Evaluating the role of microbial stress response mechanisms in causing biological treatment system upset

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Abstract It is known that microbial stress mechanisms play a significant role in short-term microbial adaptation to environmental perturbations, and activation of these mechanisms enhance a cell’s chance for surviving the perturbation with minimal damage. Although the target of these mechanisms is protective at the cellular level, the effect may be disruptive at the macroscopic level in engineered bioreactor systems. In this paper, it is proposed that these mechanisms are activated in response to wastewater influent perturbations and may be a significant cause of activated sludge treatment process upset. Selected microbial stress responses are reviewed and hypotheses indicating their potential role in treatment process upset are proposed. A research approach that was previously used to identify the mechanistic cause of deflocculation during perturbation by electrophilic chemicals is summarized, and a protocol for future experiments geared toward establishing source-cause-effect relationships for a range of wastewater upset conditions is put forth. Identifying source-cause-effect relationships will provide a basis for development of new monitoring technologies and operational strategies for systems under the influence of influent chemical perturbations.

Keywords Glutathione; GroEL; potassium efflux; source-cause-effect; stress response

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 toxic discharges. Changes in wastewater composition can impose a range of process responses. Those responses 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 BOD₅ removal, disrupted nitrification, and deflocculation of activated sludges (Love and Bott, 2000). 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) (Love and Bott, 2000). 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. Microscopic- and molecular-level mechanisms are adaptive responses that occur in response to chemical perturbations in cells. These mechanisms activate a range of functions to protect cells from toxicity. Some important stress responses occur over long time periods, but our focus is on rapid responses that are activated immediately or within a few seconds of initiation of the perturbation event. We believe that approaches are needed to evaluate the degree to which these
rapid microscopic- and molecular-level mechanisms contribute to adverse macroscopic process upsets in order to establish source-cause-effect relationships.

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 esters 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. We have 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 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.

This paper includes a review of microbial stress mechanisms that may affect activated sludge process performance, and summarizes an experimental approach that we used to establish a source-cause-effect relationship for activated sludge communities exposed to sublethal shock loads of electrophilic chemicals. We propose a protocol for future source-cause-effect relationships, and are currently using this approach to evaluate a broader range of source conditions, so that a source-cause-effect matrix can be developed.

**Microbial stress responses**

It has been well established that bacterial cells can activate stress responses that function either by protecting cells against damage (protective stress responses) or repairing cells after damage has occurred (remedial stress responses) (Hightower, 1998). 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 days 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 system induction). Consequently, the relative importance of stress responses to sudden influxes of stressors on process performance remains largely unstudied. Below, we focus on three microbial stress responses (two protective, one remedial) that we believe may have an impact on activated sludge process performance. These stress responses are summarized schematically in Figure 1.
Protective stress responses

Adaptation of the cytoplasmic membrane. The bacterial cytoplasmic membrane serves as an important protective barrier between the cytoplasm and the outside environment. Nutrients pass through this membrane in a regulated manner to sustain cell viability; however, it is known that lipophilic solvents (e.g. aromatic hydrocarbons) can dissolve into the membrane and cause serious damage by (i) allowing cytoplasmic ions and macromolecules to pass outside of the cell, (ii) dissipating the proton and electrochemical gradients, and/or (iii) altering the function of membrane-bound proteins (Sikkema et al., 1995). Some bacteria can activate long-term adaptive changes that make the membrane less permeable to solvents by changing the saturated:unsaturated fatty acid ratio in the membrane (Ingram, 1977; Segura et al., 1999), changing phospholipid headgroup composition (Weber and de Bont, 1996), or altering the rate of phospholipid biosynthesis (Pinkart and White, 1997). It is the rapid responses, however, that provide bacteria with protection in the critical first moments after being exposed to a toxic level of solvent. One such response that is known to be present in some solvent-tolerant bacteria is the isomerization of cis fatty acids to the trans form, which allows membrane fatty acid molecules to pack more tightly and membrane fluidity to decrease (Heippeper et al., 1992). In Pseudomonas putida DOT-T1, the responsible isomerase enzyme has been shown to be constitutively expressed, thus resulting in rapid responses in cells exposed to chemical perturbations (Segura et al., 1999). It has also been observed that many strains capable of activating membrane-adaptive responses to solvent stress are not capable of using the solvent as an energy or carbon source (Segura et al., 1999), thereby suggesting that catabolically incapable cells may be more likely to evolve rapid “first line of defense” protective stress responses than those that can catabolize a problematic toxin.

Efflux systems. 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 or influence cytoplasmic chemistry by removing selected cations or changing pH. A broad range of functions exist for efflux systems; however, we will focus here on those that respond to electrophilic chemicals (both organic chemicals and toxic heavy metals).

An efflux pump mechanism ubiquitous in Gram negative bacteria that protects cytoplasmic macromolecules from electrophilic (oxidative) stressors involves a cysteine-containing tripeptide called glutathione. Glutathione plays an important role in gating (or activating) K⁺ efflux (Elmore et al., 1990) (hereafter called glutathione-gated K⁺ efflux, GGKE), and is schematically described in Figure 2. The sulfhydryl substituent of reduced glutathione (GSH) is susceptible to spontaneous (nonenzymatic) electrophilic attack by many oxidants (Apontoweil and Berends, 1975a,b). A constitutive or inducible enzyme known as glutathione-S-transferase may also be present to catalytically enhance the conjugation of toxic electrophiles with GSH. 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. Researchers were able to measure significant acidification of bacterial cytoplasms synchronous with K⁺ efflux (Ferguson et al., 1995), which is consistent with potassium’s role as the major cation regulating intracellular pH (Booth, 1985) and osmotic pressure (Epstein, 1986) in Gram negative bacteria. Cytoplasmic acidification may be the mechanism that affords the cell protection from electrophilic stress (Ferguson et al., 1993, 1995, 1997). A baseline amount of glutathione is always present in Gram negative cells whether or not a stressor is present; therefore, Gram-negative cells are always poised and ready to protect against electrophilic shocks. However, differential expression of the glutathione synthesis machinery has been observed in response to oxidative stress conditions (Carmel-Harel and Storz, 2000). GGKE is not known to depend upon ATP or NADH; however, proton influx occurs as K⁺ effluxes in order to maintain a charge balance across the membrane, thereby dissipating the potential for ATP formation.

**Figure 2** 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 molecules like Dps (Ferguson et al., 1998).
Remedial stress responses

It has been well established that 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. Stress proteins are believed to help cells recover from damage upon exposure to a wide variety of stressors, such as heat (Spector et al., 1986; VanBogelen et al., 1987), starvation (Groat et al., 1986), anaerobiosis (Spector et al., 1986) and oxidative (VanBogelen et al., 1987) conditions. 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 (Georgopoulos and Welch, 1993). 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 (Keen and Jacoby, 1980; Field and Thurman, 1996); despite this broad stress-related functionality, they remain largely unstudied in bacterial systems (Vuilleumier, 1997). In addition, studies have shown that many proteins are rapidly generated in pure cultures of Escherichia coli (Blom et al., 1992; Dukan et al., 1996) and Pseudomonas putida (Lupi et al., 1995) 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.

Potential links between molecular stress responses and macroscopic indicators of process performance

Little work has been done to determine the link between molecular stress responses and macroscopic indicators of activated sludge process performance. However, hypotheses are presented that suggest how each of the stress responses discussed above might interfere with activated sludge systems.

1. Rapid cis-trans isomerization of cell membranes may adversely impact wastewater treatment processes receiving a solvent perturbation by:
   a) decreasing the rate of non-solvent substrate (active) transport through the membrane due to the decrease in membrane fluidity;
   b) changing the surface chemistry of the membrane, thereby altering the linkage between cells and extracellular polymers (ultimately, this might affect flocculation);
   c) altering the rate at which extracellular hydrolytic enzymes are transported out of the cell, thereby changing the rate at which particulate and colloidal material is biodegraded.

2. Activation of the GGKE system in flocculant bacteria may adversely impact wastewater treatment processes receiving sublethal shocks of electrophilic chemicals by causing a localized increase in K⁺ concentrations inside of flocs, thereby increasing the monovalent to divalent cation ratio. This could lead to weakened floc structure and, ultimately, deflocculation.

3. Up-regulation of stress proteins requires energy, and may lead to a reduction in BOD removal capacity in biological treatment processes due to temporary redirection of energy away from growth and towards protein biosynthesis.

   We don’t claim that all possible hypotheses are captured by this list, and other hypotheses (some, potentially conflicting) may also be justified.

   In 1995, we initiated studies to test hypothesis 3 and eventually moved toward testing hypothesis 2. The results from those experiments have been published elsewhere and are
summarized below. Based on our experience, however, we propose an approach for subsequent studies that will allow us to more fully test our global hypothesis, which states that microbial stress responses are significant causes that result in activated sludge process upset due to perturbations of sublethal levels of chemical stressors.

Evaluating source-cause-effect relationships

Summary of previous results

We initiated our evaluation of hypothesis 3 by developing a method for detecting a universal stress protein (GroEL, a member of the Hsp60 family of heat shock proteins) in activated sludge (Duncan et al., 2000). Such development was necessary because commercial antisera that were available at the time did not cross react with activated sludge protein extracts. This stress protein is known to be up-regulated in response to a broad range of chemical stressors (Blom et al., 1992) and can function during anaerobic metabolism (Spector et al., 1986), although it is not expressed in Archaea (Macario et al., 1999). We tested the expression of this stress protein in activated sludge communities fed primary effluent plus cadmium or pentachlorophenol (no chemical stressor in controls), and compared GroEL expression to macromolecular indicators of process performance, such as effluent soluble COD, effluent turbidity, and sludge volume index (Bott and Love, 2001). Up-regulation of GroEL was detected at the lowest chemical stressor concentrations tested, but process performance deterioration was not detected until stressor concentrations at least 5 times that level were applied. Although we anticipated that the process performance effect of stress protein induction would yield poorer COD removal, this effect was found to be minimal, whereas deflocculation was quite significant. In summary, GroEL was a very sensitive indicator of physiological stress in response to a chemical stressor, but up-regulation of this stress protein did not explain mechanistically why the main process effect, deflocculation, occurred.

Based on these results, we reevaluated the potential causal mechanisms and developed hypothesis number 2. Since both cadmium and pentachlorophenol are electrophilic, the GGKE mechanism seemed reasonable. To avoid conflicts associated with using charged chemical stressors, we tested a range of uncharged electrophilic chemicals and found that sublethal concentrations of these chemicals also caused deflocculation. Results with shock loads of electrophilic stressors to date have confirmed that deflocculation correlates with significant K⁺ efflux from activated sludge flocs into the bulk liquid phase, and concomitant loss of K⁺ from the flocs (Bott and Love, submitted). The effect is reversible for electrophiles that react spontaneously with glutathione (i.e. K⁺ uptake can be activated by adding a reductant, followed by re-efflux upon addition of an electrophilic chemical), indicating that the effect is not due to cell lysis. In summary, sudden increases in K⁺ in the bulk liquid of mixed liquors could indicate the presence of an electrophilic stressor and impending deflocculation. These data are in strong support of our hypothesis, and provide a good example of how understanding a biochemical/physical/chemical cause may lead to the development of helpful monitoring and operational strategies for identifying and dealing with electrophilic chemicals in wastewaters.

Based on our research experience, we propose a protocol that we think will most effectively allow for the development of other source-cause-effect relationships that are significant. The protocol is schematically shown in Figure 3. Experiments have been initiated to screen 8 distinct upset sources and determine the source-effect relationship. These experiments are on-going.

Conclusion

The wastewater treatment industry has emphasized the need for development of cost-
effective and user-friendly early warning devices that can help treatment plant operators detect toxins in wastewaters so that appropriate control actions can be implemented and environmental damage can be avoided (Love and Bott, 2000). Little work has been done to establish these relationships, yet understanding source-cause-effect relationships will lead to the development of useful, new technologies. This understanding will also lead to the development of corrective action strategies that can be integrated into decision support systems for optimization of process operation and design. These needs justify further studies of adaptive microbial stress responses.

References


