutamate and $Ca^{2+}$. The channels were activated by artificially induced osmotic down-shock, and KefB and KefC null mutations did not affect channel activity (24,25). As a result of experiments that will not be explained in this proposal, Cui *et al*. (24,25) suggested that the KefA gene product represents a regulatory protein that is bound to the mechanosensitive channel and controls the activity of the channel as a currently unknown function of internal pressure. These researchers also indicated that KefA may actually act as a "spring" within the stretch activated channel, thereby preventing physical leakage of intracellular components (24,25). It is also suspected that this system is not necessarily specific for $K^+$ efflux, and rather the observations of $K^+$ efflux are merely a result of a high steady-state $K^+$ concentration within the cell during normal growth or stationary phase.

*Evolutionary Role of Potassium Efflux - Methylglyoxal*. Many researchers have demonstrated that methylglyoxal (MG) is naturally produced by *E. coli* and other Gram-negative organisms (1,42,43,45,47,48,63,69,102). As discussed above, MG is a potent electrophile that can cause catastrophic DNA and protein damage, and which also specifically induces the KefB $K^+$ efflux pathway resulting in cytoplasmic acidification (42). The suggested reasoning for the seemingly suicidal production of MG and the MG biosynthetic and detoxification pathways are presented in a recent review by Ferguson *et al*. (45) and will not be described in this proposal. However, it is important to note that these researchers speculated that KefB represents a protective pathway for naturally produced electrophiles (MG) (39,42,44,45). Based on DNA sequences and predicted protein structure analyses, it was also suggested that KefC evolved from KefB and is responsible for protection from anthropogenic (xenobiotic) and environmentally relevant electrophilic stressors. The details of the previous system have been well reviewed, and although both the production of and the protection from MG is interesting, it will not be discussed further.

*Effect of Stationary Phase and RpoS.* As a result of excess accumulation of biomass and long SRTs, bacteria in activated sludge flocs likely exist in a physiological state of relatively slow endogenous growth, commonly represented by stationary phase pure culture experimentation. The importance of stationary phase resistance to a variety of environmental stressors has become a widely studied topic, because it has been observed that environmentally relevant bacteria very rarely exist in a state of exponential growth. As bacteria enter stationary phase, a selection of relevant proteins are produced which allow the cell to survive in nutrient limited media.

RpoS is a RNA polymerase sigma factor, $\sigma^s$, which is responsible for the expression of the stationary-phase genes that are required for survival in non-ideal conditions (99). In contrast to the $\sigma^{70}$ sigma factor which controls the constitutive expression of genes required for growth and "house-keeping", the alternative RpoS sigma factor is maximally produced at the onset of stationary-phase (99). RpoS recognizes operons, many of which are currently unknown, that transform the cell into a semi-dormant state which is better able to resist a variety of environmental challenges (99). RpoS is specifically responsible for the excess production of Dps, a protein associated with the protection of DNA from oxidative stress, as well DNA repair

enzymes such as Ada, AidB, and exonuclease III (99). Of relevance for this review, Ferguson et al. (41) demonstrated that stationary phase *E. coli* were much more resistant to NEM than exponentially growing cells. Using a variety of RpoS, Dps, and KefBC mutants, it was determined that an increased background level of RpoS and Dps, associated with stationary phase growth, was responsible for increased viability (41). The KefBC $K^+$ efflux and cytoplasmic acidification mechanism did not induce RpoS controlled expression of new proteins but rather activated existing (no induction of *de novo* proteins) protection and repair pathways (41). However, unexpected increases in RpoS levels were measured as a result of cytoplasmic acidification upon addition of weak membrane permeable acid, but not as a result of KefBC-mediated acidification (44). A ClpP protease mutant, which accumulated very high levels of RpoS, was found to be even more resistant to NEM than the original strain (41). Therefore, it appears that the KefBC protection system functions independently of RpoS gene expression, and thus KefBC-mediated cytoplasmic acidification does not result in increased RpoS controlled transcription (45). It is important to reinforce that KefBC mechanisms did provide NEM protection during stationary phase, even though it appears that the RpoS control during stationary phase tends to set the background sensitivity to a selection of electrophilic toxins (45). In other words, the protection afforded by the KefBC mechanism does not result from an increase in RpoS levels, but rather the background RpoS and protection/repair protein levels (Dps, Ada, AidB, and exonuclease III) control the amount of increased viability derived from KefB and KefC activation (45). It is therefore emphasized that, since stationary phase results in RpoS-induced production of high levels of DNA protection/repair proteins, cytoplasmic acidification is better able to protect intracellular macromolecules, because of the increased concentration of protection/repair proteins that are available for activation by acidification (KefBC induced acidification).

Based on the previous discussion, it is expected that activated sludge organisms, which exist in "stationary phase" or relatively slow growth conditions, possess a significant background tolerance to industrially relevant electrophiles and strong oxidants, simply as a result of increased levels of DNA repair/protection proteins. More importantly, KefBC mediated cytoplasmic acidification must be activated in order to take advantage of existing defense mechanisms for adequate resistance to electrophilic stress.

*Potassium Uptake Systems.* As shown above in Figure 1, there are three primary $K^+$ uptake systems in *E. coli*, the high affinity inducible Kdp-ATPase, the constitutive low affinity TrkG and TrkH PMF driven $K^+/H^+$ symporters, and the constitutive low affinity Kup (TrkD) $K^+/H^+$ symporter (5,6,108). There is also a very low affinity $K^+$ uptake system, known as TrkF, that has been poorly characterized so far, but which functions with very slow kinetics in $Kdp^-$ $TrkG^-$ $TrkH^-$ $Kup^-$ mutants. The general characteristics of these systems are given in Figure 1, and although these systems are tightly integrated with the $K^+$ efflux systems, they will not be discussed further. The reader is referred to several recent reviews on these systems (5,6,108).

Of specific interest for this proposal, Elmore et al. (31) suggested that NEM inhibits $K^+$ uptake systems (during $K^+$ efflux activation) by protein thiol modification. Circumstantial evidence suggested that exposed cysteine thiols on the $K^+$ uptake proteins (most likely TrkG and TrkH) are modified by NEM, thereby deactivating the more active uptake system during a $K^+$ efflux event (31). The reversal mechanism of the covalent protein thiol modification is unknown, but it was demonstrated that dithiothreitol was unable to reactivate $K^+$ uptake completely in the tested mutants (31). This seems to be a contradiction of the dithiothreitol induced reversal of the $K^+$ efflux mechanism for the unmutated *E. coli* strain (see above), especially since $K^+$ uptake was observed after reductant addition (8). Instead, it explains the somewhat slower uptake rate for NEM-$K^+$ depleted cells to which dithiothreitol was added, as compared to the high $K^+$ uptake rate observed for osmotic down-shock $K^+$ depleted cells (31).

**Toxicity of Electrophilic Chemicals**

In order to understand the necessity of cellular protection from electrophiles and strong oxidants, the mechanisms of cellular damage must be addressed with respect to industrially relevant organic electrophiles. A brief review of the relevant damage mechanisms will also be presented for several toxic heavy metals ($Cd^{2+}$, $Hg^{2+}$, $Zn^{2+}$) that are known to induce deflocculation. Shock loading of these metals has been specifically associated with deflocculation (87), but other toxic heavy metals (e.g. $Pb^{2+}$, $Ag^+$, $Cu^{2+}$, $As^{3+}$, $Cr^{6+}$) could also be implicated, if as discussed below, these metals are thiol reactants.

*Organic Electrophiles.* The classical electrophilic substituents, which are typically associated with industrially derived organic compounds, are defined above. This list is likely incomplete because, in many cases, the chemical structure of an organic molecule, in addition to the common electron withdrawing substituent groups, promotes increased sulfhydryl reactivity. Based on electronic assumptions alone, the electron deficiency associated with the nitro groups on CDNB likely causes the chloride substituent to be much more electrophilic than in chlorobenzene (resonance structure stabilization neglected) (107). Regardless of predicted electrophilicity of organic compounds, a better indication of $K^+$ efflux induction will be a combination of (i.) the expected thiol reactivity, (ii.) the size and hydrophobicity of the bound GSX adduct, and (iii.) the ability to oxidize GSH completely to GSSG.

Although the specific damaging mechanisms of potent organic electrophiles were previously suggested, nucleophilic DNA base substituents and exposed protein thiols are the most likely points of cell damage. Fraval and McBrien (47) indicated that the mode of MG toxicity was an inhibition of both DNA replication and protein synthesis. In most cases, it appears that intracellular electrophilic damage is very nonspecific with mutagenic reactions occurring at a variety of exposed macromolecular nucleophilic sites, such as the side chains of arginine, lysine, and cysteine and with nucleic acid bases guanine, adenine, and cytosine (45). Ferguson et al. (42) suggested that MG reacts with both proteins and DNA. At low MG concentrations, it has been difficult to determine which of these reactions caused inhibition, but at high MG levels, DNA/RNA damage likely predominates via modification (42).

*Toxic Divalent Heavy Metals.* From aquatic chemistry theory, it is known that $Hg^{2+}$ and $Cd^{2+}$, which are both soft metal cations, bind most strongly with soft ligand donors, such as R-SH, $R_2S$, $S_20_3^{2-}$ (110). $Zn^{2+}$ is typically classified as "borderline" between the soft and hard metal ions and therefore would be predicted to bind the above ligands with slightly less thermodynamic stability than $Cd^{2+}$ and $Hg^{2+}$ (110). As a result, it is expected *a priori* that $Cd^{2+}$, $Hg^{2+}$, and to a lesser degree, $Zn^{2+}$ represent active thiol reactants and tightly (bind) complex the GSH sulfhydryl substituent. In fact, as stated above, Meury et al. (74) demonstrated that $HgCl_2$ was a strong activator of both KefB and KefC.

Vallee and Ulmer (117) suggested that $Cd^{2+}$ has a very high affinity for protein thiols and can compete effectively with $Zn^{2+}$ for these ligand donors. Rayner and Sadler (98) indicated that $Cd^{2+}$ is a potent scavenger of intracellular sulfhydryl groups. $Cd^{2+}$ also binds nucleic acid bases with little specificity and typically induces DNA single strand breaks (58). In addition to thiol reactivity, $Cd^{2+}$ has been associated with enzyme inactivation as a result of histidyl and cysteinyl ligand binding (23). For these reasons, $Cd^{2+}$ toxicity is commonly associated with both enzyme inactivation and DNA mutagenesis. Although not as well defined, $Cd^{2+}$ can also complex phosphate groups, thereby affecting nucleic acids, phospholipid membrane structure, and phosphorylated metabolic intermediates (23). Bochner et al. (11,66) found that $CdCl_2$, as well as many of the organic electrophiles discussed above, induce an oxidative stress response in *S. typhimurium* that is characterized by the production of both heat shock proteins and adenylylated nucleotides (11,66). These researchers also suggested that $Cd^{2+}$ is a sulfhydryl reactant (11,66).

The toxicity of both $Hg^{2+}$ and organomercurials, such as p-chloromercuribenzoate and methylmercury, has been well studied by many researchers, and detoxification mechanisms have been elicited in both eukaryotic and prokaryotic systems. $Hg^{2+}$ is also reactive with exposed thiols, binding reduced cysteinyl sulfhydryl groups and oxidized disulfides (R-S-S-R) with considerable affinity (23,58). Highly toxic methylated-mercury species have been suggested to interact with nitrogen molecules of nucleic acid bases thus providing the commonly recognized mutagenic capability (23). As with $Cd^{2+}$, $Hg^{2+}$ can also compete for metal binding sites in metalloenzymes thereby inactivating catabolic and anabolic systems (23). Unlike $Cd^{2+}$ and $Hg^{2+}$, $Zn^{2+}$ is an essential micronutrient at low concentrations. However, at high concentrations, $Zn^{2+}$ has been shown to be a sulfhydryl reactant (23,58).

**Bioflocculation of Activated Sludge**

Bacterial cells, which have an isoelectric point of approximately pH 2-4, can be considered hydrophilic colloids stabilized in aqueous solution by a net negative surface charge at neutral pH (20,112,113). The aggregation of these cells in the form of bioflocculated particles, such as that

incurred in activated sludge systems, has been hypothesized to be governed by two related but fundamentally differing mechanisms.

*DLVO Theory*. *T*he first mechanism involves the effect of ionic strength alone on colloidal particle attachment and is typically defined by Derjaugin, Landau, Verwey, and Overbeek (DLVO) theory (119). An increase in ionic strength, regardless of the valence of the predominant cations, causes a compression of the electric double layer surrounding the bacterial cells. As typically acknowledged for the flocculation of negatively charged inorganic colloids, a decrease in the thickness of the electric double layer suppresses the electrostatic repulsion between two negatively charged particles such that attractive van der Waals interactions result in destabilization and particle aggregation (2). Zita and Hermansson (119) suggested that with increasing concentrations of either monovalent or divalent cations, the stability of activated sludge flocs increase. These researchers indicated that above an ionic strength of 0.10 M floc stability decreased, and thus DLVO theory could not be applied. After deflocculating activated sludge flocs by successive treatments with distilled water, Zita and Hermansson (119) also showed that reflocculation was a function of ionic strength and not bridging affects of multivalent cations. Although this mechanism may contribute to the overall stability of an activated sludge floc at medial ionic strength, several researchers, both before and after this investigation, have suggested that a second mechanism actually controls the flocculation of activated sludge microorganisms and thus controls floc settling and dewatering properties.

*Extracellular Polymeric Substances (EPS)*. As proposed by Novak and Haugan (89) and Tezuka (113) and as corroborated by several other researchers, the more accepted mechanism of activated sludge flocculation is the attachment of bacterial cells by extracellular polymers with multivalent cations bridging anionic sites of the biopolymers and the cell surface (19,34,35,54,55,62,82,83,91,96,113,116). Even though there may be a critical minimum ionic strength required to promote particle attachment, the strength, density, and settling and dewatering properties of an activated sludge floc are currently suspected to be controlled by the specific composition and charge of the EPS, the valence and chemical properties of the complexed cations, and the anionic surface charge density on the bacterial cell membranes. Growth of filamentous bacteria in activated sludge flocs is not addressed in this review, but the

effects of excess filaments on activated sludge settling (increase in SVI) are realized and are an obviously important aspect that must be considered with the EPS/multivalent cation flocculation model. A small number of filaments within an activated sludge floc may provide "backbone" structure to the floc and possibly increase the overall tensile strength of the floc, thereby conferring resistance to floc fracture (51). It has therefore been suggested that a reduction in floc strength, caused by a weakening of polymer binding interactions, results in the release of dispersed particles by turbulent surface erosion rather than the production of large floc fragments (94,95).

It has long been recognized that most bacterial species produce and excrete extracellular polymers upon entering stationary growth phase and are known to flocculate as a result (112,113). The evolutionary cause of this phenomena is unknown, although flocculation and/or surface adhesion may provide an individual cell selective advantages and protection during nutrient starvation. Others have suggested that these polymers are also produced during exponential growth, however the formation of a "slime layer" is not realized because of rapid cell division (112). The predominant polymers have been analyzed in both pure culture and activated sludge systems include polysaccharides, proteins, lipids, and nucleic acids (50,57,78,90,96,112,116). Experimental analysis has determined that acidic and nonionic polysaccharides and proteins are a major fraction of the EPS, with glycoproteins also being a possibly important fraction (46,54,55,57,90,109,116). In domestic treatment systems, these polymers could be derived from anthropogenic waste products, but their occurrence in activated sludge systems treating only industrially derived wastes demonstrates that the cells must be responsible for a certain fraction of the biopolymers (83). Several researchers have suggested that most of the residual COD in an industrial waste treatment system effluent can be attributed to stabilized anionic biopolymers remaining in the bulk supernatant (82).

The characteristics and interactions of the lipid fraction in the EPS are less clearly defined. Goodwin and Forster (50) detected triglycerides in an extracted EPS fraction using infrared spectroscopy. They noted a deterioration in sludge settlement (SVI) as the lipid fraction increased (50). These lipids are likely to contribute to the internal hydrophobicity within the floc polymer network and may be dynamically balanced by predominating hydrophilic interactions

(116). Although a minor component as compared to protein and polysaccharides, the EPS nucleic acid fraction is commonly associated with cell autolysis and endogenous decay, but influent sources of extracellular DNA must also be considered for systems treating domestic wastewater.

In general, most research has dealt with the protein and polysaccharide fractions of the EPS and the relationship between the type and amount of polymer extracted as well as settling and dewatering properties. It is now readily accepted that these polymers are the major components of the EPS. The polysaccharide fraction has been relatively well characterized with respect to chemical composition and ionic charge (46,57). Horan and Eccles (57) determined that polysaccharides extracted from mixed liquor were composed of five primary monomers, specifically glucose, galactose, mannose, glucuronic acid, and galacturonic acid, thus indicating that this polymer fraction should be anionic at neutral pH. Steiner et al. (109) also suggested that extracted polysaccarides were composed primarily of nonionic sugar monomers and anionic glucuronic acid. The protein fraction and the existence of anionic glycoproteins has been less well characterized, but in several cases the protein content has been found to exceed the polysaccharide content on a mass basis (9,34,55,60,116). The extracellular protein fractions are suspected to be anionic and hydrophilic, though slightly amphoteric due to positively charged amino groups (N-terminal-α-amino, ε-amino-Lys, and guanidino-Arg). Regardless, most correlations with sludge settlement and dewatering properties have been made as a function of total EPS extracted, protein concentration in the EPS, and the polysaccharide fraction in the EPS. Forster (46) initially showed that the EPS polysaccharide content could be correlated with an increase in SVI. Eriksson and Alm (34) found that floc strength and filterability increased (improved) with a decrease of extractable EPS. These researchers based their measurement of EPS on proteins and polysaccharides. Urbain et al. (116) experimentally demonstrated that both protein and polysaccharide fractions, as well as the total extractable EPS could be positively correlated to the measured SVI. Novak *et al*. (88) concluded that the thickening and dewatering properties of activated sludge are influenced by the amount and composition of biological anionic polymer present, and that the colloidal anionic polymers in the bulk solution are stabilized by anionic substituents and therefore control cationic polymer demand and filterability. It can be inferred from this review that deflocculation, induced by shock loads of toxic

compounds, could severely degrade dewatering capabilities (e.g. specific resistance to filtration (SRF)) and increase polymer demand. Interestingly, Higgins and Novak (55) found that an increase in the divalent cation feed concentration to laboratory activated sludge reactors resulted in an increase in the bound protein content, while the polysaccharide EPS content remained constant. Novak et al. (91) subsequently demonstrated that the soluble anionic biopolymer content increased in the supernatant with an increase in the monovalent to divalent cation ratio (M:D, on an equivalent basis), possibly indicating detachment from the floc surface.

*The Effect of Cations.* It has also been suggested by many researchers that, because of the anionic charge of protein carboxyl and thiol substituents and polysaccharide uronic acid groups, these polymers can be considered as naturally produced anionic polyelectrolytes (20,46,96). However, as a result of excess negative charge from polymer material within a sludge floc and primarily anionic bacterial cell surfaces, multivalent cations are required to bridge the negatively charged groups such that repulsive electrostatic interactions are minimized. The influence of cations on bridging of biopolymers and anionic cell surface groups represents the most critical part of this review and is closely related to the research hypothesis presented above. Several researchers have demonstrated the importance of divalent cations and the specific role of calcium as a requirement for efficient flocculation of bacterial cells in both pure culture and activated sludge systems (19,34,54,55,62,81,82,83,89,91,92,113). Tezuka (113) initially reported that flocculation of a *Flavobacterium* strain required both calcium and magnesium in the growth medium. Kakii et al. (62) determined that most of the $Ca^{2+}$ (and ~50% of the $Mg^{2+}$) in an activated sludge sample could be easily extracted from the floc by weak acid (pH = 3), even though little of the total $Al^{3+}$ and $Fe^{3+}$ were extracted using the same technique. Using this phenomenon, these researchers were able to show that both the SVI and the supernatant turbidity decrease with increasing amounts of $Ca^{2+}$ added (62). Eriksson and Alm (34) showed the relative importance of divalent cations on floc strength and settling/dewatering properties, using a metal chelating agent, EDTA (ethylene-diamine-tetra-acetate). Using a $Ca^{2+}$ specific complexing agent, EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N-tetra-acetate), Bruus et al. (19) demonstrated that, by extracting $Ca^{2+}$ from activated sludge floc polymers, small dispersed particles were released into the bulk supernatant thus leading to an increase in the specific resistance to filtration (SRF). Upon batch addition of $Mg^{2+}$, $K^{+}$, and $Na^{+}$, calcium ions

were released from the floc indicating an ion-exchange process (19). $Ca^{2+}$ release increased in the order, $Mg^{2+} > K^{+} > Na^{+}$, and supernatant turbidity increased as $Ca^{2+}$ was released, with the largest proportional increase in effluent turbidity for $K^{+}$ addition (19). A linearly proportional increase in the SRF was well correlated with the increase in supernatant turbidity (as measured spectrophotometrically) upon ion-exchange by cation addition (19).

Novak and coworkers (54,55,81,82,83,91,92) considered the effects of both monovalent ($K^{+}$, $Na^{+}$) and divalent cations ($Ca^{2+}$,$Mg^{2+}$) as well as the respective monovalent to divalent cation ratio (M:D) on settling/dewatering properties, filterability, biopolymer content, effluent quality, cationic polymer demand, and floc strength. These four cations were amended both in batch experiments and in the feed of lab-scale and full-scale activated sludge systems. Initially, Novak and Randall (92) demonstrated that bulk addition of $CaCl_2$ to an industrial activated sludge system reduced previously high effluent suspended solids which were suspected to have been caused by high levels of $Na^{+}$ added as NaOH to adjust the influent pH. They also suggested that transient loading of toxic compounds or $NH^{4+}$ deficiencies may have also been responsible for poor sludge settling properties (92). Using parallel laboratory-scale activated sludge systems, Higgins and Novak (55) evaluated the effect of the $Ca^{2+}$:$Mg^{2+}$ ratio and the M:D ratio. Although many conclusions were drawn from this and subsequent work, the more relevant findings, as related to this review, are presented below (54,55):

- In order to achieve good settling and dewatering properties, the minimum feed concentration of $Ca^{2+}$ and $Mg^{2+}$ is in the range from 0.72-2.0 meq/L.
- A reactor feed M:D ratio greater than about 2.0 results in significant deterioration of settling and dewatering properties, as measured by the SVI, SRF, CST, floc density, cake solids, CST versus time stirred at G = 500 $sec^{-1}$ (floc strength), and the optimum polymer conditioning dose.
- Addition of divalent cations in the reactor feed (decrease in M:D) significantly reduces the number of colloidal supernatant particles in the range from 5 to 20 μm. This results in a decrease in the polymer demand, which was suggested by the authors to be controlled by the concentration of anionic colloidal particles and EPS stabilized in the bulk supernatant.

- When $K^+$ is added to the feed at 1.5 meq/L ($Ca^{2+}$ and $Mg^{2+}$ = 3.0 meq/L), settling properties (SVI) improve, but dewatering properties (cake solids and SRF) deteriorate, as compared to a control reactor without the amended $K^+$.
- When sodium is added in a batch experiment, ion-exchange results in significant release of $Ca^{2+}$ and $Mg^{2+}$.
- For an industrial activated sludge with an M:D = 2.4, batch addition of $Ca^{2+}$ improves dewatering properties and reduces the optimum polymer dose.

In subsequent studies, Novak et al. (91) determined the effects of specific cations and cation ratios on industrial activated sludge settling and dewatering properties. The results from this study were similar to that given above (54,55), but they also demonstrated that, with small increases of feed $K^+$, (above a background of 0.12 meq $K^+$/L) settling properties unexpectedly improved. However, the effluent suspended solids increased significantly when the feed $K^+$ concentration was increased to 17 meq/L (91). Considering the potential for potassium limitation due to the relatively low background $K^+$ concentration, the authors suggested that the improvement caused by small increases in the feed $K^+$ concentration may have been a function of the physiological role of potassium in bacterial cells (91). This phenomena was further examined by Murthy and Novak (80). At relatively low M:D ratios, it was determined that added $K^+$ in the feed of laboratory reactors improved settling and dewatering properties up to approximately 1.0 meq/L (80). Above 2.0 meq/L, additional $K^+$ was detrimental to all measured settling and dewatering properties (80). Since only relatively low concentrations resulted in improved sludge properties, the author suggested that these improvements were a consequence of the minimal physiological requirements for the potassium ion (80). Novak et al. (91) also showed that, in addition to an increase in the effluent TSS, the supernatant EPS (protein and polysaccharide) increased with the M:D ratio, thus indicating detachment of soluble biopolymers from the floc surface.

Murthy and Novak (81) examined the beneficial role of cations on floc dewatering properties following batch aerobic digestion of activated sludge. Although unrelated to the conclusions presented, the soluble $K^+$ concentration approximately doubled after aerobic digestion, while the $Na^+$ increased by about 25%, and $Mg^{2+}$ and $Ca^{2+}$ remained approximately constant (81). Since

significant cell lysis is expected to occur during aerobic digestion of activated sludge, activated sludge organisms may have previously accumulated high levels of intercellular potassium and lower levels of sodium. This phenomenon was further verified in other laboratory-scale reactor experiments (unpublished data, Murthy and Novak, 1998). Finally, Murthy and Novak (82) demonstrated that soluble effluent COD was directly proportional to the M:D ratio. Using laboratory-scale reactors, these researchers determined that a large fraction of the increase in effluent COD was caused by detached floc polymers (especially polysaccharides) as a result of an increase in the feed monovalent cation concentration (82).

Based on this review of the mechanisms controlling bacterial flocculation in activated sludge systems, it seems feasible that transient increases in the extracellular potassium (and potentially sodium) concentration may cause a replacement of bridging divalent cations by ion-exchange processes and could therefore result in deflocculation. It also seems reasonable that this mechanism could increase anionic colloidal particles (detachment) to a level that would seriously alter filtering/dewatering properties and increase cationic polymer demand.

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# Chapter 2. THE IMMUNOCHEMICAL DETECTION OF STRESS PROTEINS IN ACTIVATED SLUDGE EXPOSED TO TOXIC CHEMICALS

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# Chapter 3. A PHYSIOLOGICAL MECHANISM FOR ACTIVATED SLUDGE DEFLOCCULATION CAUSED BY SHOCK LOADS OF TOXIC ELECTROPHILIC CHEMICALS

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**ABSTRACT:** It is hypothesized that physiological bacterial stress response mechanisms are involved in activated sludge process upset caused by shock loads of toxic chemicals. The goal of this research was to determine whether the bacterial glutathione-gated, electrophile-induced potassium efflux system is responsible for deflocculation observed in response to shock loads of toxic electrophilic (thiol reactive) chemicals. The results indicate significant $K^+$ efflux from the activated sludge floc structure to the bulk liquid in response to shock loads of chloro-2,4-dinitrobenzene (CDNB), N-ethylmaleimide (NEM), 2,4-dinitrotoluene (DNT), benzoquinone (BQ), and cadmium (Cd) to a bench-scale sequencing batch reactor (SBR) system. In most cases, the stressor chemicals caused significant deflocculation, as measured by an increase in effluent volatile suspended solids (VSS), at concentrations much less than that required to reduce the maximum specific oxygen uptake rate by 50% ($IC_{50}$). This suggests that toxin-induced activated sludge deflocculation may actually be caused by a protective bacterial stress mechanism and that the upset event may not be detectable by aerobic respirometry. More importantly, the amount of $K^+$ efflux appeared to correlate well with the degree of deflocculation. The transport of other cations including sodium, calcium, magnesium, iron, and aluminum, either to or from the floc structure, was negligible as compared to $K^+$ efflux. It was also determined that the $K^+$ efflux occurred immediately (within minutes) after toxin addition and then was followed by an increase in effluent turbidity. Using alkaline phosphatase as a periplasmic marker, it was determined that a negligible amount of the $K^+$ efflux response was due to lysis of activated sludge microorganisms. The current results suggest that activated sludge upset (i.e. deflocculation) may be caused by a specific protective stress response in bacteria.

**KEYWORDS:** Activated Sludge, Deflocculation, Potassium Efflux, Xenobiotic, Microbial Stress, Glutathione.

## Introduction

Both domestic and industrial activated sludge plants are plagued with problems associated with the input of toxic chemicals from sporadic industrial and commercial discharges. The random input of chemicals, which are severely toxic to activated sludge microorganisms, may cause rapid plant disruption and can lead to discharges of presumably harmful contaminants into receiving streams (22,27,34,39). Furthermore, toxic discharges may disrupt biological processes so severely that treatment performance is affected for long periods of time (weeks to months) (5,9). The likely result is a reduction in treatment efficiency, an increase in effluent toxicity, and/or process operational problems that may require a significant amount of time before complete recovery.

To address this problem, we are investigating the mechanisms of wastewater treatment process upset caused by shock loads of toxic chemicals (8,9,10,15). The underlying hypothesis of this research involves the consideration of the physiological role of microbial stress responses in process deterioration. An understanding of these mechanisms could potentially lead to upset early warning systems and mitigation strategies that will help to avoid the significant environmental problems associated with activated sludge process upset. The current investigation is focused on attempting to determine the mechanisms that cause activated sludge deflocculation in response to low concentrations (relative to specific oxygen uptake rate inhibition) of thiol-reactive organic xenobiotics and heavy metals.

Transient deflocculation in activated sludge systems is a critical issue for many municipal and industrial wastewater treatment facilities, but it is a problem for which “cause and effect” is usually not correlated (28). For example, deflocculation is not as well defined as filamentous bulking. Using microscopic examination, an operator can observe the proliferation of filamentous bacteria and then unambiguously attribute the excess filaments to sludge bulking (26). In addition, when filamentous bulking is first observed, the operator can immediately initiate mixed liquor chlorination as a temporary control of filamentous bacteria to improve settling before surface water contamination occurs.

In the case of deflocculation, the only available indication of upset conditions is usually a rapid increase in effluent suspended solids (turbidity) and a deterioration in sludge dewatering properties. If the deflocculation event is severe and prolonged, significant activated sludge system washout can occur without ever recognizing the cause of the problem or the time of initiation (23,28,31,32,37). Xenobiotic-induced deflocculation results in severe environmental problems beyond the obvious loss of biomass and the associated discharge of biochemical oxygen demand (BOD). In addition to the effluent toxicity associated with the soluble fraction, it is likely that deflocculating xenobiotics are also sorbed to the deflocculated particles and thus washed out of the system, thereby resulting in even higher effluent toxicity. In treatment plants where biological nutrient removal is employed, total phosphorous (TP) removal will be severely reduced if polyphosphate containing bacteria are among the deflocculated particles. Deflocculation also results in possible release of pathogens to receiving streams and increased $Cl_2$ demand in disinfection processes. If tertiary filtration is used, deflocculation will result in an obvious increase in backwash requirements. Investigations of both the causes of transient deflocculation and the mechanisms of floc disintegration should be undertaken so that an operator can determine the cause of process upset and initiate corrective action before process damage and surface water contamination occur.

**Research Hypothesis and Physiological Model.** The working hypothesis for this research involves a bacterial stress response which we believe causes deflocculation by acknowledged physical/chemical mechanisms. We believe that shock loading of sublethal levels of toxic thiol-reactive organics and heavy metals causes rapid up-regulation of the glutathione-gated potassium efflux (GGKE) system located in bacterial cell membranes. This biochemical mechanism has been studied in pure cultures and represents a bacterial protective stress response that is activated rapidly in the presence of electrophilic xenobiotic stressors (7,14,17,18,19,20). Booth and coworkers determined that chemical challenge by toxic electrophilic compounds (both mild thiol reactants and strong oxidants) elicits a protective mechanism that prevents macromolecular damage by rapid efflux of intracellular $K^+$ and concurrent cytoplasmic acidification (19,20). Potassium is the major cation regulating intracellular pH and osmotic turgor pressure in Gram-negative bacteria and is the predominant counter ion for many negatively charged protein residues and substrate molecules/intermediates within the cytoplasm (6,33). $K^+$ is usually highly

concentrated in the cytoplasm at up to 0.5 moles $K^+$/L of cytoplasmic fluid (4,42). Although most of the work done on the GGKE system has been performed using *Escherichia coli* strain K-12 and derived mutants, homologous efflux systems have been found in all Gram-negative organisms examined, but not in Gram-positives with the exception of *Staphylococus aureus* (14). It was determined that the GGKE system represents a glutathione-gated potassium efflux/proton uptake antiport protein which is activated by glutathione-S-conjugates (GSX) and possibly oxidized glutathione (GSSG) (7).

Glutathione (GSH) is a biosynthesized tripeptide formally known as N-(N-L-γ-glutamyl-L-cysteinyl)glycine. GSH represents a significant fraction of the low molecular weight cytoplasmic thiol pool in both eukaryotic and prokaryotic life forms (up to 95% in most Gram-negative bacteria) and is a critical component of cytoplasmic redox control in Gram-negative bacteria (2,16,44). The structures of GSH and GSSG are shown in Figure 1 (note the unusual γ peptide bond between the glutamate and cysteine residues). The thiol substituent of GSH is susceptible to spontaneous (non-enzymatic) electrophilic attack by many electrophilic oxidants, which are known to cause protein damage and DNA mutations (2,3). Although the mode of cell damage may be specific for each inhibitory electrophile, it appears that GSH acts in a sacrificial manner, scavenging harmful electrophiles to prevent macromolecular oxidations. It has also been determined that constitutively produced non-specific enzymes known as glutathione-S-transferases (GST) are capable of catalyzing the conjugation of GSH with many structurally dissimilar toxic electrophiles (30,45). The reaction between GSH and chloro-2,4-dinitrobenzene (CDNB), a well known electrophilic thiol reactant, is presented in Figure 1. It is important to note that this reaction occurs spontaneously, but the rate of GS-DNB (Figure 1) formation is significantly increased with cytoplasmic GST activity.

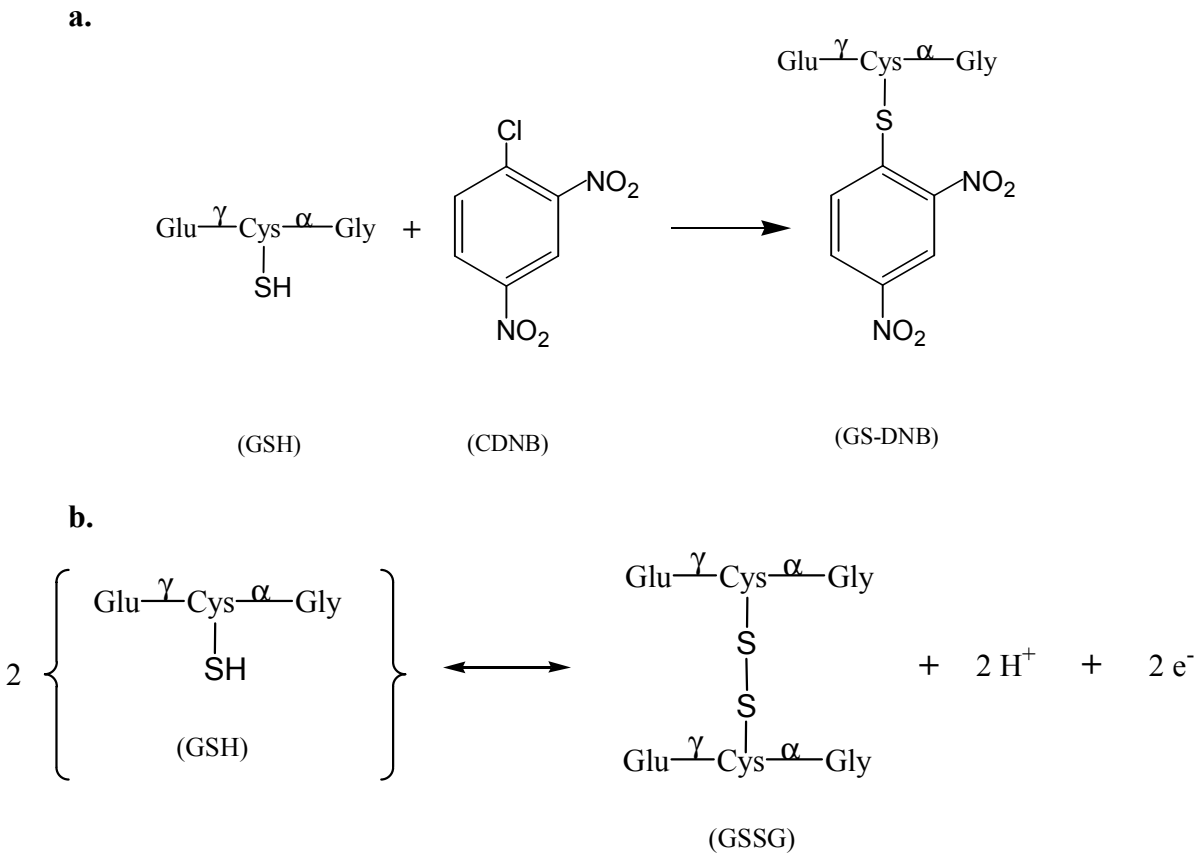


Figure 1. (a.) Reaction of GSH with CDNB to form glutathione-S-dinitrobenzene (GS-DNB). (b.) Oxidation of GSH to form oxidized glutathione (GSSG).

It is hypothesized that activation of the GGKE system results in excretion of a significant amount of $K^+$ from the bacterial cytoplasm within seconds to minutes after chemical shock loading. The sudden increase in monovalent cation levels within the extracellular polymeric substances (EPS) results in a rapid and significant increase in the localized monovalent to divalent cation ratio (M:D), which previously has been found to be an important factor controlling activated sludge floc strength (24,25,35,38). A rapid localized increase in the M:D ratio causes a reduction in floc strength and immediate deflocculation by turbulent erosion of small nonsettleable particles from the floc surface.

In order to evaluate this hypothesis, it was necessary to determine whether shock loads of chemical toxins simply caused catastrophic cell lysis (thereby releasing cytoplasmic $K^+$) or could have induced GGKE system activity. A relevant lysis indicator was monitored in the mixed

liquor bulk supernatant in response to chemical shock loading, and the marker concentration was compared to a total mixed liquor activity derived from a cell free extract (CFE). Glucose-6-phosphate dehydrogenase (G6PDH) was originally considered for this purpose since it is a strict cytoplasmic catabolic enzyme and previously has been demonstrated to be a good indicator of cell lysis in activated sludge (21). However, G6PDH contains active-site thiols that are likely vulnerable to electrophilic thiol-reactants (i.e. NEM and CDNB), and thus G6PDH could be inactivated by those chemicals of interest for evaluating the GGKE/deflocculation mechanism. Alternatively, alkaline phosphatase (AP), which is a strict periplasmic enzyme, is known to be chemically resilient (especially bacterial-derived AP) and was shown to be associated with activated sludge microorganisms with very little activity in the bulk supernatant (43). Since AP is a periplasmic enzyme, toxin-induced release of AP into the bulk liquid should be a sensitive indicator of cytoplasmic membrane damage.

**Experimental Objectives.** Several experiments have been designed to determine if the GGKE system is involved in activated sludge deflocculation caused by industrially-relevant toxic electrophiles. This paper addresses the first phase of experiments for which the objectives were to:

1. Screen an appropriate selection of toxic organic compounds for their ability to deflocculate activated sludge.

2. Evaluate $K^+$ transport (to or from floc structure) in activated sludge in response to a subgroup of deflocculating electrophilic chemicals.

3. Evaluate the transport of other relevant cations, including $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Al^{3+}$, and $Fe^{3+/2+}$ in response to a subgroup of deflocculating electrophilic chemicals.

4. Compare cation transport to activated sludge performance parameters, specifically effluent turbidity.

The results provide evidence that the GGKE system is responsible for toxin-induced activated sludge deflocculation. Specifically, it appears that the degree of $K^+$ efflux from the floc structure to the bulk liquid phase is well correlated with effluent VSS levels. It is likely that many industrially-relevant (non)electrophilic chemicals can induce deflocculation by other unrecognized physiological or physical/chemical mechanisms, and therefore the GGKE system

may not be the only cause of toxin-induced deflocculation. Even though we have considered only one group of chemical toxins, one microbial stress response, and one process upset mechanism, we contend that the GGKE system represents an important physiological stress response in environmentally relevant bacteria, and it is the first microbial stress response that has been correlated with activated sludge process upset. The implications of the results are discussed below.

## Methodology

As previously reported (9), activated sludge experiments were conducted using 3.5 L sequencing batch reactor (SBR) systems. As many as eight SBRs were operated in parallel for each stress experiment (screening and cation transport/deflocculation experiments). One reactor served as the control and the other reactors were stressed with varying concentrations of toxic chemicals. For all of the experiments discussed below, fresh publicly owned treatment works (POTW) mixed liquor and primary effluent from the Blacksburg-Virginia Tech Wastewater Treatment Plant (Blacksburg, VA) were used for each experiment. Mixed liquor was added to the SBRs, and experiments were initiated within 30 minutes of retrieving activated sludge from the local POTW. Primary effluent was obtained at the same time as the mixed liquor and stored in a 5 gallon bucket at $4^{o}C$.

**SBR System.** The SBRs consisted of eight modified 4.0 L beakers which were operated in parallel. Each reactor cycle consisted of 3 minutes of feed time, 5.25 hours reaction time with aeration and stirring, followed by 30 minutes of quiescent settling and immediate effluent sampling. Mixing was provided by 100 RPM electric motors with single blade paddles, and aeration equipment consisted of aquarium air pumps and standard air stones. Fresh and refrigerated/mixed primary effluent was used as the wastewater feed for these studies. Reactor performance was evaluated by monitoring mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI), effluent total suspended solids (TSS), and effluent volatile suspended solids (VSS), all in accordance with *Standard Methods* (1). The nominal hydraulic residence time (HRT) and solids residence time (SRT) were 24 hours and 10 days, respectively, but for most of the experiments presented herein, the system was

operated for only one 6 hour cycle. For multiple cycle experiments, the SRT was maintained by wasting a constant volume of mixed liquor in the Garrett configuration once per day. The volume of mixed liquor wasted was not corrected for increases in effluent TSS, and thus severe deflocculation decreased the actual SRT and caused a washout of mixed liquor from the stressed SBRs.

After adding mixed liquor to the SBRs, the activated sludge was stirred and aerated for approximately 10 minutes and then settled for 15 minutes. Exactly 1/4 of the total reactor volume (875 mLs) was decanted using peristaltic pumps, and aerators and mixers were restarted. Fresh primary effluent was added using peristaltic pumps to replace the decanted volume in approximately 3 minutes, thus beginning the experimental cycle. As soon as primary effluent feeding was terminated, chemical stressors were added to the experimental reactors in a single shock load, pre-dissolved in either 10 mLs of distilled/deionized water,1 to 3 mLs of ethanol (ethanol also added to control reactor, when required), or as a pure solution in case of benzene and toluene. Time-zero was defined as the point immediately after primary effluent feeding, but immediately before adding the toxic chemical. Aeration and mixing were continued for 5.25 hours after feeding/stressing, followed by exactly 30 minutes of quiescent settling and decant of effluent samples. For multiple cycle experiments, the SBR system was automated with a programmable ChronTrol® XT-4 timer (San Diego, CA).

**Deflocculation Screening.** Preliminary experiments involved screening potentially electrophilic uncharged organic compounds including N-ethylmaleimide (NEM), CDNB, benzoquinone (BQ), and 2,4-dinitrotoluene (DNT); two non-electrophilic uncharged organics (benzene and toluene), pentachlorophenol (PCP), and cadmium ($Cd^{2+}$) which was added as $CdNO_3$. In order to test the research hypothesis, it was necessary to use uncharged electrophiles so that the observation of $K^+$ efflux could not be a result of ion exchange mechanisms in the activated sludge EPS. However, as a result of previous research (8,9), we have significant experience with Cd- and PCP-induced deflocculation and therefore used these compounds as "positive controls" for deflocculation.

In order to determine a reference concentration for each chemical stressor, the $IC_{50}$ (50% inhibitory concentration) was determined using specific oxygen uptake rate (SOUR)

experiments. The $IC_{50}$ was computed as the stressor concentration resulting in a 50 percent reduction in the SOUR as compared to unstressed controls. The $IC_{25}$ and $IC_{75}$ values are also reported and represent the concentrations required to reduce the SOUR by 25 and 75 percent, respectively. For each SOUR measurement, 50 mL of fresh activated sludge, aqueous stress compound stock solution, biochemical oxygen demand (BOD) dilution water (mineral salt buffer) (1), and a biogenic carbon source (55 mg/L COD) were added to fill a 300 mL BOD bottle. The biogenic carbon source contained a defined mixture of protein extracts, sugars, and organic acids (29). The dissolved oxygen concentration profile was monitored for at least 10 minutes. It was determined that adding 55 mg/L COD to diluted (1:6 v/v) mixed liquor (MLVSS = 700 to 1000 mg/L) provided the maximum SOUR during the entire 10 to 15 minute contact period.

For the screening experiments, the stress compounds were added at several concentrations both above and below the $IC_{50}$. Deflocculation was evaluated in the SBRs by measuring effluent VSS at the end of a single 6 hour cycle. Mixed liquor properties (MLSS/MLVSS/SVI) were also evaluated to ensure similar conditions for control and stressed reactors.

**Cation Transport and Deflocculation Experiments.** In order to compare cation transport, both to and from the activated sludge floc structure, with deflocculation, reactor experiments were performed exactly as above using Cd, NEM, CDNB, BQ, and DNT. However, for these experiments, cation measurements were performed immediately before stress compound addition ($t = 0$ minutes), immediately after stress compound addition ($t \cong 3$ minutes), and 15 minutes before the initiation of the settling period ($t = 5$ hours). These results were compared to the observed increase in effluent VSS over the control reactor.

*Cation Sampling and Measurement.* Cation sampling and analysis of the mixed liquor was performed such that a mass balance could be established for each of the measured cations, $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Fe^{3+}$, and $Al^{3+}$ (Figure 2). Three mixed liquor fractions were defined including a "soluble" or bulk supernatant phase, a "floc-associated" phase, and a "total" which was simply a measurement of the total cation concentration in the mixed liquor. Using a method adapted from pure culture experiments (4), a known volume (1.3 mL) of mixed liquor from the SBRs was

immediately added to a 1.5 mL microcentrifuge tube containing 200 µLs of inert/insoluble silicone based fluid (dimethyl-tetrachlorophenyl siloxane-copolymer branches 70CS, PSO21 United Chemical Technologies, Bristol, PA). The mixed liquor was centrifuged for 5 minutes at 14,000xg, and 1.0 mL of the remaining supernatant was removed, diluted with 10 mLs of 2% $HNO_3$, and filtered using a Gelman (Ann Arbor, MI) SUPOR-200 membrane filter ("soluble"). The purpose of the hydrophobic silicone fluid is to prevent further transport of cations after centrifugation by rapidly isolating the floc material from the supernatant. The soluble phase was separated from the floc material within approximately 3 minutes after removing samples from the SBRs. The 5 minute centrifugation time was longer than that actually required to form a pellet within the silicone fluid layer, but was required to form a well-consolidated pellet that was not disturbed during silicone fluid removal. The "floc-associated" phase was obtained by carefully removing the remaining supernatant and silicone fluid. The pellet was acidified with 1.0 mL of 10% $HNO_3$, and the microcentrifuge tube was placed in a 100$^oC$ water bath for 30 minutes. The contents were diluted to 10 mLs using 2% $HNO_3$ and filtered using a Gelman (Ann Arbor, MI) SUPOR-200 membrane filter. Preliminary experiments confirmed nearly complete cation release from the floc material using the 100$^oC$ water bath extraction method described above as compared to the complete nitric acid digestion procedure required by *Standard Methods* (1) (data not shown). The "total" cation content of the mixed liquor was measured by combining 1.0 mL of the SBR sample with 100 µLs of concentrated $HNO_3$. The acidified mixed liquor was then extracted, diluted, and filtered exactly as the "floc-associated" phase.

The three mixed liquor phases were analyzed for Na, K, Mg, Ca, Fe, and Al using a Jobin Yvon 2000 Ultima (Horiba Emission Group, Longjumeau, France) inductively coupled plasma emission spectrophotometer (ICP-ES). The ICP-ES was used so that the chemical interferences associated with atomic adsorption spectrophotometric analysis, especially for Na and K, could be avoided. It was also possible to use very small sample volumes with the ICP-ES since all of the elements were measured using the same method. To check the ICP-ES results, the soluble phase (un-acidified) was also analyzed for $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$ on a Dionex DX-120 ion chromatograph (IC) with a CS12 IonPac Column, a CSRS Ultra suppressor, and an eluent of 20 mM methanesulfonic acid at flowrate of 1.0 mL/min. In all cases, the ICP-ES data was in close agreement with the IC results (data not shown).

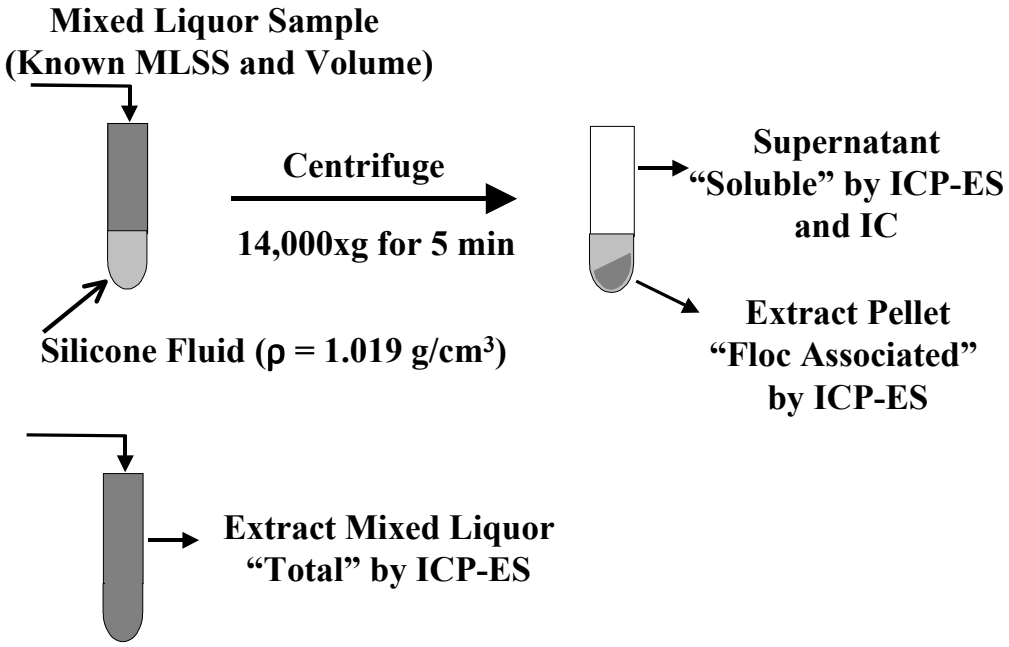


Figure 2. Schematic of mixed liquor cation measurement procedure.

*Time Dependent Cation Transport Versus Deflocculation.* Using NEM added at an initial concentration of 50 mg/L, time-dependent profiles were performed for both cation transport and deflocculation measurements. Cation samples were obtained at several times over the course of the 5.25 hour reaction period. In order to obtain effluent VSS data as a function of time, four SBRs were stressed with 50 mg/L NEM, and the remaining four SBRs served as controls. At each of four times during the reaction period, an NEM-stressed reactor and a control reactor were "sacrificed" by terminating aeration/mixing and allowing 30 minutes of quiescent settling before removing effluent samples. For this experiment, the recorded times for the effluent VSS measurements were when the aerators/mixers were turned off (i.e. not including the 30 minute settling period).

*Lysis Evaluation and Alkaline Phosphatase Assays.* Alkaline phosphatase (AP) activity was monitored both in the bulk mixed liquor supernatant and in cell free extracts (CFE) in response to NEM (0, 2, 10, 25, 50, 100, 2000 mg/L) and CDNB (0, 5, 20, 50, 100, 200 mg/L). Supernatant was collected by centrifuging 1.5 mLs of mixed liquor in a 1.5 mL microcentrifuge tube (14,000xg for 3 minutes). The pellet material was then resuspended in 1.0 mL of AP-assay glycine buffer (100 mM glycine, 1.0 mM $MgSO_4$, 0.10 mM $ZnSO_4$, pH10) and transferred to a

2.0 mL screw-cap microcentrifuge tube containing 1.0 g of glass beads (0.1 mm diameter). Cells were mechanically lysed by bead beating for 5x30 second bursts at 5000 cycles/second with rests on crushed ice using a Mini-Bead Beater (Biospec Products, Bartlesville, OK). The debris was separated by centrifugation (14,000xg for 5 minutes), and the CFE was transferred to a new tube. Both supernatant and CFE samples were held on ice until AP assays were performed (assays were initiated immediately after sampling in all cases). AP assays were conducted in 1.0 mL cuvettes containing the sample mixed liquor supernatant or CFE, pH10 glycine buffer, and 6.0 mM p-nitrophenylphosphate (pNPP), which is a standard AP substrate. The formation of the reaction product, p-nitrophenolate (at pH10), was monitored at a wavelength of 400 nm ($\varepsilon$ = 19.26 $mM^{-1}cm^{-1}$) for 3 minutes. In preliminary experiments, it was determined that neither NEM (up to 2000 mg/L) nor CDNB (up to 300 mg/L) affected the activity of commercially available AP (Type VII L from bovine intestinal mucosa, Sigma, St. Louis, MO) (data not shown). In a subsequent experiment, it was also demonstrated that NEM (up to 2000 mg/L) did not reduce the activity of activated sludge-derived AP in a mixed liquor supernatant matrix (data not shown).

## Results and Discussion

A compilation of control reactor data for 30 independent experiments indicated consistent cation levels in the primary effluent and activated sludge and relatively constant mixed liquor quality and characteristics (MLSS = 2050 ± 435 mg/L, SVI = 91.7 ± 29.6 mL/g, and effluent TSS = 16.3 ± 8.7 mg/L). During severe transitional periods when large numbers of students were either moving into or out of Blacksburg, the SBR experiments were postponed until the POTW returned to "normal" operation.

**Deflocculation Screening.** $IC_{25}$, $IC_{50}$, and $IC_{75}$ concentrations are reported in Table 1 for each of the chemical toxins discussed in this paper. Screening experiments were performed to determine if addition of toxic electrophiles resulted in activated sludge deflocculation and to compare the deflocculation response to the $IC_{50}$ concentration (Figure 3). It was also of interest to determine whether well recognized thiol-reactive GGKE system activators (i.e. NEM and CDNB) cause significant deflocculation at concentrations resulting in minimal inhibition of oxygen uptake. Furthermore, several other industrially-relevant electrophilic organics (i.e.

DNT), for which a GSX product has not been observed and cannot be predicted, were also screened for comparison with the known GGKE activators. BQ is recognized as an electrophilic thiol reactant and can form GSX conjugates and/or GSSG (BQ reduced to hydroquinone) (12), but BQ has not been demonstrated to be a GGKE system activator. A selection of the screened chemicals are shown in Figure 3 which represents a plot of the effluent VSS ratio (stressed reactor VSS/control reactor VSS) versus the added stressor concentration ratio (added stressor concentration/$IC_{50}$ concentration). A VSS ratio greater than 1.0 indicates significant deflocculation, and a concentration ratio less than 1.0 indicates that the stressor concentration was added at a level that reduces the SOUR by less than 50%.

Table 1. $IC_{25}$, $IC_{50}$, and $IC_{75}$ concentrations for chemical stressors. NA = $IC_{75}$ not measured due to low chemical solubility.

| Chemical Toxin | $IC_{25}$ (mg/L) | $IC_{50}$ (mg/L) | $IC_{75}$ (mg/L) |
|---|---|---|---|
| $Cd^{2+}$ | 13 | 110 | 410 |
| PCP | 8 | 24 | 70 |
| NEM | 8 | 97 | 270 |
| CDNB | 51 | 218 | NA |
| BQ | 0.8 | 1.5 | 2.8 |
| DNT | 45 | 118 | 425 |
| Toluene | 48 | 130 | 330 |
| Benzene | 20 | 290 | 840 |

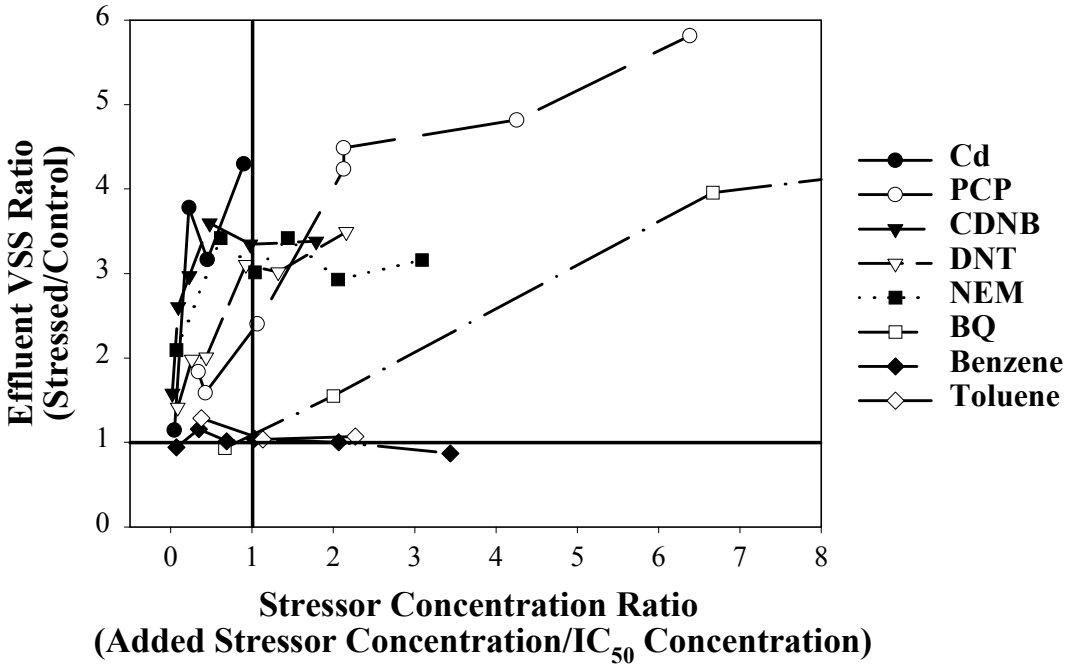


Figure 3. Deflocculation screening results are plotted as the effluent VSS ratio (stressed reactor/control reactor) versus the stressor concentration ratio (stress concentration added/$IC_{50}$ concentration).

PCP and Cd caused significant deflocculation at relatively sublethal concentrations but were not used for further experiments due to the anionic and cationic charge of the molecules, respectively. It is interesting to note, however, that $Cd^{2+}$ is a potent *in vivo* electrophile that

complexes thiol ligands with considerable thermodynamic stability and reacts with nucleophilic substituents of cellular DNA and protein (13,41). GSH is used as a reductant, with no GSX conjugate formation, in PCP dehalogenation pathways, thus producing GSSG which is also a potential activator of the GGKE system (45).

NEM and CDNB were selected for subsequent cation transport and ongoing experiments because they are known activators of the GGKE system (19,36). Although NEM is not a typical component in industrial wastewaters, CDNB, DNT, and BQ were also selected for further study based on the results observed in Figure 3 and the industrial relevance of these compounds. As seen in Figure 3, NEM, CDNB, and DNT all caused significant deflocculation at concentrations much less than the $IC_{50}$, and it appears that a maximum level of deflocculation is observed at concentrations at or below the $IC_{50}$. The $IC_{50}$ for BQ was found to be much lower than that of any other chemicals tested (1.5 mg/L). It is suspected that the $IC_{50}$ of BQ is "artificially" low, since BQ is capable of shunting electrons away from $O_2$ in the bacterial electron transport chain (i.e. BQ can serve as a temporary electron acceptor), thereby reducing BQ to hydroquinone while maintaining a significant level of ATP production and catabolic metabolism (40). Therefore, the results obtained in Figure 3 for BQ are probably shifted as a result of an extremely low $IC_{50}$ concentration, and it was observed that BQ induced significant deflocculation at concentrations within the approximate range of other stress compounds. As expected, for severe activated sludge deflocculation, it was determined that the SVI decreased slightly as compared to the control reactor, but MLSS values were similar for the stressed and control reactors over the course of the 5.25 hour reaction period (data not shown). Benzene and toluene, which have no electrophilic substituents, did not induce deflocculation even at very high initial concentrations.

**Cation Transport Versus Deflocculation.** In order to evaluate cation transport as a function of deflocculation, SBR experiments were performed with varying concentrations of NEM, CDNB, DNT, and BQ. In addition to effluent suspended solids measurements, the cation content of the soluble, floc-associated, and total phases was measured immediately after adding primary effluent feed ($t = 0$), immediately after adding stressor compounds ($t \cong 3$ minutes), and near the end of the reaction period ($t = 5$ hours). Figure 4 represents a plot of $K^+$ concentration measured in each of the three phases versus the sample times for both the control and stressed reactors. It

was shown that 50 mg/L NEM (about 1/2 of the $IC_{50}$) and 50 mg/L CDNB (about 1/4 of the $IC_{50}$) both caused a significant increase in effluent VSS above that observed in the control reactors. For each SBR mixed liquor sample, a calculated "total" (soluble + floc-associated) could be compared to a measured "total", thus providing a mass balance for all 6 cations. As seen in Figure 4, good mass balance closures were obtained for most of the mixed liquor samples, thus validating the soluble/floc-associated silicone fluid separation technique and the analytical methods. As expected, the total $K^+$ measurements remained relatively constant over time and were similar in the stressed and control reactors, even though the soluble and floc-associated $K^+$ concentrations changed significantly in the stressed reactors.

In response to 50 mg/L NEM, it was determined that the soluble $K^+$ concentration increased by approximately 20% within the 3 minutes required to process the first sample, and was nearly doubled by the end of the reaction period. The increase in the soluble phase $K^+$ concentration was coupled with a decrease in the floc-associated concentration, thus confirming significant transport of $K^+$ from the floc structure to the bulk liquid. At this stage, however, it cannot be concluded that $K^+$ was the cause of the observed deflocculation, as the $K^+$ release to the bulk liquid could simply be a consequence of deflocculation (i.e. deflocculation followed by $K^+$ efflux). For CDNB added at 50 mg/L, the soluble $K^+$ concentration increased by approximately 40% within the 3 minutes and again nearly doubled after 5 hours. Similar $K^+$ efflux trends were observed for $Cd^{2+}$, DNT, and BQ (data not shown). As expected, the BQ concentration (25 mg/L) required to induce a similar degree of $K^+$ transport from the floc structure to the bulk liquid was very high as compared to the $IC_{50}$ for BQ (1.5 mg/L). Both Cd and DNT induced significant $K^+$ efflux at concentrations much less than the $IC_{50}$s. Although it was stated that $Cd^{2+}$ could not be used to specifically address the research hypothesis, it was of interest to compare the $K^+$ efflux response of a thiol complexing heavy metal with uncharged organic thiol reactants (i.e. NEM, CDNB, and BQ).

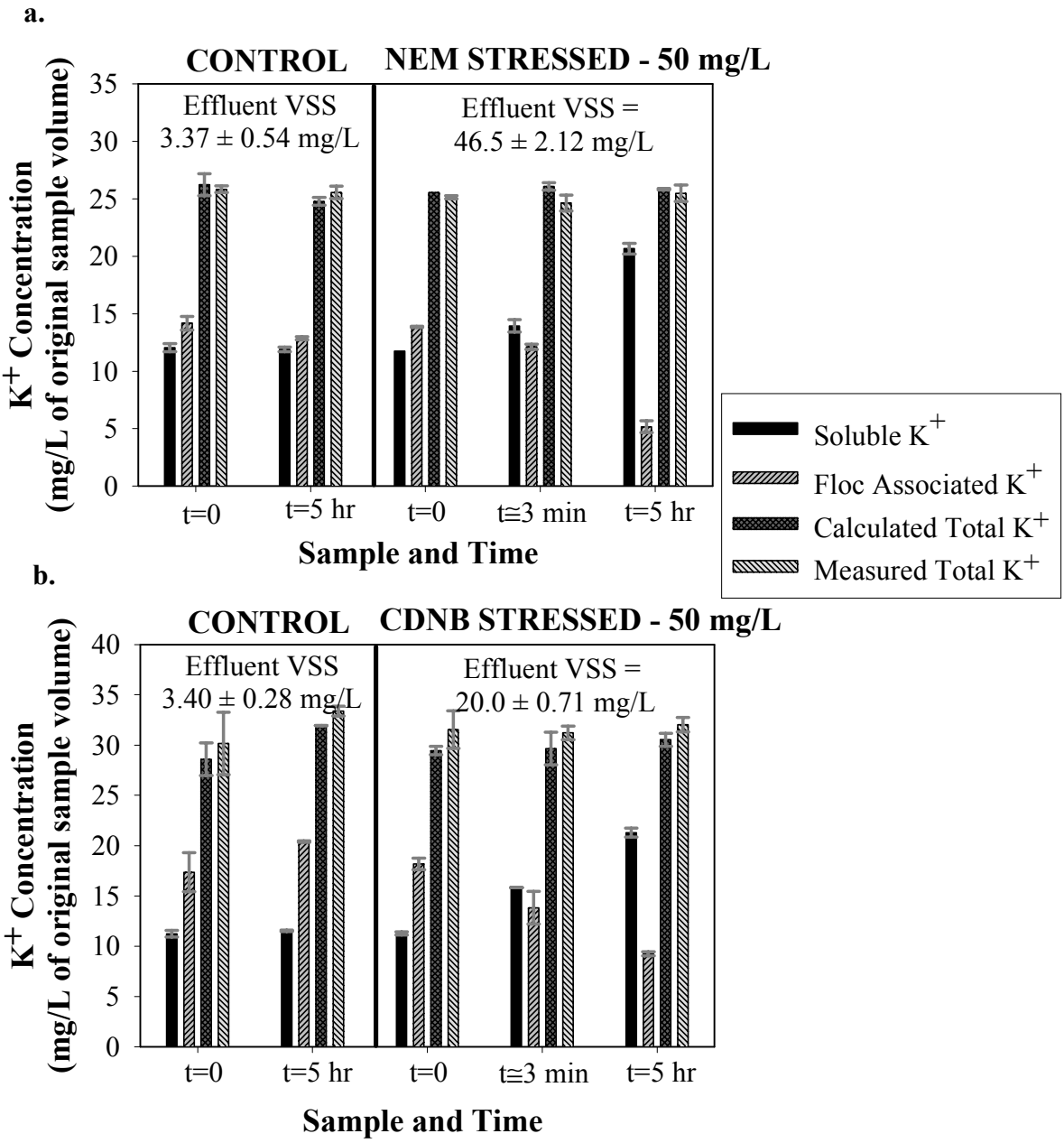


Figure 4. (a.) $K^+$ measurements for an NEM stress experiment at 50 mg/L ($IC_{50}$ for NEM = 27 mg/L). (b.) $K^+$ measurements for an CDNB stress experiment at 50 mg/L ($IC_{50}$ for CDNB = 218 mg/L). Floc associated concentrations were normalized to the original sample volume for direct comparison with soluble and total measurements. Error bars represent the standard deviation of duplicate independent SBR samples.

The cation flux experiments described above were performed for an applicable range (using results from Figure 3 and Table 1) of stressor concentrations to determine whether $K^+$ efflux was correlated with the degree of deflocculation. Figure 5 represents the concentration of $K^+$ released from the floc structure and the effluent VSS ratio as a function of the NEM or CDNB concentration added at the beginning of the reactor cycle. For NEM concentrations less than 50 mg/L, an increase in effluent suspended solids was directly proportional to an increase in $K^+$ release from the floc structure. More importantly, the degree of deflocculation and the amount of $K^+$ efflux saturated at nearly identical NEM concentrations (i.e. 50 mg/L). Although fewer concentrations were examined for CDNB, a comparable trend is observed in which $K^+$ efflux and deflocculation are well correlated and seem to saturate at similar CDNB concentrations. Once again, it is still unknown whether electrophile-induced $K^+$ efflux causes deflocculation or whether $K^+$ efflux is simply a consequence of electrophile-induced deflocculation. However, Figure 5 certainly suggests that $K^+$ transport and activated sludge deflocculation are highly interrelated phenomena, and that our research hypothesis should be thoroughly evaluated.

In all of the cation transport experiments, other relevant cations, including $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Fe^{3+/2+}$, and $Al^{3+}$ were also analyzed in each of the three activated sludge phases. Appropriate mass balance closures were obtained, and total cation measurements remained constant over time in both control and stressed reactors. $Na^+$, $Mg^{2+}$, $Fe^{3+}$, and $Al^{3+}$ transport, either to or from the floc structure, was negligible and insignificant for all of these experiments (data not shown). This is a further indication that electrophile-induced GGKE systems may be responsible for the transport of $K^+$ from the cytoplasm of activated sludge bacteria to the surrounding floc structure (EPS) and bulk liquid phase. Unexpectedly, these experiments suggested that a small amount (typically 1 to 2 mg/L) of $Ca^{2+}$ may be transported from the bulk liquid to the floc structure, but this occurs only in response to relatively high electrophile concentrations that result in significant $K^+$ efflux and effluent turbidity. It is possible that, as the floc structure weakens and as small particles are eroded from the surface of the activated sludge flocs, a large amount of negatively charged biopolymer and cellular material is exposed to the bulk liquid. The release of negatively charged particles and biopolymer could be responsible for "coagulating" some of the $Ca^{2+}$ available in the bulk liquid, and in fact, this mechanism could be responsible for overshadowing

any release of $Ca^{2+}$ from the floc structure as a result of ion exchange due to $K^+$ efflux. Previous researchers demonstrated that when $K^+$ is added externally to activated sludge at high concentrations (greater than 400-900 mg/L $K^+$), $Ca^{2+}$ and/or $Mg^{2+}$ are released from the floc structure, presumably as a result of ion exchange mechanisms, and this was followed by a rapid deterioration of settling and dewatering properties (11,38). Although the excretion of $K^+$ within the floc structure is likely a very different phenomena than externally added $K^+$ (i.e. KCl), it was previously speculated that $K^+$ efflux may be observed concurrently with a slight release of $Ca^{2+}$ or $Mg^{2+}$ from the floc structure to the bulk liquid as a result of ion exchange caused by high localized $K^+$ levels within the floc structure. As expected, external addition of KCl to an SBR, at level comparable to that produced by maximal toxin-induced $K^+$ efflux, caused no change in effluent VSS or SVI relative to a control reactor (data not shown). Specifically, 10 mg/L KCl-$K^+$ was added to a SBR exactly as described above for the toxic chemicals. Cation transport measurements indicated that the added 10 mg/L of $K^+$ was recovered completely in the soluble phase, with no uptake by the floc structure and no release or uptake of any of the other cations monitored.

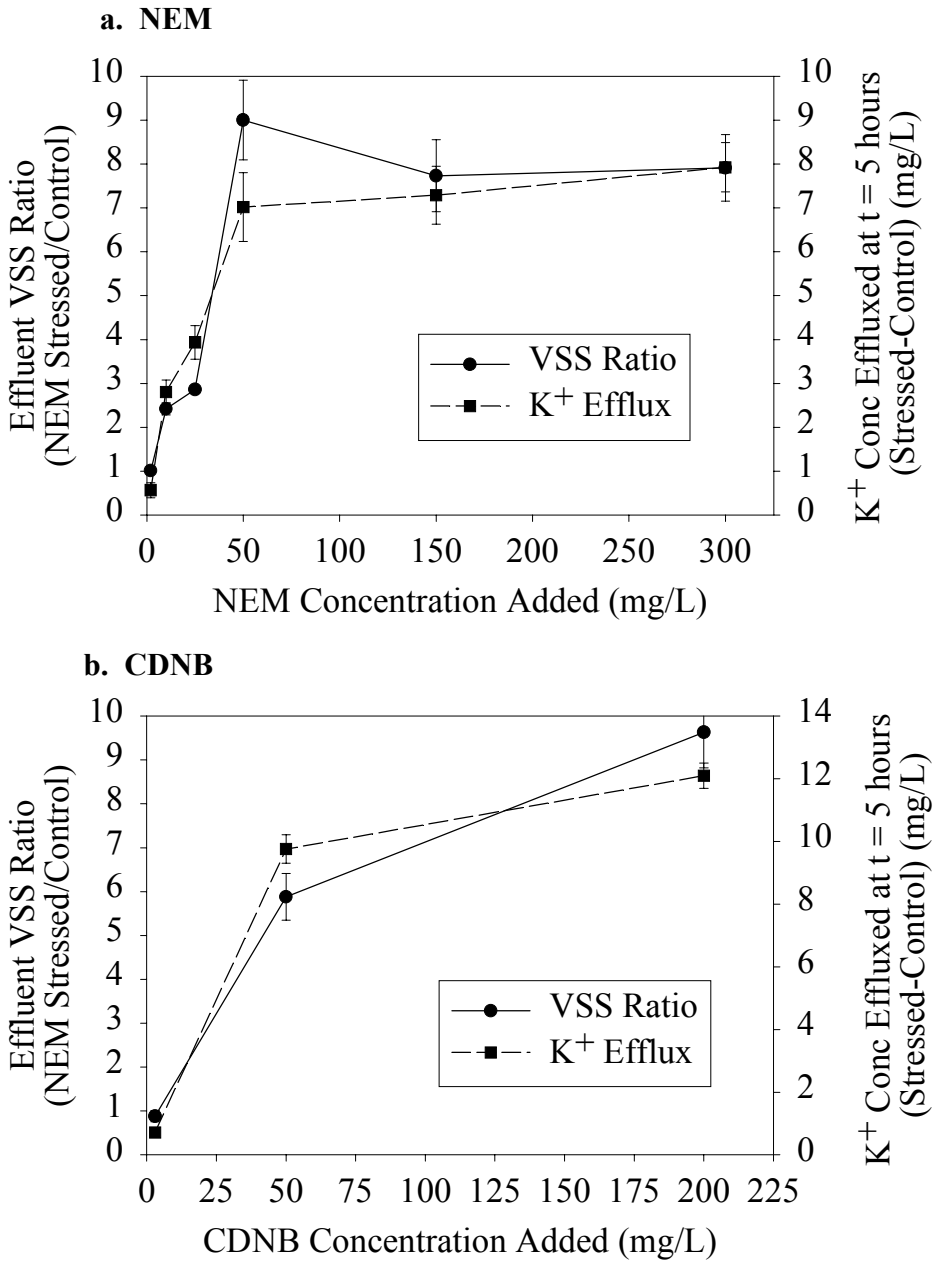


Figure 5. (a.) $K^+$ efflux and deflocculation versus NEM concentration ($IC_{50}$ for NEM = 97 mg/L). (b.) $K^+$ efflux and deflocculation versus CDNB concentration ($IC_{50}$ for CDNB = 218 mg/L). Error bars represent the standard deviation of duplicate measurements

As introduced above, it was important to determine whether shock loads of chemical toxins simply caused catastrophic cell lysis (thereby releasing cytoplasmic $K^+$). There are three distinct observations which indicate that lysis was not a significant phenomena in the activated sludge cultures. For NEM stress at 2000 mg/L, the supernatant alkaline phosphatase (AP) activity increased by approximately 24% above that observed in the control. However, the AP activity in the cell free extract (floc-associated) was approximately 5 times higher than the highest supernatant activity, suggesting that the majority of the total AP activity remained associated with the floc structure even at very high NEM concentrations. For CDNB stress, supernatant AP activity remained relatively constant up to 200 mg/L, with a statistically insignificant decrease in activity at 200 mg/L (approximately 1%). Again, the CFE activity was approximately 4.2 times the highest supernatant activity. The supernatant and CFE AP assays therefore suggest that $K^+$ release due to cell lysis was minimal. Secondly, it is apparent that both NEM and CDNB induced maximal $K^+$ efflux and deflocculation at concentrations less than the respective $IC_{50}$s (see Figure 5). Cell lysis was likely not responsible for significant amount of $K^+$ efflux, if at these stressor concentrations the SOUR was reduced by only 25 to 40%. Finally, $K^+$ efflux occurs in activated sludge cultures with no significant efflux of other cations. This observation also indicates that $K^+$ efflux was caused by a physiological response and not by cell lysis.

Studies have also been conducted to compare the cation content of the overall mixed liquor floc-associated phase with that of the deflocculated particles which remain in the reactor effluent. By collecting particles from 500 mLs of control reactor, NEM stressed (50 mg/L) reactor, and CDNB stressed (50 mg/L) reactor effluent, it was possible to perform floc-associated measurements on effluent particles that were unsettleable during the 30 minute settling period (data not shown). Even though the effluent TSS for control reactors was typically 5 to 12 mg/L, sufficient "pellet" material was collected to perform reliable floc-associated measurements. Results from a representative experiment are presented in Table 2. It is interesting to note that, the effluent particle cation content was substantially less, typically 50%, than the respective floc-associated levels for all 6 cations. This indicates that effluent particles are relatively devoid of monovalent and polyvalent cations as compared to the floc structure. It seems logical that nonsettleable particles could be stabilized in solution by increased anionic charge density, but no zeta potential or electrophoretic mobility measurements were made to confirm or deny this

suspicion. It is also interesting to recognize that there was very little difference between "control" and "deflocculated" effluent particles, although some slight trends may be significant (slightly less $K^+$ and $Fe^{2+/3+}$ and slightly more $Ca^{2+}$, $Mg^{2+}$, and $Al^{3+}$ in the "control" effluent particles as opposed to the "deflocculated" effluent particles). There is no obvious explanation for this phenomena, but the fact that NEM and CDNB stress seem to result in similar effluent particle cation levels suggests that the deflocculation event was not a function of the specific chemical but rather the degree of $K^+$ efflux resulting.

Table 2. Floc-associated cation measurements for end-cycle mixed liquor and effluent particle samples. The standard deviation represents two independent mixed liquor or effluent samples. Three replicates of each of these samples were analyzed by ICP-ES. For sample type, ML = mixed liquor and EP = effluent particle.

| SBR Reactor | Sample Time | Sample Type | Floc-Associated Cation Concentration (Std. Dev.) (mg K/ g MLSS) | (mg Na/ g MLSS) | (mg Ca/ g MLSS) | (mg Mg/ g MLSS) | (mg Fe/ g MLSS) | (mg Al/ g MLSS) |
|---|---|---|---|---|---|---|---|---|
| Control | t=5 hr | ML | 4.22 (0.19) | 3.39 (0.27) | 15.9 (0.52) | 4.46 (0.16) | 6.65 (0.22) | 5.67 (0.18) |
| NEM | t=5 hr | ML | 1.91 (0.17) | 3.94 (0.78) | 17.5 (0.02) | 4.46 (0.02) | 6.97 (0.11) | 5.86 (0.13) |
| CDNB | t=5 hr | ML | 2.01 (0.07) | 3.53 (0.13) | 16.4 (0.29) | 4.36 (0.07) | 6.72 (0.02) | 5.73 (0.15) |
| Control | t=5.75 hr | EP | 1.83 (0.08) | 1.77 (0.13) | 7.30 (0.39) | 2.05 (0.13) | 3.14 (0.28) | 2.54 (0.20) |
| NEM | t=5.75 hr | EP | 1.23 (0.02) | 1.80 (0.03) | 11.3 (0.11) | 2.68 (0.02) | 2.42 (0.00) | 3.63 (0.00) |
| CDNB | t=5.75 hr | EP | 1.43 (0.06) | 1.81 (0.01) | 10.2 (0.47) | 2.59 (0.12) | 2.36 (0.12) | 3.30 (0.20) |

The research hypothesis is critically dependent on the confirmation that transport of $K^+$ from the floc structure to the bulk liquid is responsible for deflocculation, and that $K^+$ efflux is not simply a consequence of deflocculation. In order to provide evidence supporting the research hypothesis, it was surmised that if $K^+$ efflux causes deflocculation, then $K^+$ efflux should occur immediately following electrophile addition and before deflocculation (i.e. $K^+$ efflux followed by deflocculation). If it is determined that deflocculation is followed by $K^+$ efflux, then it would suggest that $K^+$ does not cause toxin-induced deflocculation. An experiment was performed in which both cation levels and deflocculation were monitored over the course of a single SBR cycle. Figure 6 represents time-dependent profiles for both the soluble phase $K^+$ concentration and the effluent VSS concentration for both the control and NEM stressed (50 mg/L) reactors. These results suggest that a significant amount of $K^+$ efflux occurred within the first 10-15 minutes after NEM addition, followed by a continued slow release of $K^+$ for the remainder of the reaction period. More importantly, it appears that deflocculation followed a rapid and significant degree of $K^+$ efflux, thus providing further evidence supporting the research hypothesis. As with other experiments, $Na^+$, $Mg^{2+}$, $Fe^{3+}$, and $Al^{3+}$ were not transported during the reaction period, and minor $Ca^{2+}$ transport to the floc structure was not observed until the final measurement (t = 5 hours) (data not shown).

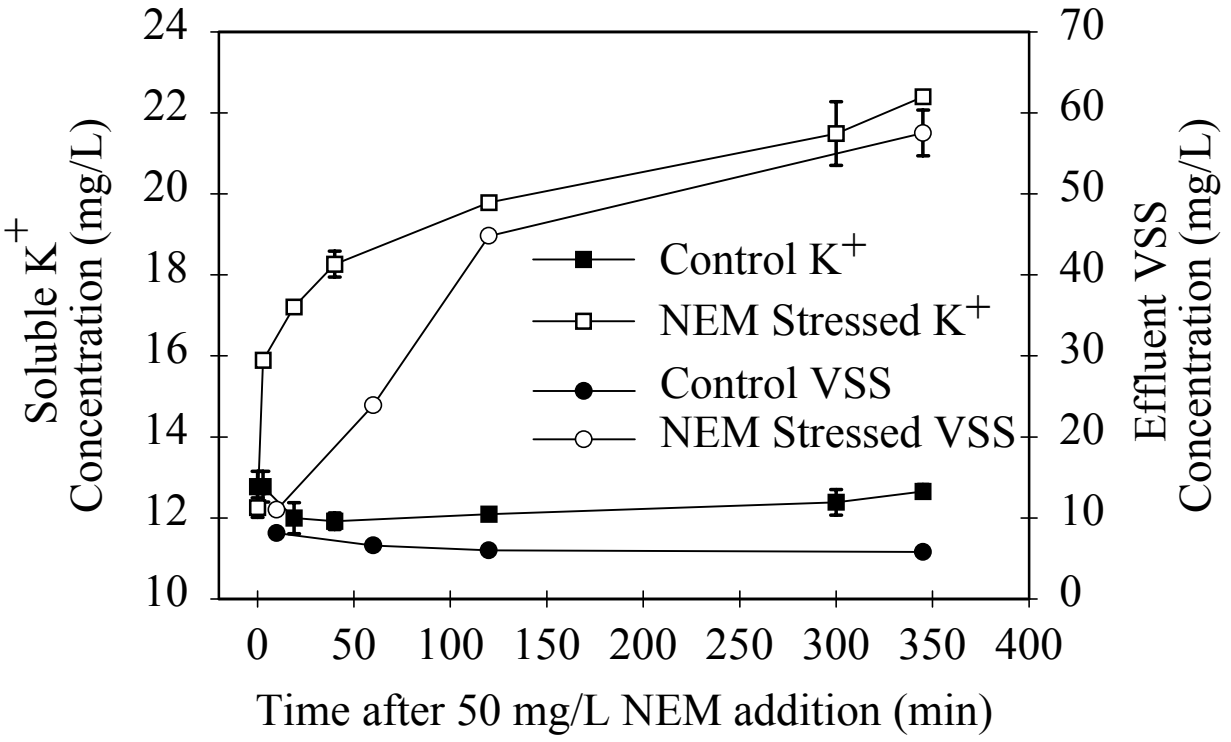


Figure 6. Soluble $K^+$ concentration and effluent VSS concentration versus time after NEM addition at 50 mg/L.

In a subsequent SBR experiment, NEM- and CDNB-stressed reactors were operated for 12 reactor cycles after the initial shock loading, with cation and performance sampling during cycles 1, 4, 8, and 13 (data not shown). During this period, effluent VSS in the toxin-stressed reactors did not decrease from that obtained at then end of cycle 1 (approximately 36 mg/L), while the control reactor VSS remained constant at about 6 to 8 mg/L. The MLSS in the stressed reactors decreased 30 to 40% by cycle 13, while the control reactor MLSS decreased by approximately 9%. This washout of biomass was expected since the wastage rate was constant throughout the experiment. As fresh primary effluent feed was obtained from the local POTW several times during the experiment, soluble cation levels fluctuated in both control and stressed reactors. However, the control and stress reactor cation levels (soluble and floc-associated) remained very similar for $Na^+$, $Mg^{2+}$, $Fe^{2+/3+}$, and $Al^{3+}$. For $K^+$ and $Ca^{2+}$, it was observed that the stressed reactor soluble phase cation levels approached that observed in the control by cycle 8, although this trend was much less apparent for $Ca^{2+}$, because the initial reduction in the soluble phase concentration was only 1 to 2 mg/L. This observation seems logical considering a 24 hour HRT,

but only if $Ca^{2+}$ and $K^{+}$ transport occurred primarily during the first cycle after chemical addition. After 13 cycles, the floc structure still contained about 50% less $K^{+}$ than the control mixed liquor and slightly elevated $Ca^{2+}$ levels. This suggests that the biomass did not immediately reaccumulate the $K^{+}$ that was lost rapidly after chemical stressor addition.

The results presented here reflect a new attempt at elucidating a specific mechanism to explain toxin-induced deflocculation and are part of a broader effort by our group to characterize the role of microbial stress responses in biological wastewater treatment process upset. We demonstrated that several electrophilic organic compounds cause significant deflocculation at concentrations which minimally impact catabolic metabolism, as determined by SOUR inhibition. As a result, deflocculation could occur with little warning from an online aerobic respirometer. This suggests that the information gained by elucidating the mechanistic cause of toxin-induced deflocculation could be very helpful in designing an upset early warning monitoring device for the upstream detection of shock loads. Mechanistic information should also assist with the development of successful methods for mitigating upset events as well as clues about the source of the problem (e.g. the chemical nature of the toxin). In many cases, the effect of a specific chemical on a biological treatment process may not be known; however, knowledge of the mechanisms of process upset may allow operators to predict the impact of a specific chemical or class of chemicals. Linking these mechanisms with appropriate mitigation strategies can help operators avoid process upset. As an example, if it is known that $K^{+}$ efflux was responsible for a deflocculation event, it may be possible to revert to normal operation by simply overcoming the $K^{+}$ efflux effect with coagulant ($FeCl_3$). If an upstream influent monitoring device can be designed to incorporate the correlation between $K^{+}$ efflux and activated sludge deflocculation, it may be possible to avoid deflocculation events by diverting harmful influent to temporary storage tanks, even if the nature of the chemical toxin is not known and/or cannot be determined. Finally, if it is known that thiol reactive electrophilic chemicals have the potential to cause serious biological process upset, then it should be possible to predict whether or not a chemical will induce deflocculation based on chemical structure.

## Conclusions

At this stage, we have provided circumstantial evidence that the GGKE system is responsible for electrophile-induced $K^+$ efflux in activated sludge. The data presented in Figures 5 and 6 represent convincing evidence that the excretion of intracellular $K^+$ from activated sludge bacteria causes deflocculation. Ongoing investigations to further address the activity of the GGKE system in activated sludge involve the use of an unflocculated, chemostatically-grown, pure culture Gram-negative bacterium that is representative of bacteria found in activated sludge cultures. The fate of thiol reactive electrophiles added to the pure culture will be determined by monitoring GSH and GSX, and the extent of GST activity will be evaluated. Finally, we will attempt to repeat the experiments conducted with the pure culture in activated sludge cultures.

Elucidation of deflocculation and other biological treatment process upset mechanisms may eventually lead to effective strategies for preventing treatment performance deterioration caused by shock loading of toxic chemicals. Although the evidence supporting the research hypothesis is circumstantial at this stage, the data presented are promising and would be difficult to explain without considering the activity of the electrophile-induced GGKE system. Based on the results presented in this paper, several specific observations can be stated with confidence:

1. CDNB and NEM, which are thiol reactants and known activators of the GGKE system, caused significant activated sludge deflocculation at concentrations which minimally impacted the SOUR.

2. CDNB, NEM, BQ, DNT, and $Cd^{2+}$ all caused significant transport of $K^+$ from the floc structure to the bulk liquid phase at concentrations that resulted in considerable increases in effluent turbidity.

3. For CDNB and NEM, it was determined that the degree of deflocculation was well correlated with the amount of $K^+$ transported from the floc structure to the bulk liquid. Both the degree of deflocculation and the amount of $K^+$ efflux appeared to saturate at similar stressor concentrations.

4. There was negligible transport of other relevant cations in response to shock loads of electrophilic stressors, with the exception of $Ca^{2+}$. A small amount of $Ca^{2+}$ was transported from the bulk liquid to the floc structure, and this transport did not occur until near the end of the first 5.25 hour reaction period.

5. Time-dependent profiles of $K^+$ efflux and effluent VSS indicated that significant $K^+$ transport occurs rapidly (within 10-15 minutes) after shock loading with 50 mg/L NEM, and that significant $K^+$ efflux precedes an increase in effluent turbidity.

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# Chapter 4. Implicating the Glutathione-Gated Potassium Efflux System as a Cause of Activated Sludge Deflocculation in Response to Shock Loads of Toxic Electrophilic Chemicals

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## Abstract

The glutathione-gated $K^+$ efflux (GGKE) system represents a protective microbial stress response that is activated in response to electrophilic or thiol reactive stressors. The hypothesis of this work states that GGKE-activated release of $K^+$ from the cytoplasm of mixed liquor microorganisms into the floc structure may be a significant cause of activated sludge deflocculation in response to shock loads of industrially relevant electrophilic chemicals. *Sphingomonas capsulata was used as* a representative activated sludge bacterium and responded to electrophilic thiol reactants with rapid efflux of up to 80% of its cytoplasmic $K^+$ pool. Furthermore, *S. capsulata* and activated sludge cultures exhibited very similar dynamic efflux/uptake/efflux responses to that previously reported for *E. coli* K-12 when exposed to the electrophilic stressors, N-ethylmaleimide (NEM) and 1-chloro-2,4-dinitrobenzene (CDNB), and the thiol reducing agent, dithiothreitol (DTT). Fluorescent membrane permeable and impermeable nucleic acid stains were used to show that cell lysis was not the cause of electrophile-induced $K^+$ efflux. Nigericin, a polyether ionophore antibiotic, was used to artificially stimulate $K^+$ efflux from *S. capsulata* and activated sludge cultures and produced results comparable to electrophile-induced $K^+$ efflux. These data suggest that GGKE activity in activated sludge bacteria exposed to electrophilic toxins causes $K^+$ release from the cytoplasm, transport into the floc structure and extracellular polymeric substances (EPS), and then diffusion into the bulk liquid. Calculations support the notion that shock loads of electrophilic chemicals result in very high $K^+$ concentrations within the activated sludge floc structure, and these $K^+$ levels are comparable to concentrations which cause deflocculation by external KCl addition.

## Introduction

This study represents an effort to explain the mechanism by which shock loads of toxic electrophilic chemicals cause deflocculation of activated sludge. Few studies have been conducted under well-controlled experimental conditions with indigenous microbial communities to show how certain wastewater conditions (source) impact activated sludge treatment processes (effect) (18). In the absence of established correlations between various sources and their effect(s), operators often cannot determine which control actions to implement in response to wastewater shock loads to most effectively mitigate process impact. To develop a better understanding of how source and effect are related, we propose identifying the predominant mechanistic biochemical, chemical and/or physical causes that link source and effect.

A range of microscopic- and molecular-level mechanisms exist that allow cells to adapt when exposed to chemical perturbations. Some important stress responses occur over long time periods, but the focus of this work is on rapid responses that are activated immediately or within a few seconds of initiation of the perturbation event. Studies are needed to evaluate the degree to which these rapid microscopic- and molecular-level mechanisms contribute to adverse process upsets in full-scale treatment systems. An understanding of source-cause-effect relationships could potentially lead to upset early warning systems and mitigation strategies that will help to avoid the significant environmental problems associated with activated sludge process upset.

Data are presented in this paper which support the hypothesis that a physiological microbial stress response, the glutathione-gated $K^+$ efflux (GGKE) system, causes activated sludge deflocculation (effect) due to sublethal shock loads of toxic electrophilic chemicals (source). The GGKE mechanism was extensively reviewed elsewhere (6) and was originally studied primarily in *E. coli* K-12 by Booth and coworkers (5,8,10,11,12,13). Ferguson et al. (12,13) determined that chemical challenge by toxic electrophilic compounds (both mild thiol reactants and strong oxidants) elicits a protective mechanism that prevents macromolecular damage by rapid efflux of intracellular $K^+$ and concurrent cytoplasmic acidification. It was determined that the GGKE system involves two independent membrane-bound potassium efflux proteins called

KefB and KefC in *E. coli*, and these proteins are gated by reduced glutathione and activated by glutathione-S-conjugates (GSX) and possibly oxidized glutathione (5).

In early work on the GGKE system, Meury et al (19) observed that NEM-induced efflux of intracellular $K^+$ was transient, and within a given period of time after NEM removal, almost all of the intracellular $K^+$ pool was recovered followed by a reinitiation of growth. Interestingly, it was determined that both the efflux and the recovery of intracellular $K^+$ were mediated without *de novo* protein synthesis (i.e. in the presence of chloramphenicol), however uptake of $K^+$ after an efflux event required a suitable exogenous carbon source (9,19,20). Meury et al. (19) and Bakker and Mangerich (3) also determined that adding a membrane-permeable thiol reductant, such as β-mercaptoethanol or dithiothreitol, after NEM-induced $K^+$ efflux resulted in recovery of cytoplasmic $K^+$. It was suggested that this was due to the activity of the $K^+$ uptake systems and inhibition of GGKE by breakdown of the GSX activator (N-ethylsuccinimido-S-glutathione in the case of NEM). However, the recovery of $K^+$ occurred at a much slower rate than KefBC efflux. Adding excess NEM after $K^+$ recovery resulted in a reactivation of the GGKE system and efflux of the previously recovered $K^+$ (efflux/uptake/efflux) (3,19). For NEM stress in *E. coli* K-12, it was possible to recover intracellular $K^+$ by adding a reductant, but CDNB-induced $K^+$ efflux *was not reversible* by adding either β-mercaptoethanol or DTT (5). In both cases, reversibility was an unexpected response based on the stability of the thioether bond formed by the addition of NEM or CDNB to the thiol of glutathione. The C-S-C bond of N-ethylsuccinimido-S-glutathione is expected to be chemically stable even in the presence of excess DTT, but using thin-layer chromatography and radioactive NEM, Elmore et al. (9) found an 80% decrease in the cytoplasmic N-ethylsuccinimido-S-glutathione concentration after addition of DTT and suggested that removal of the GGKE activator was responsible for $K^+$ recovery. Beutler et al. (4) found that N-ethylsuccinimido-S-glutathione may be unstable at physiological pH but not under acidic or basic conditions. The aromatic thioether in glutathione-S-dinitrobenzene is very stable and is not labile in the presence of excess reducing thiols. Booth et al. (5) suggested that this was the reason for the differences in $K^+$ efflux reversibility, but changes in cytoplasmic redox control or permanent inhibition of $K^+$ uptake systems by CDNB may also be involved. Regardless of the mechanism leading to recovery of cytoplasmic $K^+$, it is

well documented that NEM-induced $K^+$ efflux can be reversed with the addition of DTT, and CDNB-induced $K^+$ efflux cannot be reversed.

The presence of potassium efflux activity in activated sludge bacteria has only recently been reported (6) and represents a biochemical cause. After $K^+$ efflux occurs, it is hypothesized that the sudden increase in monovalent cation levels within the extracellular polymeric substances (EPS) results in a rapid increase in the localized monovalent to divalent cation ratio (M:D), which has been found to be an important physicochemical factor controlling activated sludge floc strength (14,15,21,22). The localized increase in the M:D ratio is believed to cause a reduction in floc strength and rapid deflocculation by turbulent erosion of small nonsettleable particles from the floc surface. The observation of $K^+$ efflux reversibility in NEM-exposed activated sludge would therefore implicate that a physiological mechanism is active and that NEM stress probably did not result in a significant cell lysis.

We recently reported that potassium is rapidly transported from activated sludge flocs to the bulk water phase in mixed liquors exposed to electrophilic chemicals (6). However, three specific questions must be further evaluated to implicate GGKE as a cause of this response. (i.) Do shock loads of toxic electrophilic chemicals activate the physiological GGKE system in activated sludge microorganisms? (ii.) Does GGKE system activity in activated sludge result in $K^+$ transport from the bacterial cytoplasm, through the EPS, and finally to the bulk liquid? (iii.) Do the increased levels of $K^+$ within the floc structure cause deflocculation by weakening the floc structure which results in turbulent erosion of particles from the floc surface? To address these questions, the specific experimental objectives for this work are:

1. Characterize electrophile-induced cation ($K^+$) transport in an environmentally relevant pure culture bacterium (*Sphingomonas capsulata*).
2. Compare dynamic $K^+$ efflux/uptake/efflux for both NEM and CDNB using a reductant (dithiothreitol) for both activated sludge and *S. capsulata*.
3. Evaluate electrophile-induced cell lysis using membrane permeable and membrane impermeable fluorescent nucleic acid stains.

4. Evaluate $K^+$ efflux due to the polyether ionophore antibiotic, nigericin, in both *S. capsulata* and activated sludge, and determine whether nigericin-induced $K^+$ efflux causes deflocculation.

5. Estimate the $K^+$ concentration within the floc structure immediately following electrophile-induced $K^+$ efflux for comparison with data suggesting that external KCl addition at high levels causes deflocculation.

The results provide evidence that the GGKE system is responsible for toxin-induced activated sludge deflocculation. We contend that the GGKE system represents an important protective physiological stress response in activated sludge bacteria, and it is the first microbial stress mechanism that has been correlated with activated sludge process upset. The implications of the results are discussed.

## Materials and Methods

**Cultures and Growth Conditions.** *Sphingomonas capsulata* (ATCC 14666) was obtained from the American Type Culture Collection, and Luria-Bertani (LB) broth (ATCC medium 1065) was used as a maintenance liquid growth medium. For all of the experiments presented here, *S. capsulata* was grown to late log or early stationary phase (approximately $4x10^9$ to $8x10^9$ CFU/mL) in a semi-defined containing glucose and Cas amino acids (Glc/Cas) media: 2.3 g/L glucose, 0.5 g/L Cas amino acids, 5.7 g/L $Na_2HPO_4$, 1.4 g/L $NaH_2PO_4$, 74.5 mg/L KCl, 0.83 g/L $NH_4Cl$, 87 mg/L $MgSO_4$, 0.8 mg/L $MnSO_4 \cdot H_2O$, 30 µg/L $Na_2MoO_4 \cdot 2H_2O$, 102 mg/L $CaCl_2 \cdot 2H_2O$, 15 mg/L $FeCl_3$, 5 µg/L $CoCl_2 \cdot 6H_2O$, 1.0 mg/L $ZnCl_2$, 0.13 mg/L $CuCl_2 \cdot 2H_2O$, and 83 µg/L $H_3BO_3$. *S. capsulata* culturable cell counts were performed by serially diluting samples in 8.5 g/l NaCl, 0.3 g/L $KH_2PO_4$, 0.6 g/L $Na_2HPO_4$, and 0.5 g/L nutrient broth (adjusted to pH 7.3 with NaOH) and plating on LB agar.

Activated sludge experiments were initiated with fresh publicly-owned treatment works (POTW) mixed liquor from the Blacksburg-Virginia Tech Wastewater Treatment Plant (Blacksburg, VA). Fresh primary effluent was obtained at the same time as mixed liquor and was used as the feed for all of the activated sludge experiments. Activated sludge bench-scale bioreactor systems consisted of either 3.5 L or 200 mL sequencing batch reactors (SBRs).

NEM, CDNB, DTT, 1,4-benzoquinone (BQ), and nigericin (Nig) were obtained in the purest form available from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA).

**Activated Sludge Experiments.** The 3.5 L SBRs were operated as described previously (6) and consisted of modified 4.0 L beakers which were operated in parallel. Each reactor cycle consisted of 3 minutes of feed time, 5.25 hours reaction time with aeration and stirring, followed by 30 minutes of quiescent settling and immediate effluent sampling. Mixing was provided by 100 RPM electric motors with single blade paddles, and aeration equipment consisted of aquarium air pumps and standard air stones. For the experiments presented here, each SBR was operated for only one 6 hour cycle since the focus was on short-term responses. Fresh mixed liquor was added to the SBRs, stirred and aerated for approximately 10 minutes, and then settled for 30 minutes. One-fourth of the total reactor volume (875 mLs) was decanted using peristaltic pumps, and aerators and mixers were restarted. Fresh primary effluent was added using peristaltic pumps to replace the decanted volume in approximately 3 minutes, thus beginning the experimental cycle. As soon as primary effluent feeding was terminated, chemical stressors were added to the experimental reactors in a single shock load, pre-dissolved in either a small volume of distilled/deionized-$H_2O$ or ethanol (ethanol also added to control reactor as necessary). It was previously determined that the stressor concentrations required to reduce the oxygen uptake rate in activated sludge by 50 percent ($IC_{50}$s) are approximately 100, 220, and 1.5 mg/l for NEM, CDNB, and BQ, respectively (6). Additionally, maximal $K^+$ efflux and deflocculation have been consistently measured in activated sludge cultures at 50 mg/L NEM, 75 mg/L CDNB, and 25 mg/L BQ (6). Time-zero was defined as the point immediately after primary effluent feeding, but immediately before adding the toxic chemical. Aeration and mixing were continued for 5.25 hours after feeding/stressing, followed by 30 minutes of quiescent settling, then decant of effluent samples.

The small 200 mL SBR reactors were required so that $K^+$ ion-selective electrodes (ISE) could be used in activated sludge cultures and so that the amount of nigericin used could be minimized due to its cost. These SBRs were operated exactly as the 3.5 L reactors and consisted of 250 mL beakers. However, for this system, mixing was provided by magnetic stir bars and aeration

consisted of small air stones. Primary effluent feed was added manually instead of with a peristaltic pump.

It was previously determined that most of the soluble chemical oxygen demand (COD) added with the primary effluent is consumed in the SBRs within about 30 minutes (data not shown). COD is a measure of available electrons in an organic compound and is expressed in terms of oxygen required to accept the electrons during complete oxidation of the compound to carbon dioxide and water. Therefore, it was necessary to amend the primary effluent feed with a readily degradable carbon source so that an exogenous substrate was present during reductant-activated $K^+$ recovery periods. $K^+$ uptake was initiated by reductant (DTT) addition following electrophile-induced efflux. Although GGKE activity is not energy demanding and requires no *de novo* protein synthesis, $K^+$ uptake requires a suitable substrate (3,19). Primary effluent was amended by adding 400 mg/L as COD of each of three substrate sources (bacto-peptone, Na-acetate, and glucose) directly to the fresh primary effluent. Since the primary effluent feed was diluted (1:4) in the SBRs, the carbon supplement resulted in an additional 300 mg/L as COD at the beginning of the reaction period.

***S. capsulata* Experiments.** *S. capsulata* was grown in batch as described above. Immediately before each experiment, cells were harvested by centrifugation at 9,500x*g* for 10 minutes (Beckman J2-HS, Fullerton, CA) and resuspended in sufficient Glc/Cas media to concentrate the cells by 3.33X, as compared to the original culture. The concentrated culture (90 mLs) was added to 150 mL beakers, and mixed with magnetic stir bars. ISE analysis was initiated, and as soon as the probes stabilized (approximately 5 minutes), time-zero samples were retrieved and processed for cation composition (to validate ISE function). Stressor chemicals were added in a single shock load pre-dissolved in a minimal amount of dd-$H_2O$ or ethanol (also added to control, as necessary).

**Analytical Procedures.** *$K^+$ and activated sludge performance measurements*. Soluble and floc- or cell-associated samples were analyzed for cations using ion chromatography (IC) and inductively coupled plasma-emission spectrophotometry (ICP-ES) for both *S. capsulata* and activated sludge experiments, as described previously (6). Activated sludge performance was

evaluated by monitoring mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI), effluent total suspended solids (TSS), and effluent volatile suspended solids (VSS) all in accordance with *Standard Methods* (2). The 200 mL SBRs did not provide enough settled decant to perform effluent suspended solids analysis, so nephelometric turbidity (NTU) measurements were used to quantify the degree of deflocculation. Multiple experiments were performed to confirm the observed trends, but results from only one trial are shown in this paper.

$K^+$ ion-selective electrodes (ISEs) were used to monitor changes in the soluble bulk liquid $K^+$ concentration in real-time with computer data acquisition. The system consisted of two Orion 9719BN (Beverly, MA) combination $K^+$ ISEs, two Accumet (Fisher Scientific, Atlanta, GA) automatic temperature compensation probes, and a two-channel Accumet AR25A pH/mV/ISE meter with serial-port data acquisition from the meter using a portable computer running LabVIEW 6.0 software (National Intsruments, Austin, TX). A data acquisition program was written to record $K^+$, temperature, and mV data in a spreadsheet file every six seconds for the duration of each experiment. Smoothing of the ISE data was performed by a 5-point moving average. The ISEs were calibrated using 1.0, 10, 50, and 100 mg/L KCl-$K^+$ standards that contained NaCl to adjust the ionic strength of the standards to appropriate levels.

*NEM analysis*. Bulk liquid NEM concentrations were measured using a Hewlett Packard 1090 high pressure liquid chromatograph (HPLC) with a UV/Visible diode-array detector and an Alltech Econosphere C-18 analytical column (250 mm x 4.6 mm x 5.0 μm) and guard column. NEM samples were processed by filtering activated sludge or *S. capsulata* supernatant using 0.2 μm Gelman (Ann Arbor, MI) SUPOR-200 membrane filters and acidifying with 50% (v/v) $H_2SO_4$ to approximately pH 2. Samples were stored in the dark at 4°C until analyzed. The HPLC eluent consisted of 90% 10 mM $H_3PO_4$ in 18 MΩ $H_2O$ and 10% HPLC-grade acetonitrile at a flowrate of 1.5 mL/min. Analysis was performed using an injection volume of 50 μLs. NEM standards were prepared from SigmaUltra (Sigma-Aldrich, St. Louis, MO) crystals dissolved in 10 mM $H_3PO_4$ in 18 MΩ $H_2O$ to concentrations ranging from 1.0 to 100 mg/L. Data acquisition and peak integration were performed using HP ChemStation software.

*Cell lysis determination*. In order to evaluate cell lysis in both *S. capsulata* and activated sludge cultures, the Molecular Probes, Inc. (Eugene, Oregon) LIVE/DEAD® BacLight™ bacterial viability system was used to assess membrane integrity. This system uses two fluorescent nucleic acid stains; the Syto® 9 (green) stain penetrates the bacterial cytoplasmic membrane under all conditions, while propidium iodide (red) can enter the cell and stain the nucleic acid only if the integrity of the membrane has been compromised. Therefore, bacteria with intact membranes fluoresce green, and lysed or "dead" cells fluoresce red. For activated sludge, the stains were used as recommended by Molecular Probes, Inc. and as previously documented by Ramirez et al. (24). Syto® 9 and propidium iodide were added directly from stocks pre-made in dimethyl sulfoxide to mixed liquor samples providing final concentrations of 5.0 and 30 μM, respectively. Staining was performed in the dark for 15 minutes before observation using a Zeiss (Carl Zeiss, Inc., Thornwood, New York) Axiovert S1D0TV epifluorescence microscope at 400X or 630X with a 500 ± 10 nm excitation filter, a 515 nm long-pass dichroic mirror, and a 520 nm emission filter. This fluorescence filter set allowed simultaneous observation of green and red fluorescence. For *S. capsulata* samples, the cells were washed and resuspended in a bicarbonate buffer solution (BCS) containing 1 mM KCl, 50 mM NaCl, and 50 mM $NaHCO_3$ adjusted to pH 7.0 with HCl. For best differentiation between green and red cells, the stain concentrations were adjusted to 0.50 and 30 μM for Syto® 9 and propidium iodide, respectively. For both activated sludge and *S. capsulata*, positive controls for lysed cells were obtained by adding 5% (v/v) iso-propanol and incubating for 1.0 hour with periodic mixing. The iso-propanol was removed by washing *S. capsulata* or mixed liquor with BCS before staining.

*Floc volume measurements*. For activated sludge stained with LIVE/DEAD, it was also possible to observe the "live" and "dead" regions within an individual floc. In order to calculate the $K^+$ concentration in the floc structure following electrophile addition, a critical parameter is the volume of an activated sludge floc that consists of intact cytoplasm relative to the volume of dead cells and EPS. Since it was observed that the entire floc stained either red or green (with varying degrees of brightness) as compare to phase contrast microscopy, it was assumed that the green and red regions represent the portion of an individual floc comprised of viable bacterial cytoplasm or lysed cells and EPS, respectively. Using the epifluorescence microscope with filter sets that allow for observation of either green or red fluorescence only, it was possible to

quantify the area and intensity of green fluorescence relative to the area and intensity of red fluorescence in an individual floc. This was accomplished using filter sets that contained either a 480 ± 20 nm excitation filter, a 505 nm long-pass dichroic mirror, and a 535 ± 25 nm emission filter (green) or a 545 ± 15 nm excitation filter, a 570 nm long-pass dichroic mirror, and a 610 ± 37 nm emission filter (red). Black and white images of a series of activated sludge flocs observed under green or red fluorescence were obtained using a computer-controlled charge-coupled device (CCD). The area of green or red fluorescence was quantified in an integrated density value using PC-based *Scion Image* release beta 3b (Fredrick, MD). The red and green integrated densities were then used to obtain percentages of green or red volume for an individual floc. Eukaryotic organisms within the activated sludge matrix did not appear to stain either red or green and were only visible under standard phase-contrast microscopy.

## Results and Discussion

**$K^+$ Efflux in *S. Capsulata* and Activated Sludge.** Booth and coworkers have extensively evaluated the GGKE mechanism in *E. coli* K-12 (and derived mutants) and have demonstrated GGKE activity in a number of other Gram-negative bacteria (8,12). However, considering the industrially relevant electrophiles used previously (e.g. CDNB and BQ) and the concentrations of these toxins that caused significant deflocculation (6), it was necessary to compare the $K^+$ efflux response observed in activated sludge with an organism that is more similar to bacteria found in biological wastewater treatment systems. Using molecular techniques, Snaidr et al. (25) determined that *S. capsulata* was present in a domestic activated sludge at approximately 3% of the total active bacterial cell count, which classifies that microorganism as being relatively prevalent. Using *S. capsulata* also provides a model for evaluating the GGKE system in the absence of flocs, thereby eliminating complications associated with electrophile or $K^+$ diffusion in and out of the floc structure.

NEM, CDNB, and BQ all resulted in significant $K^+$ efflux from *S. capsulata*, and the stressor concentrations required to achieve maximal $K^+$ efflux in *S. capsulata* were approximately 50 mg/L NEM, 100 mg/L CDNB, and 30 mg/L BQ. These results are comparable to results from activated sludge experiments (6). As shown in Figure 1, NEM was initially added at 50 mg/L

(0.40 mM), and soluble bulk liquid cations were monitored by both ISEs and IC. Cell-associated (CA) cation levels were also monitored by ICP-ES and are shown in Figure 1B normalized to the original sample volume or the CFU concentration. The $K^+$ efflux response was very rapid in the NEM-stressed culture, and most of the $K^+$ release to the soluble phase occurred within 3 to 5 minutes. During this period, soluble and CA $K^+$ levels remained constant in the control. The magnitude of the NEM-mediated increase in soluble $K^+$ in Figure 1A was typical for several independent experiments using NEM at 50 mg/L and comparable cell densities. In Figure 1A, the soluble $K^+$ concentration increased by about 11 (IC) to 14 (ISE) mg/L (reflects variation between IC and ISE measurements), and this was concurrent with a comparable decrease in the CA phase, thereby confirming transport of $K^+$ from the cells to the bulk liquid. For IC analysis, it was apparent that significant $K^+$ efflux occurred before the first sample following NEM addition could be processed, and this demonstrates the value of the ISE measurement as an indicator of the rapid rate of efflux.

Rapid $K^+$ efflux was also observed in activated sludge exposed to 50 (Figure 3) and 100 (Figure 2) mg/L NEM. However, the kinetics of the $K^+$ efflux response were much slower in activated sludge than for *S. capsulata*. Whereas maximal *S. capsulata* $K^+$ efflux to the bulk liquid typically occurred within 5 to 10 minutes, activated sludge efflux to the bulk liquid required more than 60 minutes (Figure 2). It is suspected that this kinetic difference is due to diffusion limitations caused by activated sludge flocs, both for the stressor diffusing into the interior of the floc and for the $K^+$ diffusing from the floc to the bulk liquid. ICP analysis confirming $K^+$ transport from the floc-associated phase to the bulk liquid has been reported elsewhere (6).

As shown in Figures 1A and 3, there are significant discrepancies between the ISE and IC measurements. Although the relative differences between the stressed and control samples were similar for IC and ISE measurements, the IC $K^+$ magnitude was consistently greater than that measured by ISE (typical of several independent activated sludge and *S. capsulata* experiments). This difference may be attributed to chemical or electrical interference of the ISEs, or slight aqueous $K^+$ complexing not detected by the ISEs. Nevertheless, the ISE data were very useful for assessing the actual kinetics of the $K^+$ efflux response and does seem to accurately represent the trends obtained with the IC. For both activated sludge and *S. capsulata*, the ISEs also

confirmed that efflux was initiated immediately after stressor addition and indicated the approximate time required to achieve maximal efflux.

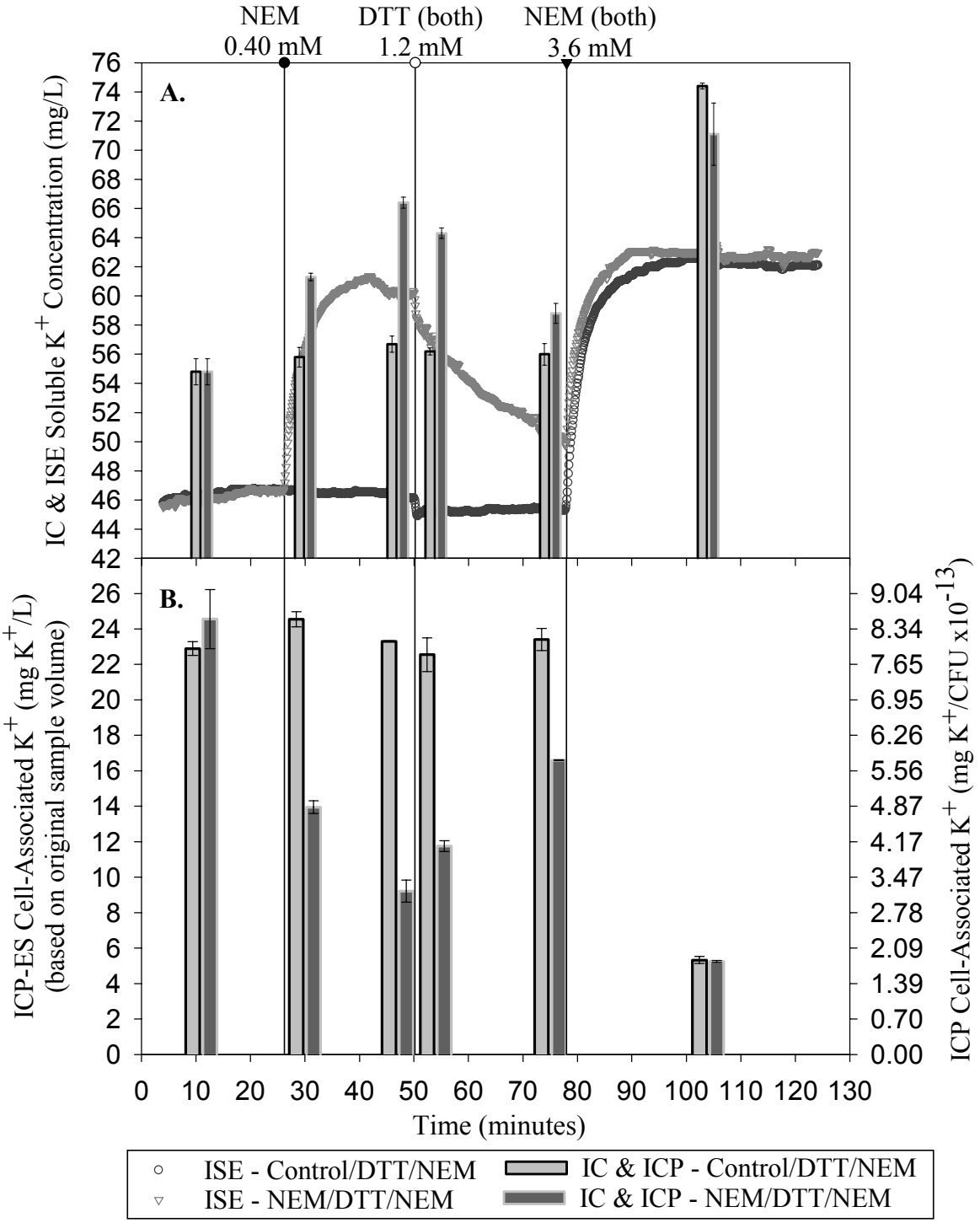


Figure 1. Batch *S. capsulata* stress experiment with (A.) ISE soluble (bulk liquid) and IC soluble (bulk liquid) $K^+$ measurements and (B.) ICP-ES cell-associated (CA) $K^+$ measurements. S. capsulata was concentrated to $2.88 \pm 0.68 \times 10^{10}$ CFU/mL, and NEM was added at 50 mg/L (0.40 mM) followed by DTT addition at 185 mg/L (1.2 mM) to both the NEM-stressed and the control beakers and a final addition of 450 mg/L (3.6 mM) NEM to both beakers. The CA fraction is presented in mg/L relative to the original sample volume and in units of mg $K^+$/CFU (CFU

measured prior to stressor addition). Error bars represent the average of duplicate analyses for one sample for the IC soluble measurements and duplicate samples for the CA measurments (± one SD).

***S. Capsulata* Dynamic Efflux/Uptake/Efflux.** The results of the dynamic efflux experiments suggest that NEM-induced efflux is reversible in *S. capsulata* and that this Gram-negative organism exhibits GGKE activity. For the *S. capsulata* stress experiment shown in Figure 1, 1.2 mM DTT was added after $K^+$ efflux, which is a concentration that is three times the molar concentration of NEM originally added. Recovery was observed with a significant decrease in the soluble bulk liquid $K^+$ concentration, a significant increase in the CA fraction, and negligible change in the control culture $K^+$ levels which also received 1.2 mM DTT. After approximately 5 mg/L of $K^+$ was recovered by the cells, NEM was added to both cultures (control and NEM-stressed) at a concentration that was three times the molar concentration of DTT (3.6 mM = 450 mg/L). It is apparent that DDT addition had little effect on bulk liquid $K^+$ levels in the control beaker which did not receive the initial 50 mg/L dose of NEM. $K^+$ "re-efflux" was immediately activated following NEM addition (450 mg/L = 3.6 mM) to the NEM/DTT/NEM beaker. The final soluble and CA $K^+$ levels were similar in the NEM/DTT/NEM and control/DTT/NEM samples despite the original 50 mg/L NEM addition. CDNB-induced $K^+$ efflux was not reversible (or even inhibited) in *S. capsulata* by DTT addition at up to three times the molar concentration of CDNB (data not shown). Reductant-provoked $K^+$ uptake following NEM-induced efflux indicates that GGKE activity (homologous to KefBC in *E. coli*) is present in *S. capsulata* and activated sludge cultures, and that the electrophile-induced increase in bulk liquid $K^+$ actually represents a physiological response in activated sludge bacteria. Furthermore, recovery of cytoplasmic $K^+$ following efflux helps to confirm that significant cell lysis did not occur as a result of electrophile addition. That is, the cells must have remained intact if $K^+$ can be re-concentrated in the cytoplasm.

**Activated Sludge Dynamic Efflux/Uptake/Efflux.** Dynamic efflux experiments were also conducted in activated sludge SBRs with NEM and CDNB. As shown in Figure 2, the addition of 100 mg/L NEM resulted in immediate $K^+$ efflux with significant release occurring before the first IC sample could be processed, and approximately 4 mg/L $K^+$ was transported to the bulk liquid after 50 minutes of contact with NEM. As soon as DTT was added to two of the SBRs, $K^+$

efflux ceased, and a slight but statistically significant ($p<0.01$) uptake of $K^+$ was measured. Reinitiation of $K^+$ efflux was achieved when 600 mg/L NEM (4.8 mM) was added to one of the reactors to which DTT had been added. The soluble $K^+$ concentration approached that for the SBR receiving only NEM by the end of the reaction period (300 minutes). The data in Figure 2 suggest that NEM-promoted $K^+$ efflux was also reversible in activated sludge, although the extent of intracellular $K^+$ recovery, as measured in the bulk liquid, was not as dramatic as observed in *S. capsulata*. Again, this may have been due to diffusion limitations that differentiate true $K^+$ concentrations between the bulk liquid and inside the floc structure.

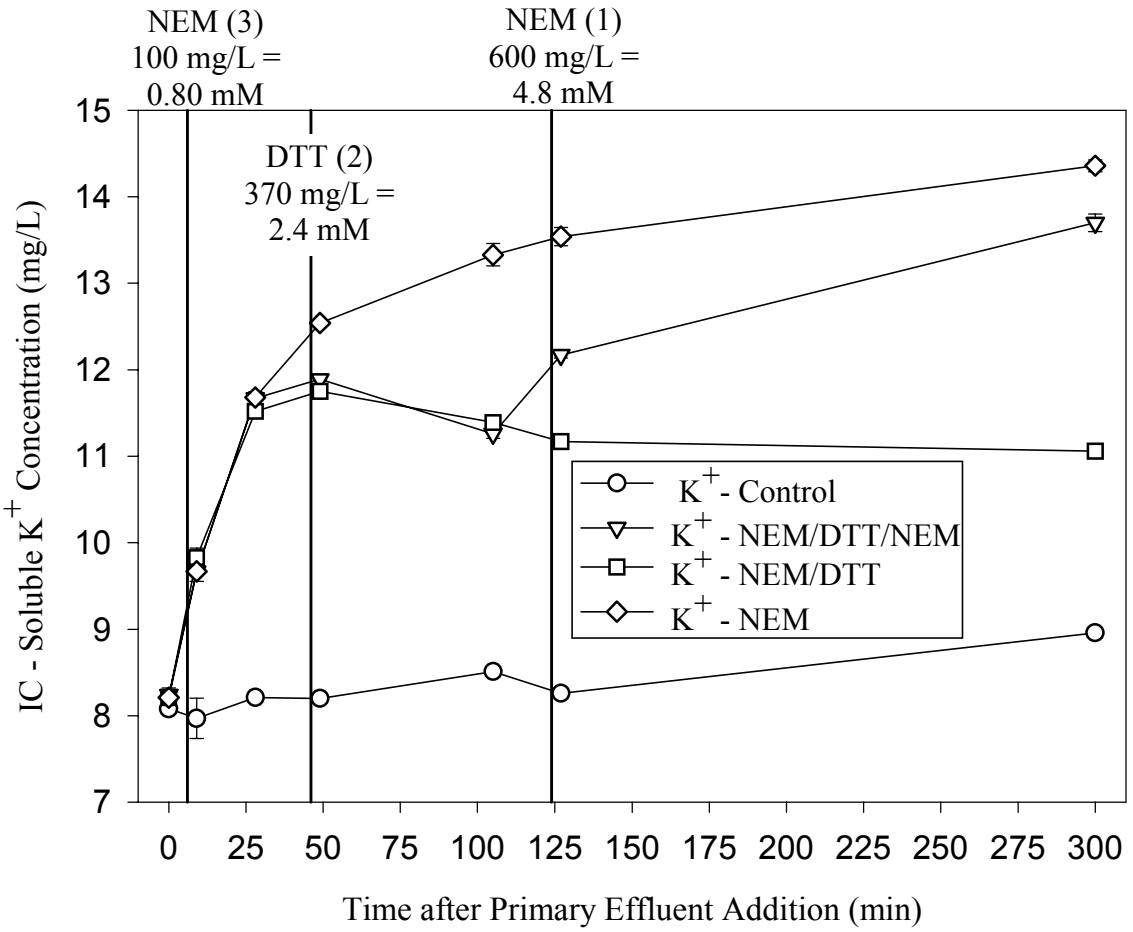


Figure 2. Soluble bulk liquid $K^+$ concentrations measured by IC for the dynamic activated sludge SBR experiment with 4 reactors operated in parallel. One SBR served as the control, one received only 0.80 mM NEM (NEM), one received 0.80 mM NEM followed by 2.4 mM DTT (NEM/DTT), and one received 0.80 mM NEM, 2.4 mM DTT, and 4.8 mM NEM

(NEM/DTT/NEM). Error bars represent the average of duplicate analyses of a single IC sample (± one SD).

VSS measurements shown in Table 1 indicate that DTT addition to activated sludge cultures (and slight $K^+$ recovery) did not alleviate deflocculation within the 5 hour reaction period. The damage within the floc structure, caused by the original NEM-induced $K^+$ efflux, was not remedied by the slight recovery of $K^+$. It is also conceivable that the slight increase in effluent VSS in NEM/DTT/NEM as compared to the NEM-only SBR may have been a result of the secondary $K^+$ efflux response. It is apparent that the initial efflux response, which is observed immediately after electrophile addition, is responsible for floc deterioration. Results from preliminary SBR experiments, in which the system was operated for several days after chemical shock loading, also indicate that the initial efflux response weakened the floc structure so severely that deflocculation continued for an extended period of time, even after the stressor had been essentially washed out of the system and the bulk liquid $K^+$ concentration returned to "normal" (6). After a significant $K^+$ efflux event, it appears that recovery from the deflocculating conditions will require either complete regrowth of the biomass (i.e. several SRTs before upset recovery) or chemical reflocculation of the mixed liquor.

NEM concentrations measured by HPLC are also presented in Table 1. As expected *a priori*, these data confirmed that the spontaneous reaction between DTT and NEM was rapid and proceeded to completion. There also appeared to be very little loss of NEM from the bulk liquid due to sorption on or reaction with the activated sludge floc material, as approximately 100 mg/L NEM was recovered in all three NEM-stressed SBRs after the first stressor addition. For the NEM-only reactor, the soluble NEM concentration was reduced by only about 28% by the end of the 300 minute reaction period. Additionally, the final NEM concentration in the NEM/DTT/NEM SBR was about 400 mg/L (3.2 mM) and is exactly what would be expected as a result of the 0.80 mM NEM, 2.4 mM DTT, and 4.8 mM NEM additions.

Table 1. NEM concentration and effluent VSS for the dynamic activated sludge efflux/uptake/efflux experiment conducted in the 3.5 L SBR reactors.

| Sample Time (minutes) | Soluble NEM Concentration (mg/L)[1,3] Control Measured | Control Expected | NEM/DTT/NEM Measured | NEM/DTT/NEM Expected | NEM/DTT Measured | NEM/DTT Expected | NEM Measured | NEM Expected |
|---|---|---|---|---|---|---|---|---|
| 0 | NPD | 0 | NPD | 0 | NPD | 0 | NPD | 0 |
| 28 | NPD | 0 | 99.3 (0.21) | 100 | 99.7 (0.12) | 100 | 101 (0.11) | 100 |
| 49 | NPD | 0 | NPD | 0 | NPD | 0 | 97.3 (0.08) | 100 |
| 105 | NPD | 0 | NPD | 0 | NPD | 0 | 90.9 (1.8) | 100 |
| 127 | NPD | 0 | 390 (7.0) | 400 | NPD | 0 | 88.9 (0.05) | 100 |
| 300 | NPD | 0 | 397 (0.31) | 400 | NPD | 0 | 73.0 (4.2) | 100 |
| | Effluent VSS Concentration (mg/L)[2,3] | | | | | | | |
| 345 | 8.71 (0.16) | | 116 (14) | | 82.0 (2.8) | | 76.7 (0.9) | |

NPD = No NEM peak detected

1. Expected NEM concentrations are reported assuming complete equimolar reaction between NEM and DTT, and no sorptive or reactive losses of NEM.
2. Effluent VSS was measured at the end of the 5.25 hour reaction period following 30 minutes of quiescent setting.
3. For both NEM and VSS measurements, the reported values represent the average of duplicate analyses of single samples with the SDs in parentheses.

Figures 3 and 4 represent dynamic activated sludge experiments conducted in the smaller SBR systems with IC and ISE measurement of soluble bulk liquid $K^+$ concentration. After NEM stress at 50 mg/L, approximately 1 mg/L of intracellular $K^+$ was recovered following DTT addition (Figure 3). After a period of relatively slow uptake of $K^+$ by the floc structure, $K^+$ efflux was reinitiated by a subsequent NEM addition, and about 2 mg/L $K^+$ was released from the floc structure. However, as shown in Figure 4, CDNB-induced $K^+$ efflux was not recoverable, or even inhibited, by adding DTT at a level three times the molar amount of CDNB added. Subsequent addition of 608 mg/L CDNB did not increase the rate of $K^+$ efflux or cause any additional efflux. The final CDNB addition was well above the aqueous solubility of CDNB (about 300-400 mg/L), and some small CDNB particles were observed after addition of the ethanol stock solution. Although the recovery of $K^+$ after NEM-induced efflux was kinetically very slow and relatively insignificant compared to the efflux response, there is a distinct difference in the recovery responses shown in Figures 3 and 4. Finally, the efflux/uptake/efflux responses obtained in activated sludge and *S. capsulata* were similar to that observed in *E. coli* K-12 (5,9) when comparing DTT-promoted recovery of cytoplasmic $K^+$ following NEM- and CDNB-induced efflux.

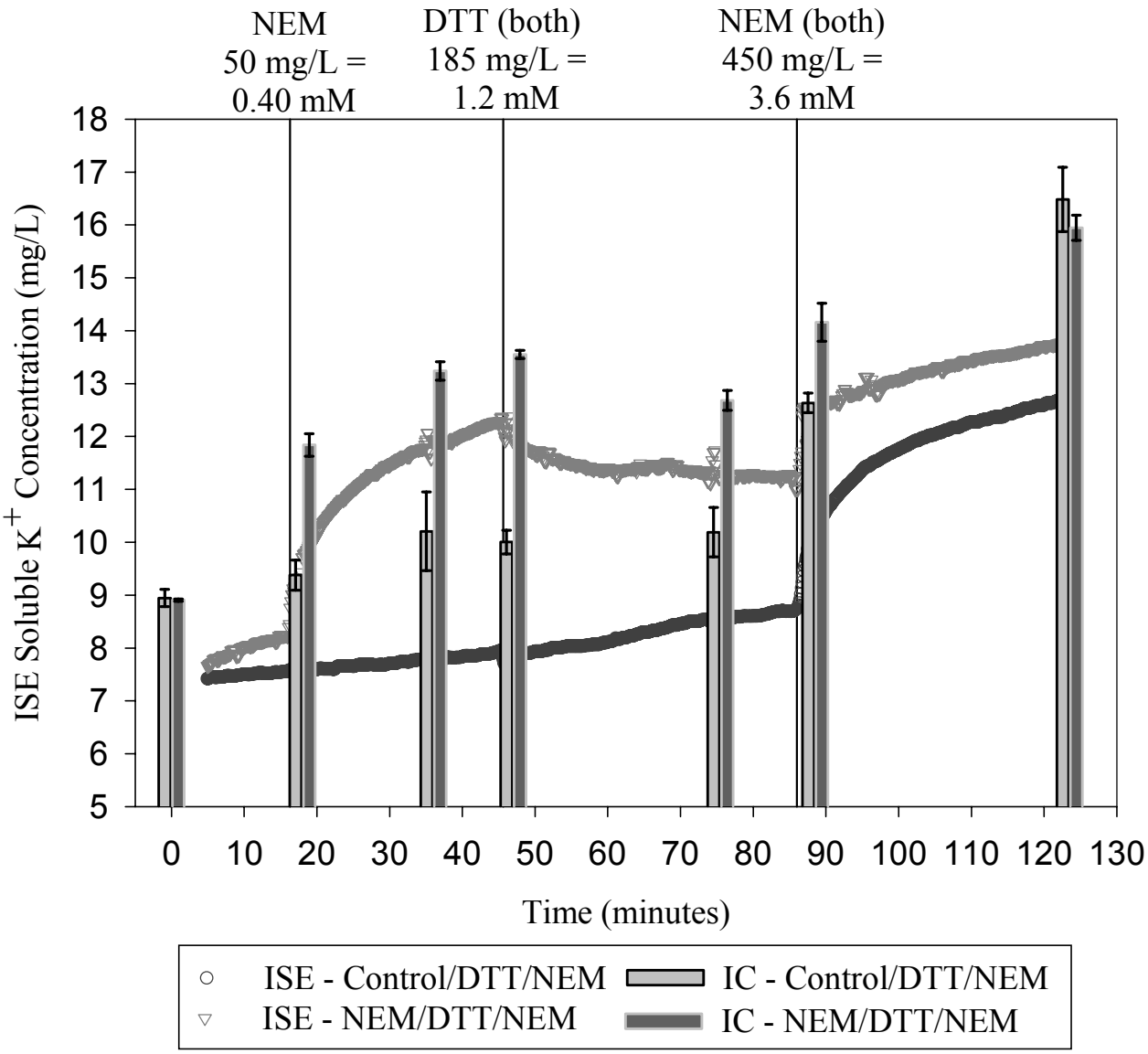


Figure 3. Dynamic activated sludge efflux experiment conducted in small SBRs operated in parallel. One reactor served as the control but subsequently received 1.2 mM DTT followed by 3.6 mM NEM. The other reactor was stressed with NEM at 50 mg/L (0.40 mM) followed by 1.2 mM DTT and 3.6 mM NEM. The soluble bulk liquid $K^+$ concentrations were measured by IC and ISEs. Error bars represent the average of duplicate analyses of a single IC sample (± one SD).

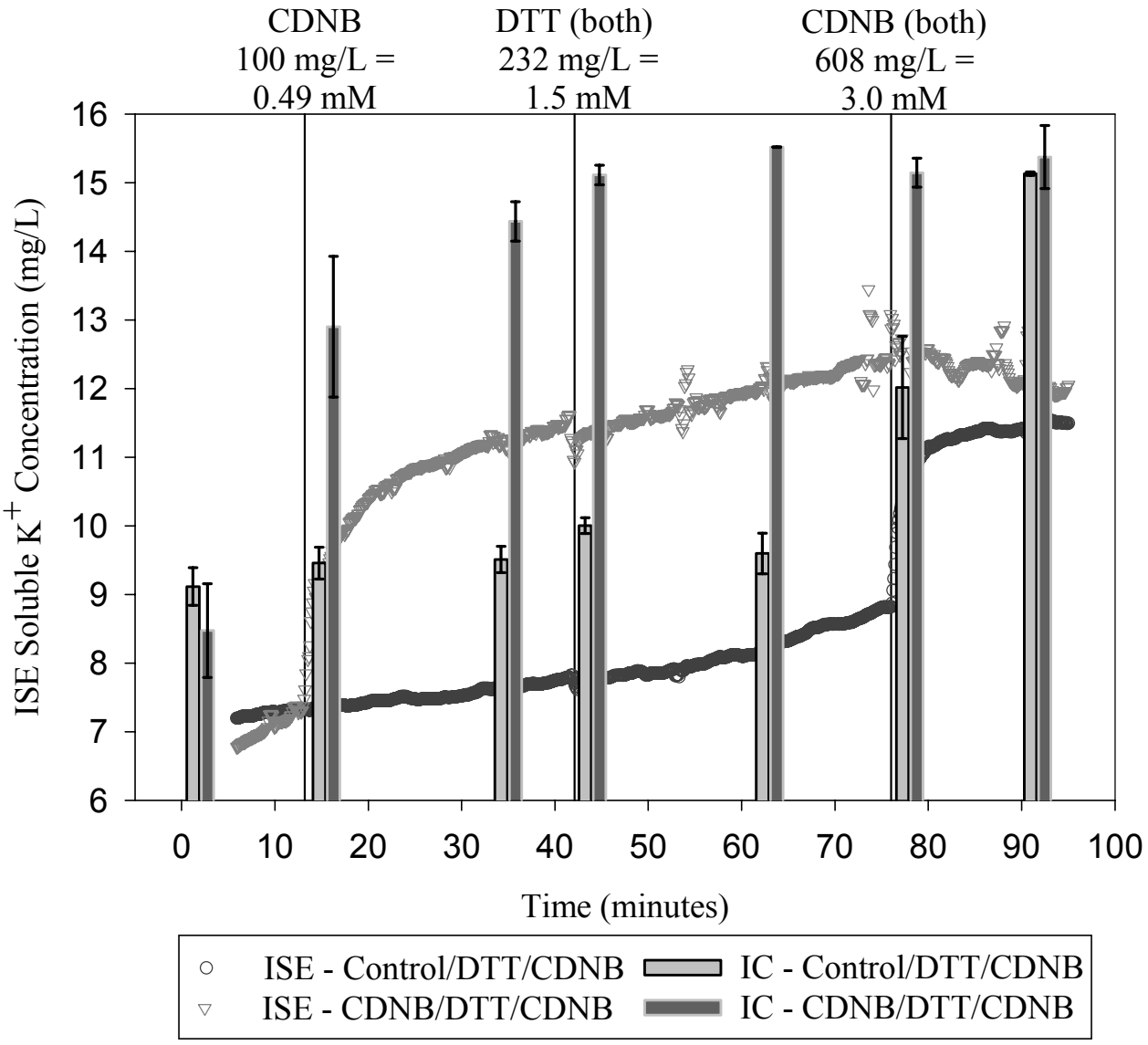


Figure 4. Dynamic activated sludge efflux experiment conducted in small SBRs operated in parallel. One reactor served as the control but subsequently received 1.5 mM DTT followed by 3.0 mM CDNB. The other reactor was stressed with CDNB at 100 mg/L (0.49 mM) followed by 1.5 mM DTT and 3.0 mM CDNB. Error bars represent the average of duplicate analyses of a single IC sample (± one SD).

It has been demonstrated that *S. capsulata* exhibits electrophile-induced $K^+$ efflux activity that is similar to that observed in *E. coli* K-12. It was also determined that electrophile concentrations required to induced $K^+$ efflux in *S. capsulata* were very similar to that for activated sludge cultures. The observation that effluxed $K^+$ was recovered by activated sludge following DTT addition represents convincing evidence that electrophilic stress causes negligible cell lysis

within the concentration range of interest. Furthermore, the similarities between the responses with activated sludge and *S. capsulata* for DTT-mediated $K^+$ recovery following either NEM- or CDNB-stress are evidence that GGKE activity was responsible for electrophile-induced $K^+$ efflux in activated sludge.

**$K^+$ Efflux Due to Cell Lysis.** There are several observations which suggest that cell lysis was not responsible for electrophile-induced $K^+$ efflux. We previously demonstrated that (i.) maximal $K^+$ efflux and deflocculation occurred at electrophile concentrations significantly less than that required to reduce the oxygen uptake rate by 50%, (ii.) electrophilic stress resulted in significant $K^+$ efflux but no efflux of other cations, including $Fe^{2+/3+}$, $Al^{3+}$, $Ca^{2+}$, $Mg^{2+}$, and $Na^+$, (iii.) and electrophile addition caused negligible release of alkaline phosphatase from activated sludge flocs (6). In this work, the Molecular Probes, Inc. (Eugene, Oregon) LIVE/DEAD® BacLight™ system was used to assess membrane integrity after addition of NEM to *S. capsulata* and activated sludge cultures. Epifluorescence microscopic observations revealed that NEM stress did not result in the formation of red cells in excess of that observed in controls for *S. capsulata* (data not shown). For activated sludge, multiple flocs were observed for both control and NEM-stressed mixed liquor. As expected, some "red zones" were observed within individual flocs even for unstressed mixed liquor, but there was an obvious predominance of "green area". After NEM stress at 50 mg/L for 60 minutes and observation of red and green fluorescence simultaneously, there was no discernable increase in red area within the observed flocs (data not shown). Therefore, it has been concluded that cell lysis was not responsible for a significant amount of electrophile-induced K+ efflux in either activated sludge or *S. capsulata*.

**Effect of Nigericin of $K^+$ Efflux.** In order to evaluate the effect of $K^+$ efflux on floc integrity, nigericin was used to induce $K^+$ release, independent of the KefBC GGKE system and without the addition of electrophilic toxins. Nigericin is a cyclic polyether ionophore which is closed by a carboxylate group hydrogen bonded to a hydroxyl substituent (16). This molecule is absorbed into the cytoplasmic phospholipid membrane and very selectively transports $K^+$ down a concentration gradient. Nigericin actually serves as an artificial $K^+/H^+$ antiport (16). In bacteria, nigericin eliminates both the catabolic $\Delta pH$ gradient and the significant outwardly directed $K^+$ concentration gradient by essentially making the membrane permeable to only $H^+$ and $K^+$. The

benefit of using this antibiotic is to simulate the mechanism of the GGKE system without requiring electrophile addition. Nigericin has often been used in eukaryotic cells to equilibrate intracellular $K^+$ levels with that in the bulk liquid (17), but there are few reports that discuss the use of nigericin in bacterial systems (1,23). The use of this antibiotic in an activated sludge matrix has not been reported.

Nigericin addition (50 μM) to *S. capsulata* resulted in significant $K^+$ efflux and was comparable to the efflux derived from 100 mg/L NEM (Figure 5). Measurable $K^+$ efflux occurred rapidly and immediately after chemical addition, but before the first sample could be processed. More importantly, DTT caused recovery from NEM-induced $K^+$ efflux but not from nigericin-promoted $K^+$ release. This was as expected since nigericin-induced efflux should not depend on the redox state of the cytoplasm or the presence of excess reductant. Following nigericin addition, subsequent NEM stress (900 mg/L) resulted in negligible further $K^+$ release, suggesting that nigericin addition caused complete $K^+$ equilibration of the bacterial cytoplasm with the bulk liquid. ISE data are not presented because nigericin caused severe probe interference that was probably due to nigericin partitioning into the organophilic ion-exchange membrane of the ISEs.

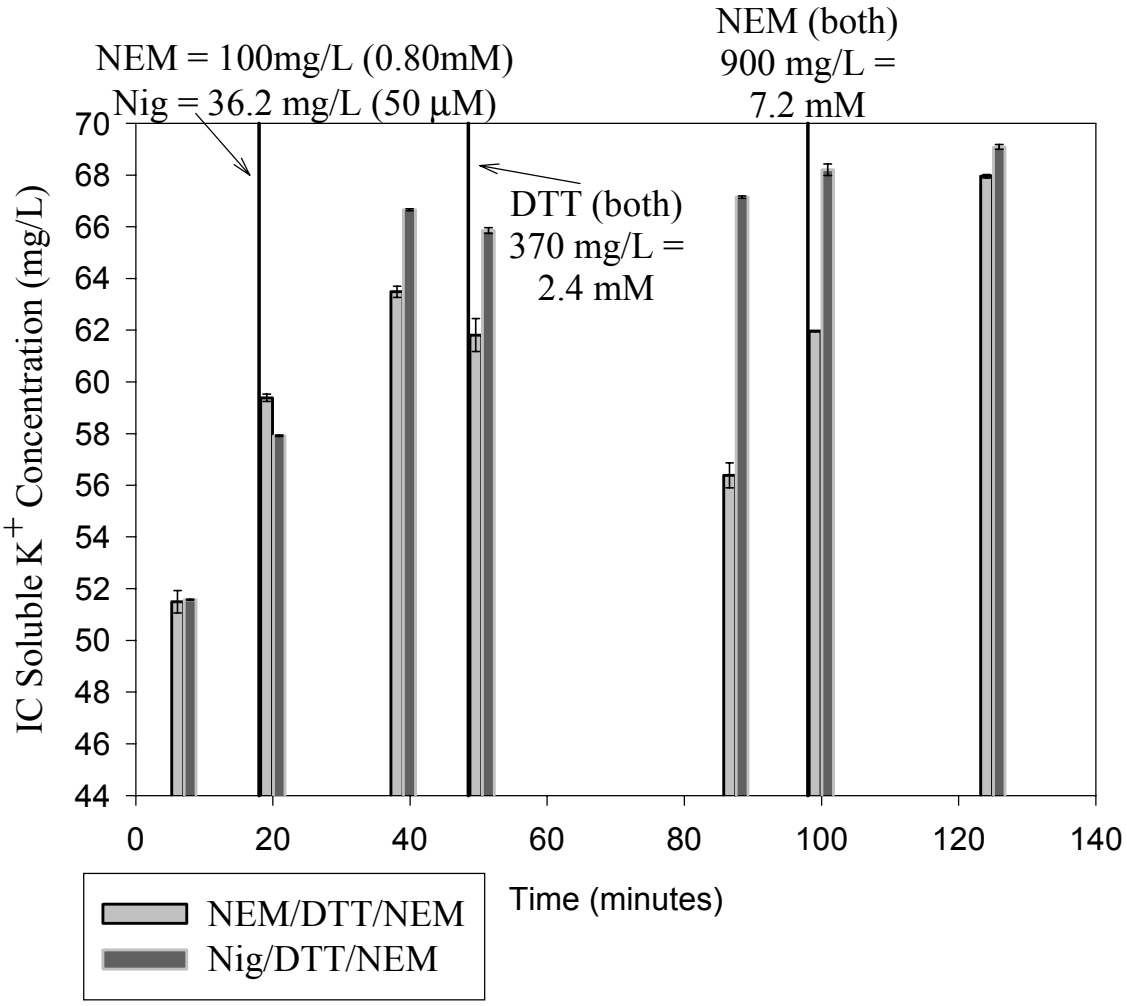


Figure 5. Nigericin, 50 µM (36.2 mg/L), and NEM, 0.80 mM (100 mg/L), addition to *S. capsulata* at cell density of $(1.8 \pm 1.1)x10^{10}$. After the initial addition of Nig or NEM, both systems received DTT at a concentration of 370 mg/L (2.4 mM) and a subsequent addition of NEM at 900 mg/L (7.2 mM). Soluble bulk liquid $K^+$ concentrations were measured using IC. Error bars represent the average of duplicate analyses of a single IC sample (± one SD).

Nigericin was also added at increasing concentrations to small activated sludge SBRs operated in parallel (Figure 6). Nigericin caused rapid and significant $K^+$ efflux from activated sludge to the bulk liquid at a faster rate than for 50 mg/L NEM. Increasing concentrations of nigericin caused enhanced $K^+$ release to the bulk liquid. Based on the average floc-associated $K^+$ concentrations typically observed in this domestic mixed liquor (data not shown), the degree of efflux obtained for 100 µM nigericin approximately represents the point of complete equilibration of the floc structure $K^+$ with the bulk liquid (maximal $K^+$ release from the floc).

Figure 7 shows that nigericin-induced $K^+$ efflux resulted in significant deflocculation. The degree of deflocculation obtained with 50 mg/L NEM was similar to that for 50 to 100 μM nigericin; however, the $K^+$ efflux response for these nigericin concentrations was 46 to 61% greater. Previous results indicate that the degree of deflocculation is well correlated with the amount of $K^+$ efflux for several electrophilic chemicals (6). The explanation for the discrepancy between electrophile-induced GGKE activity (presumed) and nigericin addition may be associated with the observed differences in the kinetics of the $K^+$ efflux response. This issue deserves further attention.

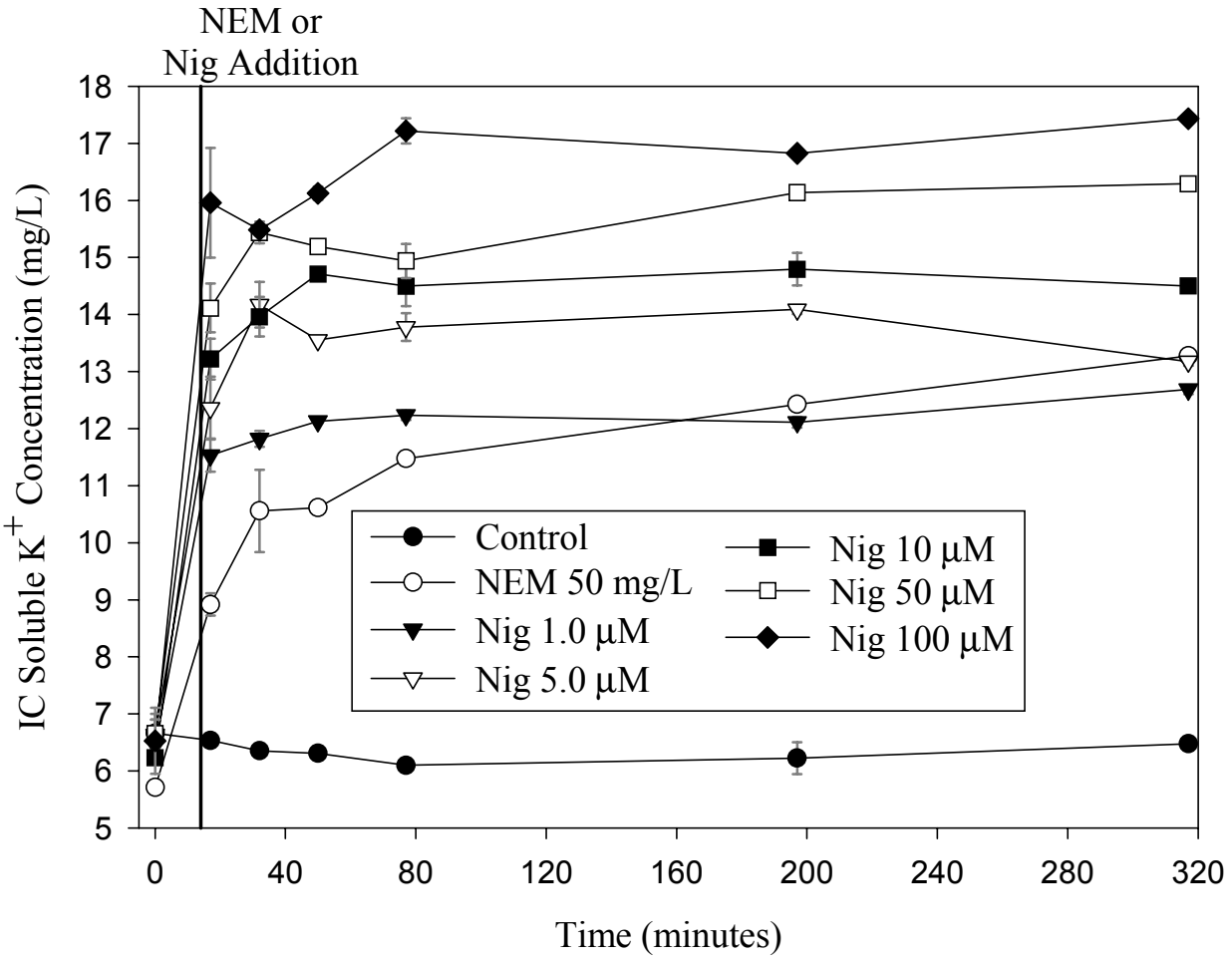


Figure 6. Nigericin was added to 5 small activated sludge SBRs a concentrations ranging from 1.0 to 100 µM. NEM (50 mg/L) was added to a sixth SBR as a positive control for deflocculation and $K^+$ efflux, and a seventh SBR served as the control. Soluble bulk liquid $K^+$ concentrations were measured using IC. Error bars represent the average of duplicate analyses of a single IC sample (± one SD).

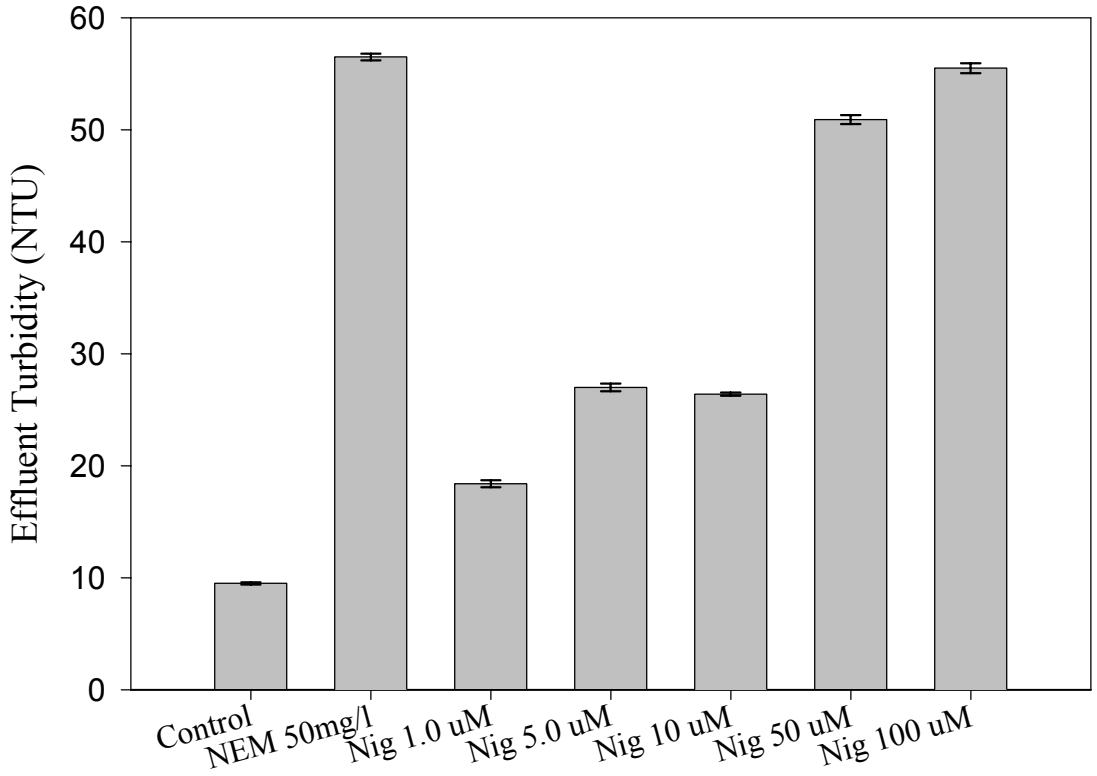


Figure 7. Effluent turbidities measured after 30 minutes of quiescent settling following addition of either Nig or NEM and a 5.25 hour SBR reaction period. Error bars represent the average of duplicate measurements ± one SD.

It is recognized that nigericin is relatively hydrophobic and is preferentially absorbed into the cytoplasmic membrane of bacteria. Therefore, nigericin-induced $K^+$ efflux in activated sludge was a good indication that the increase in bulk liquid $K^+$ was caused by a release from the cytoplasm of activated sludge bacteria. In addition, since nigericin results in nearly complete release of $K^+$ from activated sludge flocs, it is suspected that, prior to electrophile addition, most of the floc-associated $K^+$ is located in the cytoplasm of viable bacteria, and that the pre-stress levels of $K^+$ in the floc-structure EPS (outside of the cells but within the floc) are similar to that observed in the bulk liquid. These observations provide evidence that the GGKE system could be responsible for electrophile-induced $K^+$ efflux in mixed liquor microorganisms. Nigericin experiments also suggested that electrophile-induced deflocculation was caused by a rapid and significant increase in monovalent cation levels in the floc structure. However, the transport of $H^+$, due to both nigericin and GGKE, also deserves further attention. At this stage, we have not

been able to resolve the effect of the GGKE system on bulk liquid or floc-associated pH, but regardless, there is no convincing evidence that mild changes in pH affect floc strength.

**$K^+$ Efflux Calculations.** It was previously demonstrated that external addition of $K^+$ as KCl to activated sludge at concentrations in the range of 600-2000 mg/L $K^+$ caused deflocculation of the mixed liquor occurred (7,22). It was hypothesized that GGKE activity results in temporally and locally very high $K^+$ levels within the floc structure immediately after electrophilic stress. The high levels of $K^+$ (outside the cell, but still within the floc structure) then diffuse slowly to the bulk liquid. Therefore, to implicate GGKE-derived $K^+$ efflux as a possible cause of activated sludge deflocculation, the $K^+$ concentration that would be expected in the floc structure (i.e. outside of the cells, but within the floc structure) immediately following electrophilic-stressor addition was calculated. There are several key parameters that are necessary to complete this calculation, and the derivation of each will now be discussed.

A series of 8 activated sludge flocs were observed after LIVE/DEAD staining under both green-only and red-only fluorescence. Independent quantification of red and green signals indicated an average of 70.2 % green ($f_{cyto}$) and 29.8% red ($f_{EPS}$) with a standard deviation of 9.0 %. For these calculations, it was assumed that the percentages, $f_{cyto}$ and $f_{EPS}$, represent the volume fractions of activated sludge floc that are comprised of viable cytoplasm (capable of concentrating $K^+$) or EPS, respectively. This is represented symbolically as:

$$V_{floc} = V_{cyto} + V_{EPS} \tag{1}$$

$$\frac{V_{cyto}}{V_{floc}} = f_{cyto} \tag{2}$$

$$\frac{V_{EPS}}{V_{floc}} = f_{EPS} \tag{3}$$

where $V_{floc}$ is the total floc volume (mL floc/L mixed liquor), $V_{cyto}$ is the floc volume that is comprised of viable cytoplasm, and $V_{EPS}$ is the floc volume that represents dead cells or EPS.

In order to determine $V_{floc}$, the MLSS concentration and the floc density ($\rho_{floc}$) were used. An average MLSS of 2014 ± 505 was calculated from 10 independent SBR activated sludge experiments in which NEM was added at 50 mg/L. The activated sludge floc density is only slightly more than 1.0 g/mL for any given mixed liquor, and the overall calculation was found to be relatively insensitive to $\rho_{floc}$. It was previously determined that the floc density for the same domestic mixed liquor varied only from 1.008 to 1.024 g/mL under a variety of experimental conditions (15,21). It is also recognized, based on the silicone fluid separation method used to rapidly separate floc material from the bulk liquid (6), that the floc density was never less than 1.019 g/mL. Therefore, a floc density of 1.021 g/mL was used to calculate $V_{floc}$:

$$V_{floc} = \frac{MLSS}{\rho_{floc}} = 1.97 \pm 0.50 \text{ mL floc/L} \quad (4)$$

Using this value and Equations 1-3, $V_{cyto}$ and $V_{EPS}$ were calculated as 1.385 mL cytoplasm/L mixed liquor and 0.588 mL EPS/L mixed liquor, respectively.

From the *S. capsulata* experiment described in Figure 1 (50 mg/L NEM), it was observed that the CA $K^+$ concentration decreased by 80% from about 24 mg/L to 5 mg/L at a cell concentration of $2.88 \pm 0.68 \times 10^{10}$ CFU/mL. Therefore, *S. capsulata* cells contained approximately $8.25 \times 10^{-13}$ mg $K^+$/cell before efflux and about $1.72 \times 10^{-13}$ mg $K^+$/cell after electrophile addition. For calculation purposes, it was assumed that $V_{cyto}$ in the activated sludge was composed entirely of cells that have a $K^+$ efflux potential that is similar to *S. capsulata*. This assumption indicates that $C_{cyto}$, and the decrease in $C_{cyto}$ following electrophile addition in activated sludge are approximately equal to the $K^+$ concentration in *S. capsulata* both before and after NEM addition. Considering the prevalence of Gram-negative bacteria in activated sludge (25,26) and wide-spread existence of electrophile-induced GGKE activity in Gram-negative bacteria (8), the assumption is reasonable. Using the measured diameter of *S. capsulata*, it was possible to compute the $K^+$ concentration in the cytoplasm of *S. capsulata.* It was determined that the overall calculation is most sensitive to the cell diameter, simply because the cell volume is function of the radius cubed. As a result, the diameter of *S. capsulata* was carefully determined by quantifying green-only fluorescence after exposure to 50 mg/L NEM and LIVE/DEAD staining (note that there was no detectable increase in the number of red cells due to NEM exposure at this level). Using

*Scion Image* analysis software, it was possible to determine the average cell diameter by quantifying the "green area" for nearly all of the cells in a single spatially-calibrated digital image. The average cell diameter was 0.682 ± 0.047 μm, which gives an average cell volume of 0.166 $\mu m^3$. Therefore, $C_{cyto}$ is 4.96 mg $K^+$/mL cytoplasm and 1.03 mg $K^+$/mL cytoplasm before and after NEM addition, respectively (80% decrease). This corresponds to a release of about 3.93 mg $K^+$/mL cytoplasm ($C'_{cyto}$). Assuming that the $K^+$ is released from the bacterial cytoplasm into the floc structure EPS, the decrease in $C_{cyto}$ represents the increase in the $K^+$ concentration in the EPS ($C_{EPS}$). $C_{EPS}$ can thus be calculated as follows:

$$C_{EPS} = \frac{C'_{cyto}\ V_{cyto}}{V_{EPS}} = \frac{C'_{cyto} f_{cyto}}{f_{EPS}} \quad (5)$$

Using the terms as indicated above, the concentration of $K^+$ in the floc structure EPS ($C_{EPS}$) was determined to be approximately 9,300 mg/L immediately following electrophile addition, much more than that known to induce floc deterioration as a result of external KCl addition. However, if the errors associated with each of the estimated parameters are applied (i.e. term ± 1 SD) such that $C_{EPS}$ is minimized, the lowest estimate of $C_{EPS}$ following NEM addition is 3,240 mg/L. In either case, this calculation assumes that, as soon as the electrophile is added to activated sludge, 80% of the cytoplasmic $K^+$ pool is instantaneously released from the bacteria into the EPS, before any $K^+$ diffuses to the bulk liquid. Given the observation of much faster rates of $K^+$ release to the bulk liquid for *S. capsulata* than for activated sludge, this assumption is reasonable. This calculation probably overestimates the actual $C_{EPS}$ considerably, but even if $C_{EPS}$ has been overestimated by an order of magnitude, the predicted $C_{EPS}$ is very similar to the 600-2000 mg/L KCl-$K^+$ range known to induce deflocculation (7,22).

Instead of assuming that activated sludge bacteria are similar to *S. capsulata*, $C_{EPS}$ can also be calculated based on the $K^+$ efflux response measured in activated sludge. For the 10 activated sludge experiments with 50 mg/L NEM, the average increase in the bulk liquid $K^+$ concentration was 7.4 ± 2.5 mg/L after 5 hours of contact with NEM. As expected, it was determined that the amount of $K^+$ efflux derived from 50 mg/L NEM varied slightly over time, but most critically depends on the MLSS concentration ($R^2 = 0.75$ and $p<0.01$). The calculation for $C_{EPS}$ can also be performed using the average activated sludge $K^+$ efflux value, if it is assumed that all of the

$K^+$ which ended up in bulk liquid (7.4 mg/L) was originally from the bacterial cytoplasm (in the activated sludge floc). It also must be assumed that the $K^+$ release into the floc structure EPS was instantaneous and was followed by diffusion of all of the $C_{EPS}$ to the bulk liquid. These calculations provide a $C_{EPS}$ of 12,500 mg/L and a minimum $C_{EPS}$ of 6,350 mg/L if the error associated with each parameter is applied.

## Conclusions

The results presented here represent convincing evidence that GGKE activity was observed in activated sludge due to shock loading of electrophilic chemicals and that $K^+$ efflux was responsible for floc deterioration. This evidence is based on multiple independent experiments that provide evidence in favor of the research hypothesis. Elucidation of deflocculation and other biological treatment process upset mechanisms may eventually lead to effective strategies for preventing treatment performance deterioration caused by shock loading of toxic chemicals. At this stage, it is reasonable to assume that toxic electrophilic chemicals activate the GGKE system in Gram-negative activated sludge microorganisms. It is also likely that the increase in bulk liquid $K^+$ is derived from GGKE-mediated transport of $K^+$ from the bacterial cytoplasm, into the floc structure, and finally diffusion into the bulk liquid. Based on the results presented in this paper, several specific observations can be stated:

1. *S. capsulata* and activated sludge exhibited very similar dynamic $K^+$ efflux/uptake/efflux responses when exposed to the electrophilic stressors, NEM or CDNB, and the thiol reductant, DTT. This suggests that GGKE activity may be present in activated sludge bacteria and that lysis was not a significant mechanisms of $K^+$ efflux.

2. It was observed that NEM stress resulted in much faster rates of $K^+$ release to the bulk liquid for *S. capsulata* than for activated sludge. It was speculated that diffusion limitations within the floc structure were responsible for this effect.

3. Nigericin addition caused $K^+$ efflux in *S. capsulata* that was not reversible by DTT addition. For activated sludge, nigericin resulted in nearly complete $K^+$ efflux to the bulk liquid, as compared to typical floc-associated measurements, and deflocculation comparable to that obtained with NEM. This indicates that $K^+$ was released from the cytoplasm of activated sludge bacteria and that the release of $K^+$ into the floc structure may be responsible for deflocculation.

4. Using fluorescent membrane permeable and impermeable nucleic acid stains, it was determined that cell lysis did not contribute significantly to $K^+$ efflux observed in response to electrophile addition.

5. Immediately after shock loading with toxic electrophilic chemicals, the estimated $K^+$ concentration in the floc structure EPS was found to be similar to that known to induce deflocculation by external KCl addition. This further suggests that GGKE activity results in a weakening of the floc that leads to deflocculation.

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## Appendix A. Data for Chapter 2 - THE IMMUNOCHEMICAL DETECTION OF STRESS PROTEINS IN ACTIVATED SLUDGE EXPOSED TO TOXIC CHEMICALS

Figure 1.
GroEL Western Blot (p54-57b2)

| Sample | Integrated Density Original Value | Integrated Density Value/μg Protein |
|---|---|---|
| Control | 24453 | 97812 |
| Heat Shock | 39273 | 157092 |
| 200 mg/L Chl | 24453 | 97812 |
| 132 mg/L Ace | 31122 | 124488 |
| 8 mg/L PCP | 34827 | 139308 |
| 5 mg/L Cd | 28899 | 115596 |

Constant Protein Loading = 0.25 μg/Lane

Figure 2.
SBR Stress Experiment - 25 mg/L $Cd^{2+}$ - operated for 16 cycles.
p88-92b1

| SBR Cycle Number | $Cd^{2+}$ VSS (mg/L) | Con VSS (mg/L) | $Cd^{2+}$ sCOD (mg/L) | $Cd^{2+}$ sCOD SD (mg/L) | Con sCOD (mg/L) | Con sCOD SD (mg/L) |
|---|---|---|---|---|---|---|
| 1 | 40.7 | 26.0 | 29.1 | 2.12 | 21.1 | 0.57 |
| 2 | 52.0 | 27.2 | 26.0 | 0.00 | 21.9 | 0.49 |
| 4 | 55.3 | 25.6 | 24.5 | 0.00 | 18.4 | 0.00 |
| 5 | 50.0 | 29.2 | 26.8 | 0.00 | 22.6 | 0.57 |
| 8 | 40.5 | 30.8 | 24.9 | 0.57 | 17.3 | 0.49 |
| 12 | 56.7 | 32.0 | 23.4 | 0.57 | 15.0 | 1.63 |
| 16 | 29.3 | 25.2 | 22.3 | 2.26 | 13.8 | 0.00 |

Figure 3.
GroEL Western Blot - 50 mg/L $Cd^{2+}$ - SBR Profile
p66-68b2

| Sample Time (minutes) | Integrated Density Original Value | | Integrated Density Value/µg Protein | |
|---|---|---|---|---|
| | Control | 50 mg/L $Cd^{2+}$ | Control | 50 mg/L $Cd^{2+}$ |
| 1 | 333.7 | 397.8 | 953 | 1137 |
| 8 | 543.2 | 1037.7 | 1552 | 2965 |
| 23 | 575.7 | 880.3 | 1645 | 2515 |
| 60 | 298.0 | 361.7 | 851 | 1033 |
| 180 | 658.9 | 841.1 | 1883 | 2403 |
| 300 | 698.4 | 814.8 | 1995 | 2328 |

Constant Protein Loading = 0.35 µg/Lane

Figure 4.
GroEL Western Blot - 5, 25, & 50 mg/L $Cd^{2+}$ - SBR Profiles
p58-71b2

| Sample Time (minutes) | Integrated Density Value/µg Protein Loaded | | | | | |
|---|---|---|---|---|---|---|
| | Control | 5 mg/L $Cd^{2+}$ | Control | 25 mg/L $Cd^{2+}$ | Control | 50 mg/L $Cd^{2+}$ |
| 1 | 1149 | 2487 | 761 | 1464 | 953 | 1137 |
| 8 | 1069 | 2015 | 950 | 1160 | 1552 | 2965 |
| 23 | 955 | 1338 | 831 | 1332 | 1645 | 2515 |
| 60 | 1053 | 1534 | 1497 | 1391 | 851 | 1033 |
| 180 | 635 | 910 | 1707 | 1654 | 1883 | 2403 |
| 300 | 379 | 1011 | 1961 | 1244 | 1995 | 2328 |

| Sample Time (minutes) | Average Control | Std. Dev. Control | Average $Cd^{2+}$ Stress | Std. Dev. $Cd^{2+}$ Stress | Stress/Con Ratio | Stress/Con Std. Dev. |
|---|---|---|---|---|---|---|
| 1 | 954 | 194 | 1696 | 705 | 1.777 | 0.463 |
| 8 | 1190 | 319 | 2047 | 903 | 1.719 | 0.516 |
| 23 | 1144 | 439 | 1728 | 682 | 1.511 | 0.550 |
| 60 | 1134 | 330 | 1320 | 258 | 1.164 | 0.351 |
| 180 | 1408 | 675 | 1656 | 747 | 1.176 | 0.658 |
| 300 | 1445 | 924 | 1528 | 703 | 1.057 | 0.787 |

Constant Protein Loading = 0.35 µg/Lane

# Appendix B. Data for Chapter 3. - A PHYSIOLOGICAL MECHANISM FOR ACTIVATED SLUDGE DEFLOCCULATION CAUSED BY SHOCK LOADS OF TOXIC ELECTROPHILIC CHEMICALS

Figure 3.
Deflocculation Screening (p72-82b3, p19-45b4)

| | | Ratio | Control VSS | | | Stressed VSS | | | % Incr. | VSS |
|---|---|---|---|---|---|---|---|---|---|---|
| IC50 | Conc | Conc/IC50 | 1 | 2 | Average | 1 | 2 | Average | VSS | Ratio |
| **Cd** | | | | | | | | | | |
| 111 | 5 | 0.045 | 15.2 | 18.0 | 16.6 | 19.0 | 19.0 | 19.0 | 12.6 | 1.145 |
| 111 | 25 | 0.225 | 15.2 | 18.0 | 16.6 | 32.7 | 32.0 | 32.4 | 48.7 | 1.949 |
| 111 | 25 | 0.225 | 7.8 | 9.2 | 8.5 | 46.5 | 48.8 | 47.7 | 82.2 | 5.606 |
| 111 | 50 - NEM | 0.450 | 17.1 | 17.4 | 17.3 | 54.0 | 49.0 | 51.5 | 66.5 | 2.986 |
| 111 | 50 - Chl | 0.450 | 17.4 | 18.6 | 18.0 | 49.0 | 47.0 | 48.0 | 62.5 | 2.667 |
| 111 | 50 - MeCl | 0.450 | 14.6 | 13.7 | 14.2 | 42.0 | 49.0 | 45.5 | 68.9 | 3.216 |
| 111 | 50 - IOAM | 0.450 | 17.7 | 17.1 | 17.4 | 49.2 | 51.5 | 50.4 | 65.4 | 2.894 |
| 111 | 50 - CDNB | 0.450 | 16.0 | 16.0 | 16.0 | 47.3 | 47.3 | 47.3 | 66.2 | 2.956 |
| 111 | 50 - CB | 0.450 | 16.0 | 17.1 | 16.6 | 45.0 | 46.0 | 45.5 | 63.6 | 2.749 |
| 111 | 50 - Anil | 0.450 | 19.6 | 17.2 | 18.4 | 53.0 | 50.0 | 51.5 | 64.3 | 2.799 |
| 111 | 50 - Benz | 0.450 | 14.0 | 14.0 | 14.0 | 41.0 | 41.0 | 41.0 | 65.9 | 2.929 |
| 111 | 50 - DNT | 0.450 | 12.0 | 11.2 | 11.6 | 43.0 | 45.0 | 44.0 | 73.6 | 3.793 |
| 111 | 50 - NT | 0.450 | 9.6 | 9.6 | 9.6 | 45.0 | 44.0 | 44.5 | 78.4 | 4.635 |
| 111 | 100 | 0.901 | 15.2 | 18.0 | 16.6 | 74.7 | 68.0 | 71.4 | 76.7 | 4.298 |
| **PCP** | | | | | | | | | | |
| 23.5 | 8 | 0.340 | 16.8 | 18.8 | 17.8 | 31.0 | 34.3 | 32.7 | 45.5 | 1.834 |
| 23.5 | 10 | 0.426 | 24.3 | 21.5 | 22.9 | 35.9 | 36.7 | 36.3 | 36.9 | 1.585 |
| 23.5 | 25 | 1.064 | 24.3 | 21.5 | 22.9 | 54.0 | 56.0 | 55.0 | 58.4 | 2.402 |
| 23.5 | 50 | 2.128 | 24.3 | 21.5 | 22.9 | 98.0 | 96.0 | 97.0 | 76.4 | 4.236 |
| 23.5 | 50 | 2.128 | 25.4 | 23.2 | 24.3 | 102.0 | 116.0 | 109.0 | 77.7 | 4.486 |
| 23.5 | 100 | 4.255 | 25.4 | 23.2 | 24.3 | 118.0 | 116.0 | 117.0 | 79.2 | 4.815 |
| 23.5 | 150 | 6.383 | 25.4 | 23.2 | 24.3 | 137.5 | 145.0 | 141.3 | 82.8 | 5.813 |
| **BQ** | | | | | | | | | | |
| 1.5 | 1 | 0.667 | 16.8 | 18.8 | 17.8 | 15.6 | 17.6 | 16.6 | -7.2 | 0.933 |
| 1.5 | 3 | 2.000 | 22.5 | 23.5 | 23.0 | 35.3 | 36.0 | 35.7 | 35.5 | 1.550 |
| 1.5 | 10 | 6.667 | 22.5 | 23.5 | 23.0 | 88.0 | 94.0 | 91.0 | 74.7 | 3.957 |
| 1.5 | 25 | 16.667 | 22.5 | 23.5 | 23.0 | 115.0 | 120.0 | 117.5 | 80.4 | 5.109 |
| **NEM** | | | | | | | | | | |
| 96.5 | 6.77 | 0.070 | 17.1 | 17.4 | 17.3 | 35.2 | 37.0 | 36.1 | 52.2 | 2.093 |
| 96.5 | 59.49 | 0.616 | 17.1 | 17.4 | 17.3 | 57.0 | 61.0 | 59.0 | 70.8 | 3.420 |
| 96.5 | 100.1 | 1.037 | 17.1 | 17.4 | 17.3 | 53.0 | 51.0 | 52.0 | 66.8 | 3.014 |
| 96.5 | 138.9 | 1.439 | 17.1 | 17.4 | 17.3 | 58.0 | 60.0 | 59.0 | 70.8 | 3.420 |
| 96.5 | 198.6 | 2.058 | 17.1 | 17.4 | 17.3 | 50.0 | 51.0 | 50.5 | 65.8 | 2.928 |
| 96.5 | 298.3 | 3.091 | 17.1 | 17.4 | 17.3 | 54.0 | 55.0 | 54.5 | 68.3 | 3.159 |
| **CDNB** | | | | | | | | | | |
| 218 | 4.37 | 0.020 | 16.0 | 16.0 | 16.0 | 24.5 | 26.0 | 25.3 | 36.6 | 1.578 |
| 218 | 20.3 | 0.093 | 16.0 | 16.0 | 16.0 | 40.0 | 43.3 | 41.7 | 61.6 | 2.603 |
| 218 | 49.9 | 0.229 | 16.0 | 16.0 | 16.0 | 46.7 | 48.3 | 47.5 | 66.3 | 2.969 |
| 218 | 104.2 | 0.478 | 16.0 | 16.0 | 16.0 | 55.8 | 59.2 | 57.5 | 72.2 | 3.594 |
| 218 | 213.3 | 0.978 | 16.0 | 16.0 | 16.0 | 54.0 | 53.0 | 53.5 | 70.1 | 3.344 |
| 218 | 389.9 | 1.789 | 16.0 | 16.0 | 16.0 | 52.0 | 56.2 | 54.1 | 70.4 | 3.381 |
| **Benzene** | | | | | | | | | | |
| 290.6 | 20 | 0.069 | 14.0 | 14.0 | 14.0 | 13.6 | 12.8 | 13.2 | -6.1 | 0.943 |
| 290.6 | 100 | 0.344 | 14.0 | 14.0 | 14.0 | 16.0 | 16.4 | 16.2 | 13.6 | 1.157 |
| 290.6 | 200 | 0.688 | 14.0 | 14.0 | 14.0 | 13.6 | 14.8 | 14.2 | 1.4 | 1.014 |
| 290.6 | 300 | 1.032 | 14.0 | 14.0 | 14.0 | 14.0 | 15.2 | 14.6 | 4.1 | 1.043 |
| 290.6 | 600 | 2.065 | 14.0 | 14.0 | 14.0 | 14.4 | 13.6 | 14.0 | 0.0 | 1.000 |
| 290.6 | 1000 | 3.441 | 14.0 | 14.0 | 14.0 | 12.4 | 12.0 | 12.2 | -14.8 | 0.871 |

Figure 4.
SBR Cation Flux - 50 mg/LNEM & 50 mg/L CDNB (p89-97b4, p17-21b5)

| SBR Sample | Sample Time | Soluble $K^+$ (mg/L) | Soluble $K^+$ SD (mg/L) | FA $K^+$ (mg/L) | FA $K^+$ SD (mg/L) | Calc. Tot. $K^+$ (mg/L) | Calc. Tot. $K^+$ SD (mg/L) | Meas. Tot. $K^+$ (mg/L) | Meas. Tot. $K^+$ SD (mg/L) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 0 | 12.0 | 0.35 | 14.2 | 0.59 | 26.2 | 0.94 | 25.8 | 0.29 |
| Control | 5 hours | 11.9 | 0.20 | 12.9 | 0.14 | 24.8 | 0.34 | 25.6 | 0.54 |
| NEM | 0 | 11.7 | 0.00 | 13.9 | 0.06 | 25.6 | 0.00 | 25.1 | 0.13 |
| NEM | ~ 3 min | 13.9 | 0.54 | 12.1 | 0.22 | 26.1 | 0.32 | 24.6 | 0.69 |
| NEM | 5 hours | 20.7 | 0.46 | 5.2 | 0.52 | 25.8 | 0.06 | 25.5 | 0.71 |
| Control | 0 | 11.2 | 0.33 | 17.4 | 1.95 | 28.6 | 1.62 | 30.2 | 3.10 |
| Control | 5 hours | 11.5 | 0.07 | 20.4 | 0.07 | 31.9 | 0.01 | 33.4 | 0.50 |
| CDNB | 0 | 11.3 | 0.16 | 18.2 | 0.58 | 29.4 | 0.42 | 31.5 | 1.86 |
| CDNB | ~ 3 min | 15.8 | 0.00 | 13.8 | 1.62 | 29.6 | 1.62 | 31.2 | 0.67 |
| CDNB | 5 hours | 21.3 | 0.45 | 9.2 | 0.20 | 30.5 | 0.65 | 32.0 | 0.72 |

Figure 5.
SBR Cation Flux - NEM & CDNB (p2-21b5)

| NEM (mg/L) | Effl. VSS Ratio | Effl. VSS Ratio SD | Soluble $K^+$ Effluxed (mg/L) | Soluble $K^+$ SD (mg/L) |
|---|---|---|---|---|
| 2 | 1.01 | 0.02 | 0.57 | 0.17 |
| 10 | 2.42 | 0.14 | 2.81 | 0.27 |
| 25 | 2.86 | 0.03 | 3.94 | 0.38 |
| 50 | 9.00 | 0.91 | 7.02 | 0.78 |
| 150 | 7.73 | 0.82 | 7.29 | 0.66 |
| 300 | 7.91 | 0.76 | 7.92 | 0.56 |
| CDNB (mg/L) | Effl. VSS Ratio | Effl. VSS Ratio SD | Soluble $K^+$ Effluxed (mg/L) | Soluble $K^+$ SD (mg/L) |
| 3.00 | 0.9 | 0.13 | 0.7 | 0.14 |
| 50.00 | 5.9 | 0.53 | 9.8 | 0.45 |
| 200.00 | 9.6 | 0.81 | 12.1 | 0.40 |

Figure 6.
SBR Cation Flux Profile - 50 mg/L NEM (p35-45b5)

| Sample Time (min) | Control Sol $K^+$ (mg/L) | Control SD (mg/L) | NEM Sol $K^+$ (mg/L) | NEM SD (mg/L) | Control VSS (mg/L) | Control SD (mg/L) | NEM VSS (mg/L) | NEM SD (mg/L) |
|---|---|---|---|---|---|---|---|---|
| 0 | 12.8 | 0.38 | 12.3 | 0.24 | | | | |
| 3 | 12.8 | 0.38 | 15.9 | 0.03 | | | | |
| 10 | | | | | 8.1 | 0.18 | 11.0 | 0.28 |
| 19 | 12.0 | 0.38 | 17.2 | 0.03 | | | | |
| 40 | 11.9 | 0.20 | 18.3 | 0.32 | | | | |
| 60 | | | | | 6.6 | 0.11 | 23.9 | 0.18 |
| 120 | 12.1 | 0.08 | 19.8 | 0.14 | 6.0 | 0.35 | 44.8 | 0.00 |
| 300 | 12.4 | 0.32 | 21.5 | 0.79 | | | | |
| 345 | 12.7 | 0.17 | 22.4 | 0.06 | 5.8 | 0.28 | 57.5 | 2.83 |

# Appendix C. Data for Chapter 4. - IMPLICATING THE GLUTATHIONE-GATED POTASSIUM EFFLUX SYSTEM AS A CAUSE OF ACTIVATED SLUDGE DEFLOCCULATION IN RESPONSE TO SHOCK LOADS OF TOXIC ELECTROPHILIC CHEMICALS

Figure 1.

NEM/DTT/NEM - *S. capsulata* - IC Soluble $K^+$, ISE Soluble $K^+$, ICP CA $K^+$ (p39-52b7)

First NEM addition - 26.2 min
DTT addition - 50.2 min
Second NEM addition - 78.0 min

| A. | IC Soluble $K^+$ Concentration (mg/L) | | | |
|---|---|---|---|---|
| Sample | Con/DTT/NEM | | NEM/DTT/NEM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. |
| 11 | 54.8 | 0.90 | 54.8 | 0.89 |
| 30 | 55.8 | 0.68 | 61.3 | 0.26 |
| 47 | 56.7 | 0.56 | 66.4 | 0.38 |
| 54 | 56.2 | 0.24 | 64.3 | 0.36 |
| 75 | 56.0 | 0.74 | 58.8 | 0.69 |
| 104 | 74.4 | 0.20 | 71.1 | 2.13 |

| B. | ICP Cell-Associated $K^+$ Concentration (mg/L) | | | |
|---|---|---|---|---|
| Sample | Con/DTT/NEM | | NEM/DTT/NEM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. |
| 11 | 22.9 | 0.39 | 24.6 | 1.67 |
| 30 | 24.5 | 0.42 | 13.9 | 0.36 |
| 47 | 23.3 | 0.02 | 9.21 | 0.62 |
| 54 | 22.5 | 0.95 | 11.8 | 0.31 |
| 75 | 23.4 | 0.62 | 16.6 | 0.02 |
| 104 | 5.33 | 0.20 | 5.25 | 0.05 |

Figure 2.
NEM/DTT/NEM - Activated Sludge SBR
IC Soluble $K^+$, Effluent VSS, HPLC NEM (p24-38b7)

| SBR Code | NEM Addition | DTT Addition | NEM Addition |
|---|---|---|---|
| Control | 0 | 0 | 0 |
| NEM/DTT/NEM | 100 mg/L | 370 mg/L | 600 mg/L |
| NEM/DTT | 100 mg/L | 370 mg/L | 0 |
| NEM | 100 mg/L | 0 | 0 |
| Time (min) = | 6 | 46 | 124 |

| | IC Soluble Bulk Liquid $K^+$ Concentration (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Control | | NEM/DTT/NEM | | NEM/DTT | | NEM | |
| Time (minutes) | Average | Std. Dev. | Average | Std. Dev. | Average | Std. Dev. | Average | Std. Dev. |
| 0 | 8.08 | 0.05 | 8.24 | 0.08 | 8.21 | 0.01 | 8.21 | 0.01 |
| 9 | 7.97 | 0.24 | 9.67 | 0.12 | 9.83 | 0.11 | 9.67 | 0.03 |
| 28 | 8.21 | 0.05 | 11.7 | 0.08 | 11.5 | 0.00 | 11.7 | 0.02 |
| 49 | 8.20 | 0.03 | 11.9 | 0.00 | 11.7 | 0.06 | 12.5 | 0.03 |
| 105 | 8.51 | 0.06 | 11.3 | 0.05 | 11.4 | 0.02 | 13.3 | 0.13 |
| 127 | 8.26 | 0.04 | 12.2 | 0.03 | 11.2 | 0.00 | 13.5 | 0.11 |
| 300 | 8.96 | 0.03 | 13.7 | 0.10 | 11.1 | 0.03 | 14.4 | 0.07 |

Figure 3.
NEM/DTT/NEM - Activated Sludge SBR
IC Soluble $K^+$, ISE Soluble $K^+$ (p53-58b7)

First NEM addition - 16.3 min
DTT addition - 45.6 min
Second NEM addition - 86.0 min

| | IC Soluble $K^+$ Concentration (mg/L) | | | |
|---|---|---|---|---|
| Sample | Con/DTT/NEM | | NEM/DTT/NEM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. |
| 0 | 8.94 | 0.16 | 8.91 | 0.02 |
| 18 | 9.38 | 0.29 | 11.8 | 0.21 |
| 36 | 10.2 | 0.75 | 13.2 | 0.17 |
| 47 | 10.0 | 0.22 | 13.6 | 0.08 |
| 75.5 | 10.2 | 0.47 | 12.7 | 0.19 |
| 88.5 | 12.6 | 0.19 | 14.2 | 0.36 |
| 123.5 | 16.5 | 0.61 | 15.9 | 0.24 |

Figure 4.

CDNB/DTT/CDNB - Activated Sludge SBR
IC Soluble $K^+$, ISE Soluble $K^+$ (p53-58b7)

First CDNB addition - 13.2 min
DTT addition - 42.1 min
Second CDNB addition - 76.0 min

| | IC Soluble $K^+$ Concentration (mg/L) | | | |
|---|---|---|---|---|
| Sample | Con/DTT/CDNB | | CDNB/DTT/CDNB | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. |
| 2 | 9.12 | 0.27 | 8.47 | 0.68 |
| 15.5 | 9.46 | 0.23 | 12.9 | 1.02 |
| 35 | 9.51 | 0.19 | 14.4 | 0.29 |
| 44 | 10.0 | 0.12 | 15.1 | 0.14 |
| 63 | 9.60 | 0.29 | 15.5 | 0.00 |
| 78 | 12.0 | 0.74 | 15.1 | 0.21 |
| 91.7 | 15.1 | 0.03 | 15.4 | 0.46 |

Figure 5.

Nigericin - *S. capsulata* - IC Soluble $K^+$ (p76-81b7)
Nigericin or NEM with DTT Addition and NEM again

Nig or NEM addition - 18.0 min
DTT addition - 48.5 min
Second NEM addition - 98.0 min

| | IC Soluble $K^+$ Concentration (mg/L) | | | |
|---|---|---|---|---|
| Sample | Nig/DTT/NEM | | NEM/DTT/NEM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. |
| 7 | 51.5 | 0.43 | 51.6 | 0.01 |
| 20 | 59.4 | 0.15 | 57.9 | 0.03 |
| 39 | 63.5 | 0.22 | 66.7 | 0.04 |
| 50.5 | 61.8 | 0.64 | 65.8 | 0.11 |
| 87.5 | 56.4 | 0.48 | 67.2 | 0.05 |
| 100 | 62.0 | 0.02 | 68.2 | 0.22 |
| 125 | 68.0 | 0.07 | 69.1 | 0.10 |

Figure 6.
NEM 50 mg/L or Nigericin Addition - Activated Sludge SBR
IC Soluble $K^+$, Effluent NTU (p6-12b8) NEM or Nig addition - 14 min

| | IC Soluble Bulk Liquid $K^+$ Concentration (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Control | | NEM 50 mg/L | | Nigercin 1.0 μM | | Nigercin 5.0 μM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. | Average | Std. Dev. | Average | Std. Dev. |
| 0 | 6.66 | 0.34 | 5.71 | 0.09 | 6.45 | 0.09 | 6.63 | 0.27 |
| 17 | 6.54 | 0.08 | 8.92 | 0.19 | 11.5 | 0.29 | 12.4 | 0.55 |
| 32 | 6.35 | 0.03 | 10.6 | 0.72 | 11.8 | 0.14 | 14.2 | 0.40 |
| 50 | 6.31 | 0.08 | 10.6 | 0.04 | 12.1 | 0.03 | 13.6 | 0.00 |
| 77 | 6.10 | 0.11 | 11.5 | 0.12 | 12.2 | 0.09 | 13.8 | 0.25 |
| 197 | 6.22 | 0.28 | 12.4 | 0.03 | 12.1 | 0.09 | 14.1 | 0.08 |
| 317 | 6.47 | 0.06 | 13.3 | 0.02 | 12.7 | 0.08 | 13.2 | 0.07 |

| | IC Soluble Bulk Liquid $K^+$ Concentration (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| Sample | Nigercin 10 μM | | Nigercin 50 μM | | Nigercin 100 μM | |
| Time (min) | Average | Std. Dev. | Average | Std. Dev. | Average | Std. Dev. |
| 0 | 6.23 | 0.10 | 6.66 | 0.24 | 6.53 | 0.58 |
| 17 | 13.2 | 0.36 | 14.1 | 0.43 | 16.0 | 0.96 |
| 32 | 14.0 | 0.35 | 15.4 | 0.19 | 15.5 | 0.11 |
| 50 | 14.7 | 0.03 | 15.2 | 0.03 | 16.1 | 0.12 |
| 77 | 14.5 | 0.35 | 14.9 | 0.30 | 17.2 | 0.22 |
| 197 | 14.8 | 0.28 | 16.1 | 0.12 | 16.8 | 0.05 |
| 317 | 14.5 | 0.00 | 16.3 | 0.10 | 17.4 | 0.01 |

Figure 7.
NEM 50 mg/L or Nigericin Addition - Activated Sludge SBR
IC Soluble $K^+$, Effluent NTU (p6-12b8)
NEM or Nig addition - 14 min

| SBR | Nig or NEM | Effluent Turbidity (NTU) | |
|---|---|---|---|
| Name | Added | Average | Std. Dev. |
| Control | | 9.50 | 0.10 |
| NEM | 50 mg/L | 56.5 | 0.29 |
| Nig1 | 1.0 μM | 18.4 | 0.31 |
| Nig2 | 5.0 μM | 27.0 | 0.35 |
| Nig3 | 10 μM | 26.4 | 0.14 |
| Nig4 | 50 μM | 50.9 | 0.41 |
| Nig5 | 100 μM | 55.5 | 0.44 |

# CHARLES B. BOTT

VITA

Charles Bott is a native of the Eastern Shore of Virginia and was raised on Nassawadox Creek, a tributary of the Chesapeake Bay. He became interested in environmental engineering and related topics because of issues associated with the environmental protection and remediation of the Chesapeake Bay and surrounding watershed. Charles' undergraduate studies were completed at the Virginia Military Institute, and he received a B.S. in Civil Engineering in 1996 with specialization in Environmental Engineering. During summer furloughs, Charles worked for Combined Technologies, Inc., a geotechnical/geo-environmental consulting and engineering firm in Richmond, Virginia now owned by McKinney and Company, Ashland, Virginia. Charles attended the Johns Hopkins University for his Masters degree and received a M.S.E. in Environmental Engineering in 1997. Charles initiated his Ph.D. work in the Fall of 1997 in the Department of Civil and Environmental Engineering at Virginia Tech. The emphasis of his Ph.D. education was water and wastewater treatment engineering, and the research conducted at Virginia Tech focused on industrial and domestic biological wastewater treatment processes. Charles defended this dissertation on April 4, 2001.