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IMPACT OF SODIUM TUNGSTATE AND TUNGSTEN ALLOYS ON THE GROWTH OF SELECTED MICROORGANISMS WITH ENVIRONMENTAL SIGNIFICANCE

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Tungsten is a transition metal with unique properties that permit its use in a range of applications, including household products and small caliber ammunition. Increased use has restored interest in tungsten and tungsten-based products in determining not only their impacts upon human health, but also on the environment. Given the dependence on microbes for environmental processes, it is appropriate to evaluate tungsten effect on microorganisms. The goal of this study was to investigate the impact of sodium tungstate (Na_2WO_4) and tungsten alloys on the growth of selected environmental microorganisms that play roles in metal reduction, biogeochemical cycling and biodegradation. A soil community was also evaluated for its tolerance to Na_2WO_4. *Shewanella*, a strong metal reducer, displayed the most robust ability to grow in the highest concentrations of Na_2WO_4 evaluated, whereas *Pseudomonas* displayed lower tolerances. Interestingly, bacteria cultivated from the environment displayed only minor delays and reduction in growth relative to pure cultures, suggesting that such a microbial consortium is better suited to cope with Na_2WO_4 exposure. Tungsten alloys also had profound effects on bacterial growth, however, these were dependent on the metals and nutrients present, suggesting the effect may be exacerbated in certain environmental settings where nutrients may be limited.

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Tungsten, sodium tungstate, microbial growth, environmental microbiology, bacteria, *Shewanella*, *Pseudomonas*

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Abstract

Tungsten is a transition metal with unique properties that permit its use in a wide range of applications, including household products and small caliber ammunition. Increased use, and therefore exposure, has restored interest in tungsten and tungsten-based products in determining not only their impacts upon human health, but also on the environment. Given the critical dependence on microbes for many environmental processes, it is appropriate to evaluate the effect of tungsten on microorganisms. The goal of this study was to investigate the impact of sodium tungstate (Na_2WO_4) and tungsten alloys (W-Ni-Co and W-Ni-Fe) on the growth of a select group of environmental microorganisms that play key roles in metal reduction, biogeochemical cycling and biodegradation. In addition, a soil community was also evaluated for its tolerance to Na_2WO_4. *Shewanella*, a strong metal reducer, displayed the most robust ability to grow even in the highest concentrations of Na_2WO_4 evaluated (250 mM), whereas *Pseudomonas* displayed much lower tolerances. Interestingly, bacteria cultivated directly from the environment displayed only minor delays and reduction in growth relative to the pure cultures, suggesting that such a microbial consortium is better suited to cope with sodium tungsten exposure. Tungsten alloys tested also had profound effects on bacterial growth, however, these were highly dependent on the metals and nutrients present, suggesting the effect may be exacerbated in certain environmental settings where nutrients may be limited. It is unclear whether bacteria can develop or enhance tolerance to tungsten with slow and increased exposure.

Keywords: Tungsten, sodium tungstate, microbial growth, environmental microbiology, bacteria, *Shewanella*, *Pseudomonas*

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Introduction

Tungsten is a transition metal with unique properties that permit its use in a wide range of applications. It has extraordinary chemical and physical properties, including the highest melting point of all elements (excluding carbon) and the lowest vapor pressure and expansion coefficient of all metals. Tungsten also displays high density, thermal and electrical conductivity (Koutsospyros et al., 2006). It has only limited chemical activity and is considered to be non-reactive with strong mineral acids and water. These properties allow tungsten to be highly desirable for applications. The many extensive uses span from household items including television sets and lighting filaments/coils to more specialized products such as X-ray tubes and turbine blades (Strigul et al., 2005).

In the mid 1990s, due to increased concerns regarding the environmental impacts of lead and depleted uranium, the military began to employ tungsten in military applications as an alternative to these elements. As part of the U.S. Army pollution prevention initiative, The Green Armament Technology (GAT) program called for the replacement of lead with tungsten in small caliber ammunition and replacement of depleted uranium in kinetic energy penetrators with tungsten often occurring in alloys comprised of tungsten-nickel-cobalt or tungsten-nickel-iron. These initiatives, in addition to the recent finding of tungsten in groundwater (Seiler et al., 2005), have restored interest in tungsten and tungsten-based products in determining not only their impacts upon human health, but also on the environment. Despite its widespread use, the biological and chemical implications of tungsten and tungsten-containing compounds on the environment are not well understood (Tajima, 2001; Koutsospyros et al., 2006). Given that microbes play key roles in environmental processes from biogeochemical cycling to biodegradation and bioremediation, one aspect that is important to evaluate is the effect of tungsten on microorganisms.

The geochemistry of dissolved tungsten species in environmental settings is complicated. Tungsten occurs in surface soils at concentrations ranging from 0.68 to 2.7 mg kg^{-1}

(Senesi et al., 1988; Senesi et al., 1999). However, it exists in nature primarily as the tungstate anion and is generally considered to be thermodynamically stable (Gustafsson, 2003; Seiler et al., 2005; Koutsospyros et al., 2006). Tungsten is able to polymerize with itself and other anions thereby generating poly- and heteropoly-tungstates with variable geochemical and toxicological properties (Seiler et al., 2005). While many of the element's chemical properties are well defined, a better understanding of tungsten speciation and geochemistry is vital to discerning its sorption to soil, mobility, bioavailability, and toxicity in environmental systems.

The effect of tungsten on microorganisms and microbial communities is poorly defined. There are many potential mechanisms by which tungsten may physiologically impact microorganisms either directly or indirectly. Studies suggest that tungsten may lower soil pH in the environment, thereby altering growth and activity (Dermatas et al., 2004). On a more molecular level, tungsten oxidation to poly-tungstate may block phosphate regulated biochemical process and alter metabolic pathways. Tungsten may also replace molybdenum at enzymatic catalytic centers, thereby deactivating key enzymes, including nitrogenase and nitrate reductase (Kletzin and Adams, 1996). Such deactivation would interfere with microbial nitrogen fixation and denitrification activities, thereby disrupting these critical processes. Substitution of tungsten for molybdenum in catalytic centers may also affect other enzymes including perchlorate reductase, a critical enzyme in the conversion of perchlorate to chloride (Bender et al., 2005). Interference in such pathways highlights the potential of tungsten to not only disrupt environmental microbial communities, but also greatly hamper bioremediation efforts.

Limited reports of tungsten's effects on individual species of bacteria suggest the inability to survive in the presence of even low levels of tungsten/tungstate anion (Sugio et al., 2001; Strigul et al., 2005). However, observations at community levels imply increases in biomass and function with shifts in microbial community structure (Ringelberg et al., 2009). The goal of this study was to investigate the impact of sodium tungstate (Na_2WO_4) and tungsten

alloys (W-Ni-Co and W-Ni-Fe) on the growth of a select group of environmental microorganisms that play key roles in metal reduction, biogeochemical cycling and biodegradation. In addition, a soil community was also evaluated for its ability, as a whole, to withstand increased concentrations of Na_2WO_4.

Materials and Methods

Bacterial strains and media

Shewanella oneidensis MR-1 (ATCC 700550) was obtained from American Type Culture Collection (ATCC, Manassas, VA). *S. oneidensis* is a Gram-negative bacillus primarily found in deep sea anaerobic habitats, but also resides in soils and sediments. It grows both aerobically and anaerobically and displays a broad range of electron acceptors and therefore plays an important role in metal reduction and biogeochemical cycles (Fredrickson et al., 2008). *S. oneidensis* was propagated aerobically in Difco nutrient broth (pH 6.8; BD, Franklin Lakes, NJ) at 30°C.

Pseudomonas putida KT2440 was a gift of Dr. Daniella Regenhardt (German Research Center for Biotechnology, Braunschweig, Germany). *P. putida* is a Gram-negative, metabolically diverse bacillus that colonizes many different environments and has potential application in agriculture, biocatalysis and bioremediation. Strain KT2440 is a plasmid-free derivative of the toluene-degrading parent strain (Nelson et al., 2002). While strain KT2440 is cured of the TOL plasmid and is unable to use phenol as a carbon source, it is still suitable for evaluating the ability of this environmentally significant species to grow in the presence of tungsten. *P. putida* was propagated aerobically at 30°C in nutrient broth or M9 minimal media (12.8 g Na_2HPO_4-$7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl per liter; pH 7.5) supplemented with trace mineral supplement (ATCC; 10 ml per liter) and glucose (final concentration of 5%).

Pseudomonas aeruginosa O1 (PAO1) was a gift of Dr. John Rowe (University of Dayton, Dayton, OH). *P. aeruginosa* is a Gram-negative bacillus that displays vast ecological versatility, while also having significant clinical ramifications and playing a role in the degradation of cyclic

carbon compounds, including benzoate (Jeffrey et al., 1992). *P. aeruginosa* was propagated aerobically at 30°C or 37°C in nutrient broth or M9 minimal media supplemented with trace mineral supplement and glucose (final concentration of 5%), as described above.

Collection and preparation of soil sample

To assess the ability of natural microbial communities to withstand/grow in the presence tungsten, soil samples were collected in mid-June (2009) from the top 2 cm of soil on Wright-Patterson Air Force Base, Dayton, OH. One gram of soil was used to inoculate 10 ml nutrient broth or M9 mineral media and the cultures were incubated aerobically at 30°C for 72 h to establish a microbial consortium. To remove soil particles and nutrients, 50 µl of each culture was used to inoculate 2 ml of fresh media and cultures were incubated aerobically at 30°C overnight. The undefined microbial consortia established in these cultures were then used as a representation of a natural community.

In vitro growth inhibition assays with sodium tungstate

To evaluate the effect of tungsten (W) on the growth of *S. oneidensis, P. putida* and *P. aeruginosa*, sodium tungstate dehydrate (Na_2WO_4-$2H_20$; EM Science, Darmstadt, Germany) was used as a W source (stock concentration of 500 mM) and was added to the growth media at final concentrations of 0, 0.5, 1, 10, 25, 50, 75, 100, 200 and 250 mM. To prepare the bacteria for exposure to W, pure bacterial cultures were grown overnight aerobically in either M9 minimal media with supplements or nutrient broth at the indicated temperature (30°C or 37°C) in the absence of W. The natural microbial community isolated from soil was prepared as described above. Following overnight aerobic incubation, each culture was diluted 1/100 into fresh media with the indicated amount of Na_2WO_4. 400 µl of each inoculated culture was added to a sterile 100-well plate (Growth Curves USA, Piscataway, NJ). Cultures were monitored spectrophotometrically using the Bioscreen C (Growth Curves USA) at 30 min intervals at 600 nm. Growth assays were carried out at the indicated temperature (30°C or 37°C) for 48 or 72 h,

as indicated in the figure legend. All growth curves were performed four times and showed similar trends, with one representative curve shown for each experimental condition.

In vitro growth inhibition assays with water-soluble components of tungsten alloys

Given that Na_2WO_4 serves as a bioavailable form of tungsten, while the military relevant form of tungsten also exists as part of an alloy, it is appropriate to assess the effect of tungsten containing alloys on microbial growth. To this end, two common tungsten alloys were investigated: tungsten-nickel-cobalt (W-Ni-Co; 91.1%, 6%, 2.9%) and tungsten-nickel-iron (W-Ni-Fe; 97.1%, 1.7%, 1.2%). To closer simulate the manner in which the tungsten alloys would likely be presented to microorganisms in the environment the alloys were suspended in water to "naturally" release components of the alloy. Shavings of each alloy were obtained from Aerojet Ordnance Tennessee, Inc. (Jonesborough, TN) and 1 g of shavings was autoclaved and suspended in 9 ml sterile water for 1, 2 or 3 days at room temperature with intermittent mixing. The shavings were removed following 1, 2 or 3 days. The remaining water contained any metal complexes that were released during the process, presuming that the amount of soluble components increased with soaking time.

These water samples, and their undefined constituents, were diluted 1:1 with 2x nutrient broth or M9 minimal media (with supplements). Overnight pure cultures of *P. aeruginosa* were prepared as described above and used to inoculate fresh nutrient broth and M9 minimal media containing water exposed to the alloys. Growth assays were carried out aerobically for 72 h at 30°C to better mimic an environmental temperature. Growth curves were performed four times and showed similar trends, with one representative curve shown for each experimental condition.

Results

During this study, selected microorganisms were used as models to evaluate the impact of sodium tungstate on microbial growth. In addition, a microbial community was cultured from a soil sample to assess the ability of a natural microbial consortium to withstand increasing

concentrations of tungsten. As an alternative to sodium tungstate exposure, water-soluble components of tungsten alloys (W-Ni-Co and W-Ni-Fe) were evaluated for their ability to affect bacterial growth under nutrient rich (nutrient broth) and nutrient limiting (M9 minimal media) conditions.

Growth of pure cultures in the presence of Na_2WO_4

Shewanella oneidensis is an environmentally significant bacterial species due to its ability to display a broad range of electron acceptors and therefore play an important role in metal reduction and biogeochemical cycles (Fredrickson et al., 2008). *S. oneidensis* remained viable at the highest concentration of Na_2WO_4 tested (250 mM) (Figure 1). However, the maximum densities of the cultures were reduced with increased concentration of Na_2WO_4. Despite the reduction in maximum density, *S. oneidensis* displayed the most robust ability to grow in the presence of Na_2WO_4 of the three bacterial species evaluated.

Pseudomonas putida is a metabolically diverse microorganism that colonizes many different environments and plays a critical role in biocatalysis and bioremediation. The ability of *P. putida* to grow in the presence of Na_2WO_4 varied not only on the concentration of Na_2WO_4, but also the type of media. Under nutrient rich conditions (i.e., nutrient broth) *P. putida* tolerated as much as 50 mM Na_2WO_4 (Figure 2). However, the lag phase was considerably longer at this concentration (16 h vs. 3 h in control media) and the culture did not reach the same density as those exposed to less or no Na_2WO_4. Interestingly, *P. putida* was only able to grow in as much as 25 mM Na_2WO_4 when grown in minimal media while also displaying an increase in the lag phase (36 h) at this concentration (Figure 3). As observed in nutrient broth, the higher concentrations of Na_2WO_4 that supported growth also resulted in a reduction in maximum culture density in comparison to cultures exposed to less or no Na_2WO_4.

Pseudomonas aeruginosa is capable of vast ecological versatility. While its most referenced traits involve those that contribute to it being an opportunistic pathogen able to infect

multiple tissues, it also has the capacity to play a role in degradation processes (Jeffrey et al., 1992). Similar to *P. putida*, *P. aeruginosa* was unable to grow in the presence of any greater than 75 mM Na_2WO_4, while also showing large increases in the lag phase when exposed to 50 and 75 mM Na_2WO_4 (7 and 21 h, respectively; Figure 4). Identical to *P. putida, P. aeruginosa* was unable to grow in any concentration of Na_2WO_4 greater than 25 mM when grown in minimal media (Figure 5). However, only slight increases in the lag phase at 10 and 25 mM Na_2WO_4 were observed.

Growth of a soil microbial community in the presence of Na_2WO_4

An undefined culture of soil bacteria was grown from a fresh soil sample in either nutrient broth or M9 minimal media to assess the ability of microorganisms directly isolated from the environment to tolerate Na_2WO_4. As shown in Figure 6, the microbial consortium grown in nutrient broth was able to tolerate all concentrations of Na_2WO_4 tested (0-250 mM) with only minor delays and reduction in growth. The consortium isolated in minimal media was also able to grow in concentrations of Na_2WO_4 up to 250 mM, with significantly longer lag phases (1 h vs. 36 h) necessary to support growth relative to the increase in Na_2WO_4 concentration (Figure 7).

Growth of P. aeruginosa *in the presence of water-soluble components of tungsten alloys*

Tungsten alloy (W-Ni-Co and W-Ni-Fe) shavings were suspended in water for increasing lengths of time to allow any water soluble components to dissolve into the water. The water was then used to prepare the nutrient broth and M9 minimal media and the growth *P. aeruginosa* was evaluated. The water soluble components of the W-Ni-Co alloy in nutrient broth did not affect *P. aeruginosa* to grow, however there was a substantial reduction in culture density following 12 h of growth for those cultures exposed to the W-Ni-Co components (Figure 8). Conversely, cultures exposed to the water-soluble components of the W-Ni-Fe alloy in nutrient broth, while also not experiencing inhibition of growth, maintained their densities over the remainder of the experiment, with the higher densities correlating to the length of time the shavings were immersed in water (Figure 9). Interestingly, M9 minimal media containing water-

soluble components of either alloy was unable to support any growth of *P. aeruginosa* over the course of 72 h (Figures 10 and 11).

Discussion

Given the vast application of tungsten and tungsten alloys, the effect of this heavy metal on human health and the environment has become a rising concern. Microorganisms are key to environmental processes and carry out critical events, including nitrogen fixation, decomposition and degradation of harmful compounds. Highly polluted environments are often characterized by harsh conditions preventing or enhancing the activity/growth of most potentially useful microorganisms. Previous studies reveal limited and conflicting data regarding the effect of tungsten on microorganisms, where it has been attributed to both positive and negative effects on microbial growth (Sugio et al., 2001, Strigul et al., 2005, Andreesen and Makdessi, 2008).

The data presented in this study highlight the vast capacities that different microorganisms have for growth conditions. *S. oneidensis*, a bacterium renowned for its broad range of electron acceptors and role in metal reduction and biogeochemical cycles (Fredrickson et al., 2008), showed the greatest resilience towards Na_2WO_4 over either *Pseudomonas* species evaluated. *S. oneidensis* growth was reduced, although not prevented, at the highest concentration of Na_2WO_4 evaluated (250 mM) while the growth of *P. putida* and *P. aeruginosa* was completely inhibited at concentrations higher than 25-75 mM, depending on the limitation of nutrients within the media. In addition, increased concentrations of Na_2WO_4 often resulted in longer lag phases, suggesting that there may be an adjustment period for the bacteria when exposed to Na_2WO_4. It is possible that this delay in growth, and lower culture densities, may be attributed to the expression of stress genes, as has been reported for *Escherichia coli* exposed to polytungstates (Tajima, 2003). Furthermore, tungsten is known to replace molybdenum at the enzymatic catalytic center of certain enzymes, thereby deactivating critical enzymes (Kletzin and Adams, 1996). The efficiency of this substitution, which likely increases with increased

concentration of Na_2WO_4, may contribute not only to delayed growth, but also in the reduction in overall growth.

It should be noted that incorporation of tungsten into enzymes does not always have a negative impact. Some bacterial species are suggested to thrive in the presence of tungsten, including some Clostridia and methanogenic archaea (Andreesen and Makdessi, 2008; Klezin and Adams, 1996). This positive effect may be attributed to enzymes, including those of the aldehyde oxidoreductase (AOR) family, that require tungsten (Andreesen and Makdessi, 2008). The microbial community cultured from a soil sample was well adept to grow in the presence of Na_2WO_4, as indicated by slight increases in culture density at given Na_2WO_4 concentrations in nutrient broth. Under more limiting nutrient conditions (minimal media), the soil bacteria did display significant lag phases with the highest Na_2WO_4 concentrations, however, they still reached very high densities at all concentrations tested. This suggests that environmental organisms may have a higher capacity to tolerate, are already primed to survive in, or have a requirement for some level of tungsten.

One coping mechanism that bacteria have for exposure to tungsten is that of the discrimination of catechol siderophores, small chelating compounds that serve to hunt and return iron to the bacteria. The N_2-fixing bacterium, *Azotobacter vinelandii*, produces catechol siderophores in response to the presence of tungsten. These siderophores can distinguish targets (i.e., iron and molybdenum) from tungsten that would be toxic to the bacterium due to deactivation of nitrogenase (Wichard et al., 2008). It is possible that some members of the soil microbial community isolated during this study may also produce these catechol siderophores and could therefore discriminate between tungsten and desired elements. If this were the case, over time there would be a shift in the population from those unable to or less capable of surviving in the presence tungsten, to those that have the support mechanisms for tolerating or using tungsten. Such shifts in community structure in tungsten-containing soils have been observed elsewhere (Ringelberg et al., 2009).

One highly novel aspect to this study was the evaluation of water-soluble components from the tungsten alloys on microbial growth. In high nutrient media, the water-soluble components from W-Ni-Co and W-Ni-Fe had opposite effects on the growth of *P. aeruginosa* once the bacteria reached stationary phase. The nutrient broth containing components of the tungsten alloy with cobalt caused a faster decrease in culture density (i.e., death) compared to the media control. This may be due to cobalt interference in the uptake of iron (supplied in the media), as iron became more scarce at the later stage of experiment (Kothamasi and Kothamasi, 2004). Conversely, the nutrient broth containing components of the tungsten alloy with iron allowed the cultures to remain at higher densities for longer periods of time. One explanation for this effect may be the presence of supplemental iron from the alloy that could serve to support additional survival. Whether or not either of these effects would be an issue in environmental settings is unclear.

The water-soluble components of both alloys inhibited bacterial growth when nutrients were limiting. Given this common observation in minimal media, whereas conflicting results were obtained when growth was assessed under high nutrient conditions, suggests that this effect may be due in part to a second component of the solution. One such explanation may lie in bacterial sensitivity to nickel, a common element to both alloys. This is supported by the fact that *P. aeruginosa* in minimal media has been found to be 40-fold more sensitive to the toxic effects of nickel than bacteria growing under high nutrient conditions (Sar et al., 1998). Such results may suggest a protective role of nutrients when nickel is present. Again, it is unclear whether or not this would play a role under environmental conditions. It is difficult to ascertain the exact mechanism for these growth effects, positive and negative, without a better understanding of the forms and amounts released into the water by the alloys.

In conclusion, this study demonstrates the potential for environmentally significant bacteria, namely *Pseudomonas* and *Shewanella* species, to withstand various levels of tungsten. *Shewanella*, a strong metal reducer, had the most robust ability to grow even in the

highest concentrations of Na_2WO_4 evaluated, whereas *Pseudomonas* displayed much lower tolerances. Interestingly, bacteria cultivated directly from the environment displayed only minor delays and reduction in growth relative to the pure cultures, suggesting that such a microbial consortium is better suited to exposure. Reasons for such an observation may include a reliance on tungsten in enzymes or mechanisms that selectively exclude tungsten from entering the cell, such as catechol siderophores.

Tungsten alloys also had profound effects on the ability of *Pseudomonas* cultures to grow or maintain their cell densities. However, the effects were highly dependent on the types of metals and nutrients present, suggesting the effect may be exacerbated in certain environmental settings where nutrients can be limited. It remains to be seen whether bacteria can develop or enhance tolerance to tungsten with slow and increased exposure.

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Figure 1: Growth of *Shewanella oneidensis* in nutrient broth with sodium tungstate.

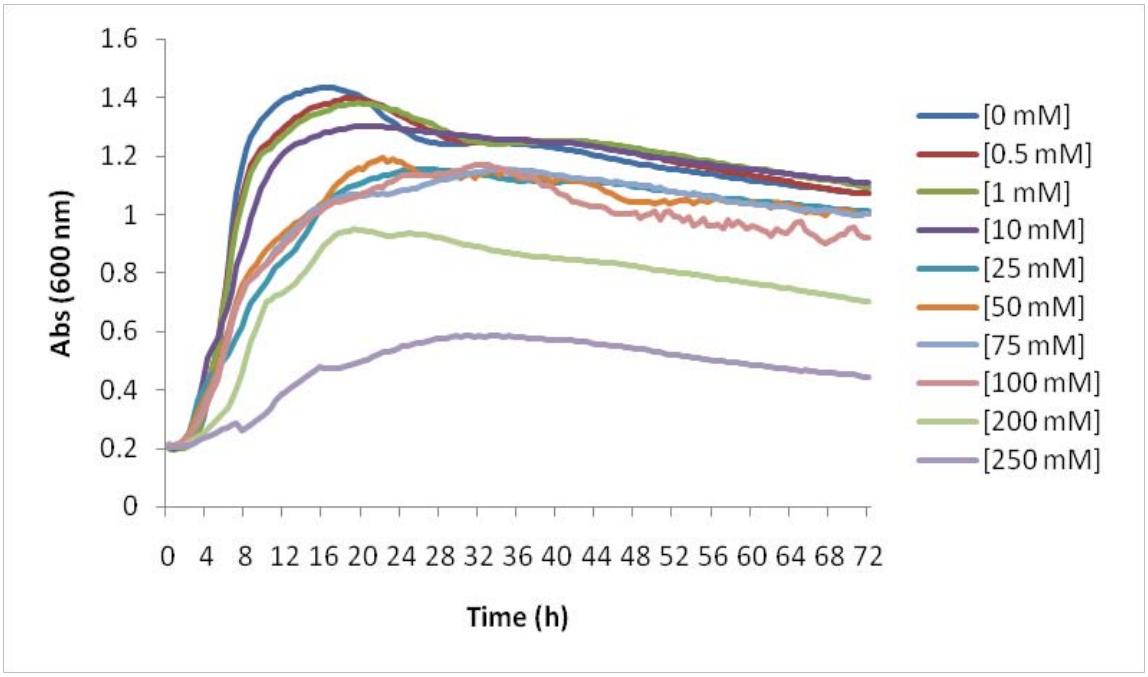

Figure 1: Growth of *Shewanella oneidensis* in nutrient broth with sodium tungstate. *Shewanella oneidensis* was aerobically grown at 30°C for 72 h in nutrient broth supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. While *S. oneidensis* was able to grow in the presence of the highest concentration of Na_2WO_4, it displayed decreased growth with increased concentration of Na_2WO_4. Additionally, the cell densities of both 200 mM and 250 mM Na_2WO_4 concentrations did not reach that of the lower concentration exposures. Data are representative of at least three independent experiments.

Figure 2: Growth of *Pseudomonas putida* in nutrient broth with sodium tungstate.

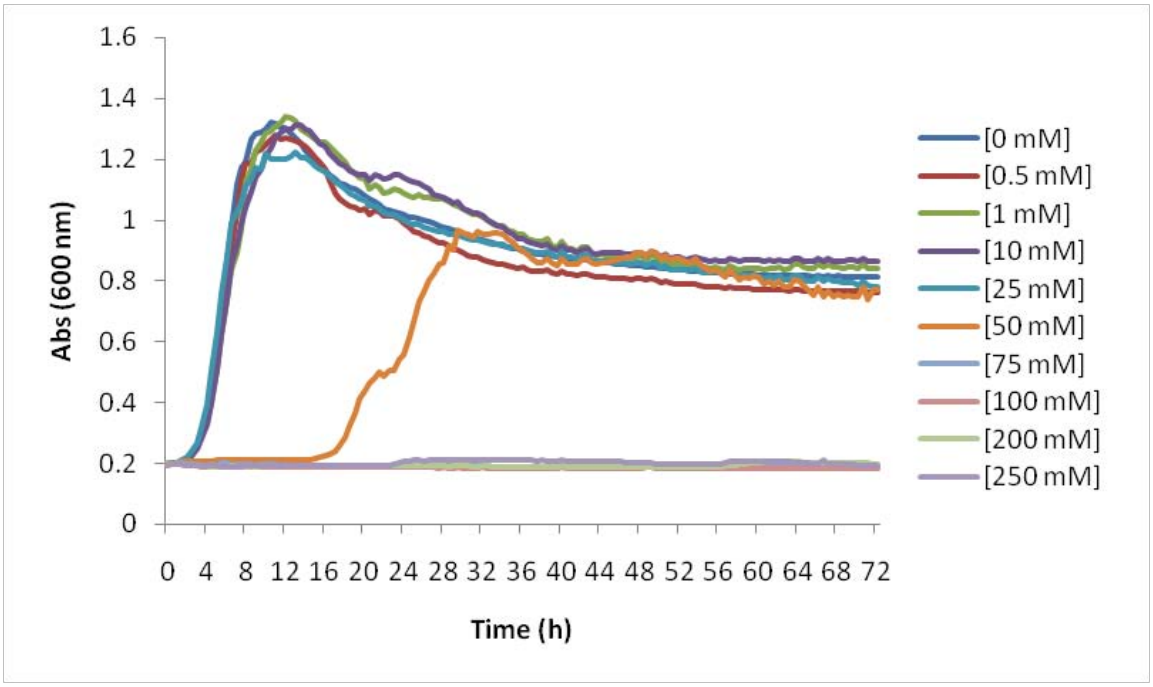

Figure 2: Growth of *Pseudomonas putida* in nutrient broth with sodium tungstate. *Pseudomonas putida* was aerobically grown at 30°C for 72 h in nutrient broth supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. *P. putida* was able to grow in Na_2WO_4 concentrations up to 25 mM similar to growth in the absence of Na_2WO_4. The lag phase was considerably longer in the presence of 50 mM Na_2WO_4 compared with lower concentrations (16 vs. 3 h) and the overall density of the culture did not reach that of cultures grown in lower concentrations of Na_2WO_4. Na_2WO_4 concentrations greater than 50 mM completely inhibited growth of the bacteria. Data are representative of at least three independent experiments.

Figure 3: Growth of *Pseudomonas putida* in M9 minimal media with sodium tungstate.

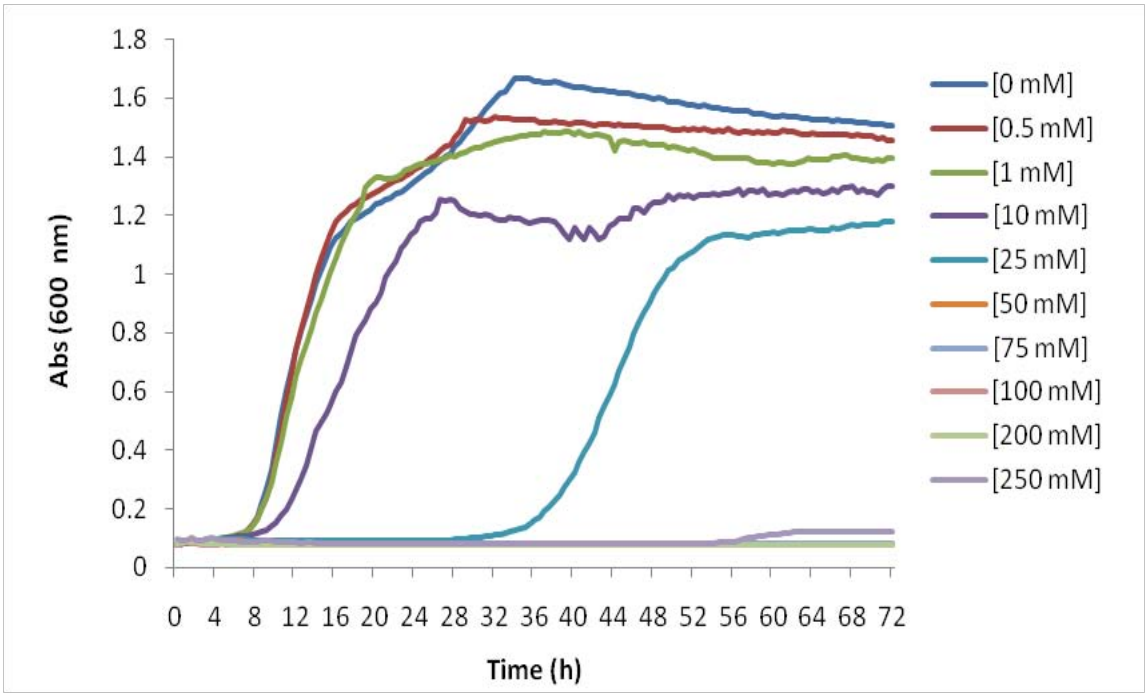

Figure 3: Growth of *Pseudomonas putida* in M9 minimal media with sodium tungstate. *Pseudomonas putida* was aerobically grown at 30°C for 72 h in M9 minimal media supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. *P. putida* was able to grow in Na_2WO_4 concentrations up to 1 mM similar to growth in the absence of Na_2WO_4, Bacterial growth was inhibited at 10 mM Na_2WO_4, and further at 25 mM, as evidenced by an increase in the lag phase (36 h). Cultures exposed to Na_2WO_4 were unable to reach the density of the unexposed culture over the course of 72 h, with density inversely proportional to Na_2WO_4 concentration. Na_2WO_4 concentrations greater than 25 mM completely inhibited growth of the bacteria. Data are representative of at least three independent experiments.

Figure 4: Growth of *Pseudomonas aeruginosa* in nutrient broth with sodium tungstate.

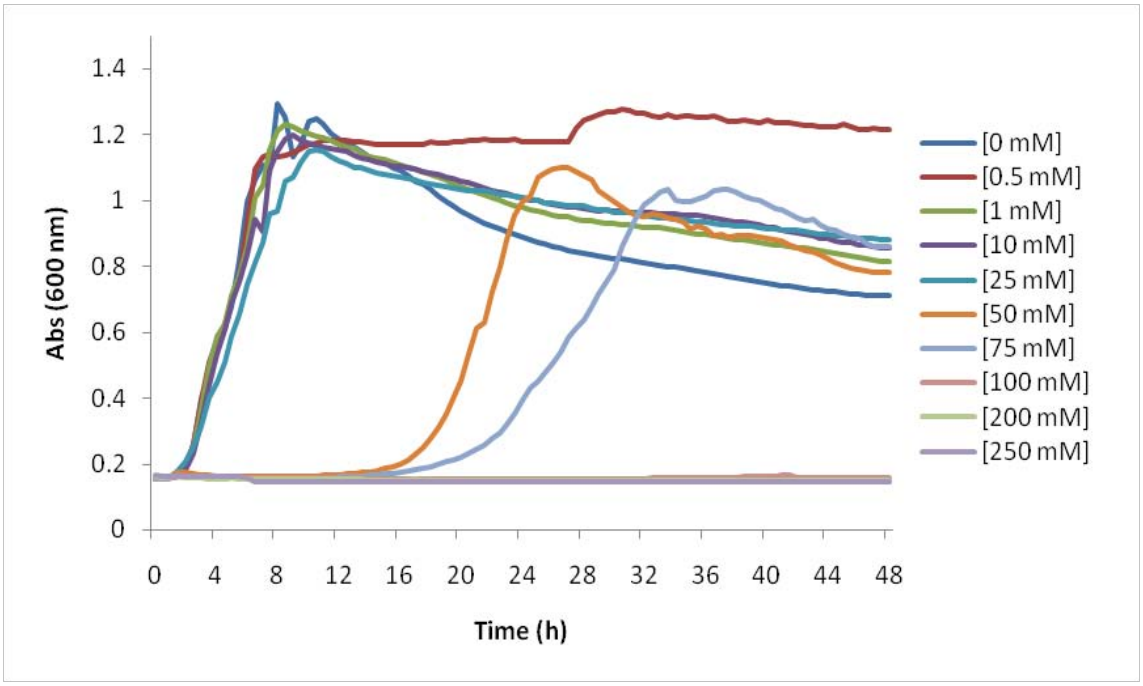

Figure 4: Growth of *Pseudomonas aeruginosa* in nutrient broth with sodium tungstate. *Pseudomonas aeruginosa* was aerobically grown at 37°C for 48 h in nutrient broth supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. *P. aeruginosa* displayed equivalent lag and exponential phase with Na_2WO_4 exposures up to 25 mM, after which cultures exposed to 0.5 to 25 mM displayed higher densities than cultures not exposed to Na_2WO_4. The lag phase was considerably longer for cultures grown in 50 and 75 mM Na_2WO_4 (16 vs. 2 h) with the overall densities not reaching that of cultures grown in lower concentrations of Na_2WO_4. Na_2WO_4 concentrations greater than 75 mM completely inhibited growth of the bacteria. Data are representative of at least three independent experiments.

Figure 5: Growth of *Pseudomonas aeruginosa* in M9 minimal media with sodium tungstate.

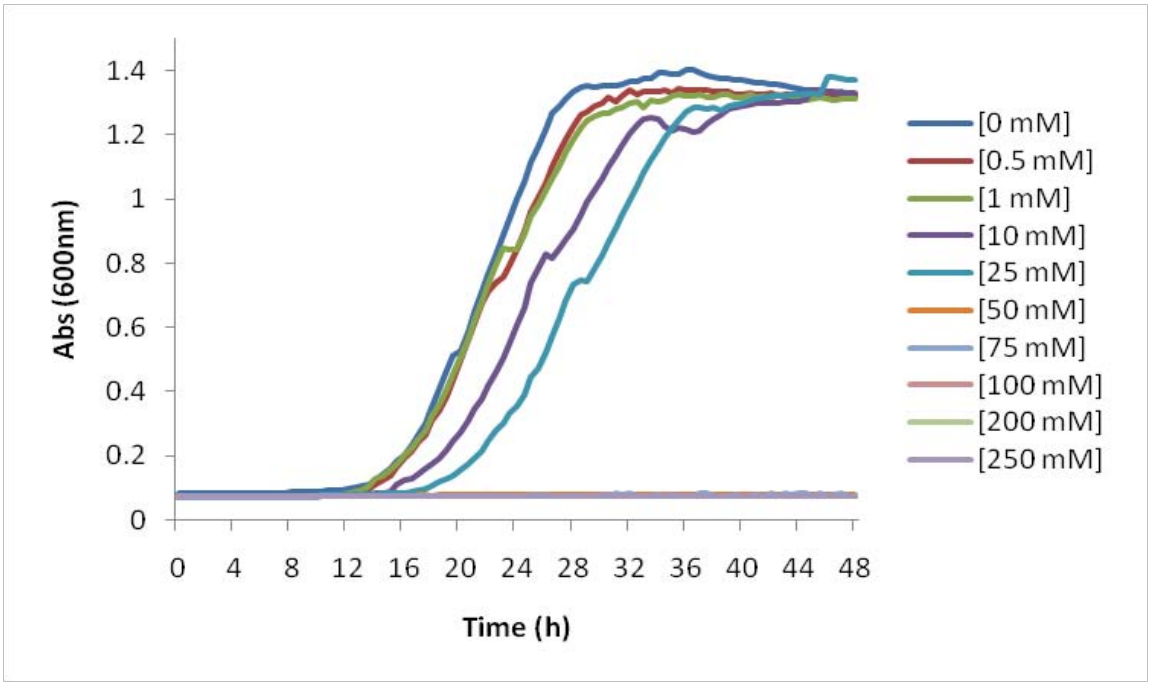

Figure 5: Growth of *Pseudomonas aeruginosa* in M9 minimal media with sodium tungstate. *Pseudomonas aeruginosa* was aerobically grown at 37°C for 48 h in M9 minimal media supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. *P. aeruginosa* displayed increases in lag phase with increased Na_2WO_4 concentration. Na_2WO_4 concentrations greater than 25 mM completely inhibited growth of the bacteria. Data are representative of at least three independent experiments.

Figure 6: Soil microbial community growth in nutrient broth with sodium tungstate.

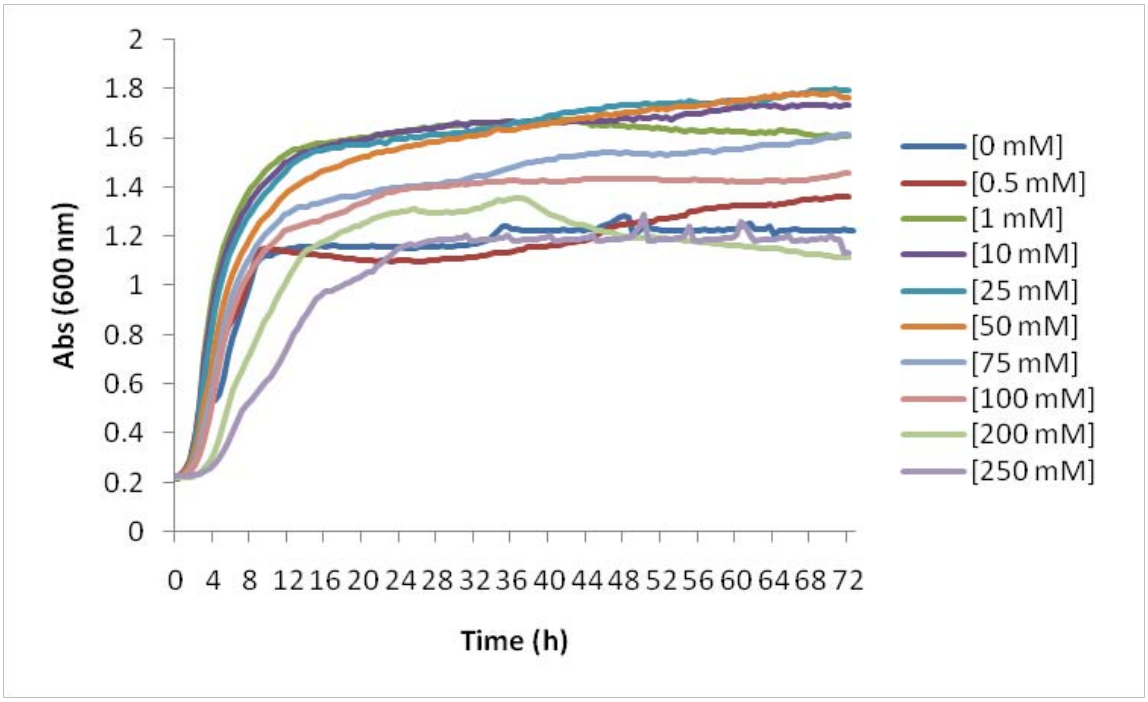

Figure 6: Soil microbial community growth in nutrient broth with sodium tungstate. An undefined culture of soil bacteria was grown aerobically grown at 30°C for 72 h in nutrient broth supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. The soil community was able to grow in all Na_2WO_4 concentrations tested, with only minor increases in lag time observed with 200 and 250 mM of Na_2WO_4. Cultures exposed to Na_2WO_4 at 10 to 100 mM reached higher densities than cultures with no and lower Na_2WO_4 concentrations. Data are representative of at least three independent experiments.

Figure 7: Soil microbial community growth in M9 minimal media with sodium tungstate.

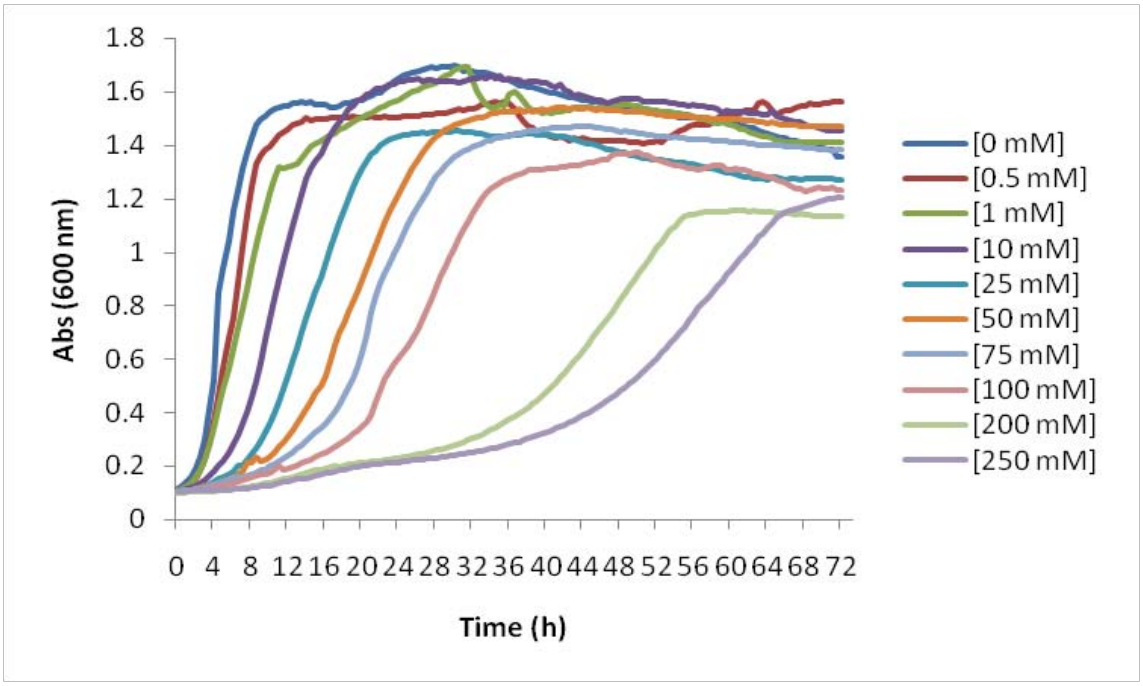

Figure 7: Soil microbial community growth in M9 minimal media with sodium tungstate. An undefined culture of soil bacteria was grown aerobically grown at 30°C for 72 h in M9 minimal media supplemented with sodium tungstate (Na_2WO_4) in concentrations ranging from 0 to 250 mM. Bacterial growth was measured by absorbance at 600 nm. The soil community was able to grow in all Na_2WO_4 concentrations tested, with substantial increases in lag time with increased concentration and only minor reductions in culture density at the highest concentrations. Data are representative of at least three independent experiments.

Figure 8: Growth of *Pseudomonas aeruginosa* in nutrient broth containing water-soluble components of tungsten-nickel-cobalt alloy shavings.

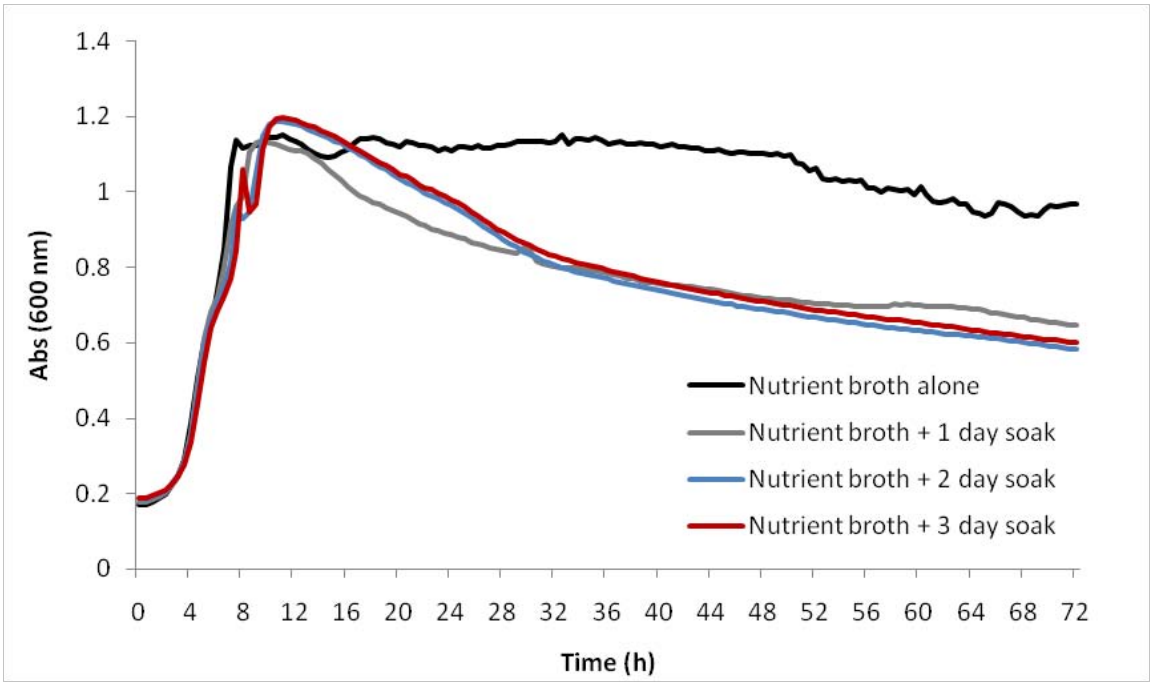

Figure 8: Growth of *Pseudomonas aeruginosa* in nutrient broth containing water-soluble components of tungsten-nickel-cobalt alloy shavings. Tungsten-nickel-cobalt (W-Ni-Co) alloy shavings were suspended in water at room temperature for 1, 2 or 3 days. This water, with the undefined components, was used to dilute 2x nutrient broth to the proper concentration of media. *Pseudomonas aeruginosa* was aerobically grown at 30°C for 72 h in nutrient broth alone (black line) or in nutrient broth with the undefined components following 1, 2 or 3 days of suspension in water (gray, blue and red lines, respectively). Bacterial growth was measured by absorbance at 600 nm. Growth of *P. aeruginosa* was initially uninhibited by the presence of the W-Ni-Co components. However, there was a substantial decrease in culture density following 12 h of growth for those cultures exposed to the W-Ni-Co components. Data are representative of at least three independent experiments.

Figure 9: Growth of *Pseudomonas aeruginosa* in nutrient broth containing water-soluble components of tungsten-nickel-iron alloy shavings.

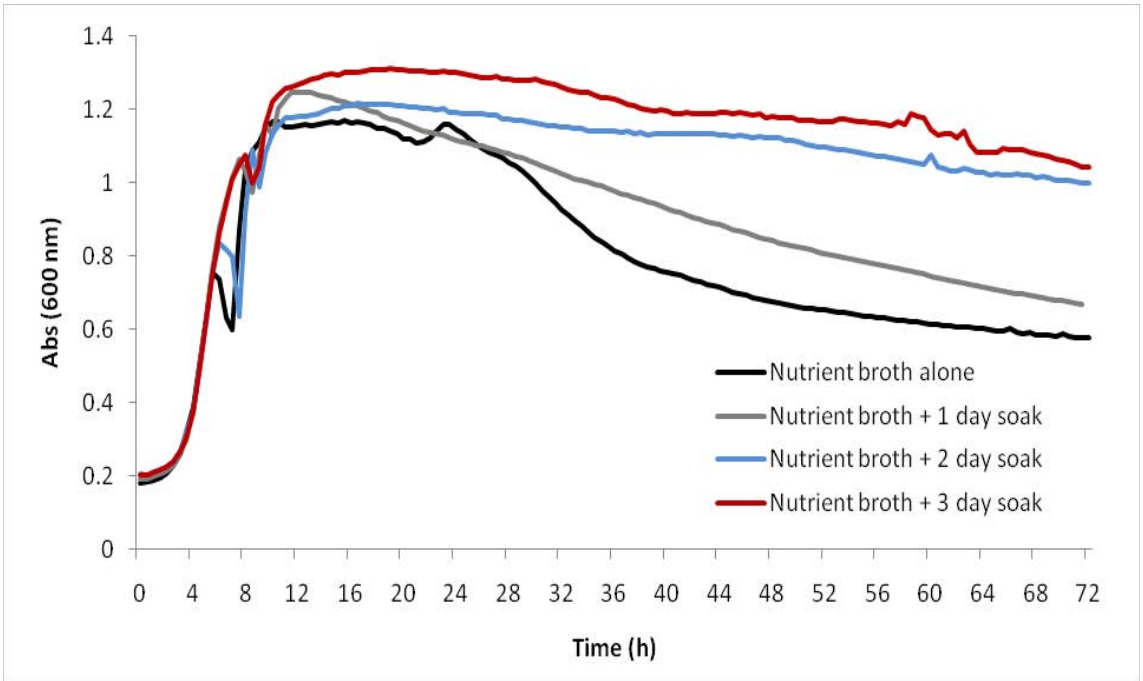

Figure 9: Growth of *Pseudomonas aeruginosa* in nutrient broth containing water-soluble components of tungsten-nickel-iron alloy shavings. Tungsten-nickel-iron (W-Ni-Fe) alloy shavings were suspended in water at room temperature for 1, 2 or 3 days. This water, with the undefined components, was used to dilute 2x nutrient broth to the proper concentration of media. *Pseudomonas aeruginosa* was aerobically grown at 30°C for 72 h in nutrient broth alone (black line) or in nutrient broth with the undefined components following 1, 2 or 3 days of suspension in water (gray, blue and red lines, respectively). Bacterial growth was measured by absorbance at 600 nm. Growth of *P. aeruginosa* was uninhibited by the presence of the W-Ni-Fe components. After 32 h the cultures grown in nutrient broth alone displayed a decrease in density (i.e., death) whereas cultures exposed to the W-Ni-Fe components were able to maintain their densities over the remainder of the experiment, with the higher densities correlating to the amount of time the shavings were immersed in water. Data are representative of at least three independent experiments.

Figure 10: Growth of *Pseudomonas aeruginosa* in M9 minimal media containing water-soluble components of tungsten-nickel-cobalt alloy shavings.

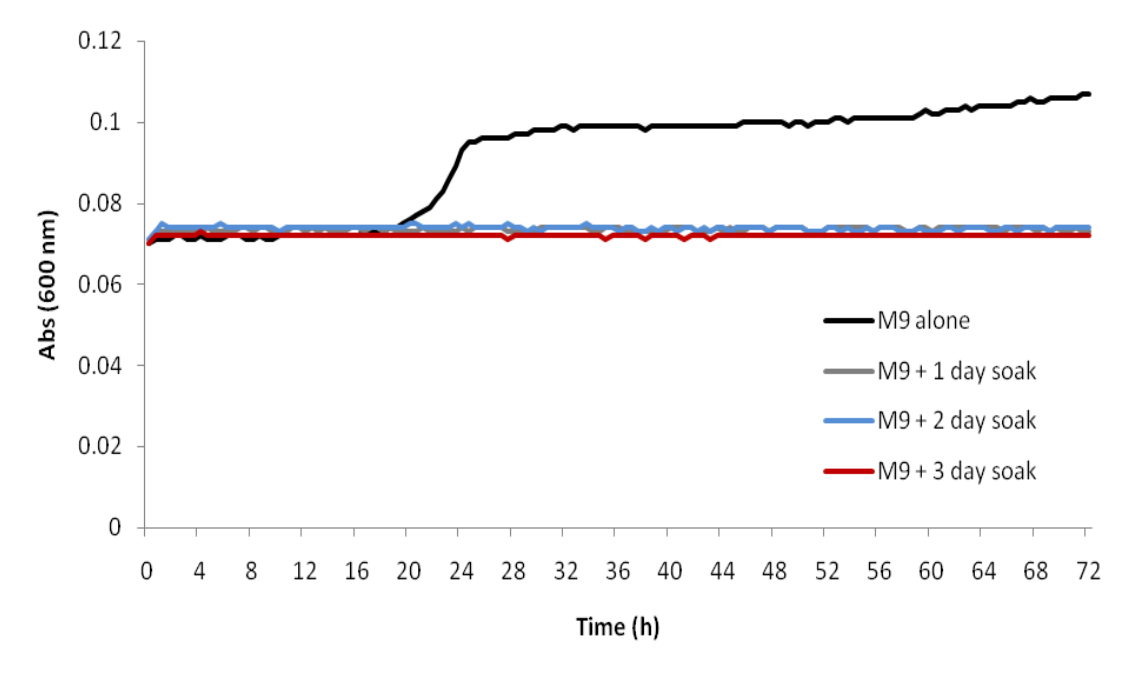

Figure 10: Growth of *Pseudomonas aeruginosa* in M9 minimal media containing water-soluble components of tungsten-nickel-cobalt alloy shavings. Tungsten-nickel-cobalt (W-Ni-Co) alloy shavings were suspended in water at room temperature for 1, 2 or 3 days. This water, with the undefined components, was used to dilute 2x nutrient broth to the proper concentration of media. *Pseudomonas aeruginosa* was aerobically grown at 30°C for 72 h in M9 minimal media alone (black line) or in M9 minimal media with the undefined components following 1, 2 or 3 days of suspension in water (gray, blue and red lines, respectively). Bacterial growth was measured by absorbance at 600 nm. Growth of *P. aeruginosa* was completely inhibited by the presence of the W-Ni-Co components. Data are representative of at least three independent experiments.

Figure 11: Growth of *Pseudomonas aeruginosa* in M9 minimal media containing water-soluble components of tungsten-nickel-iron alloy shavings.

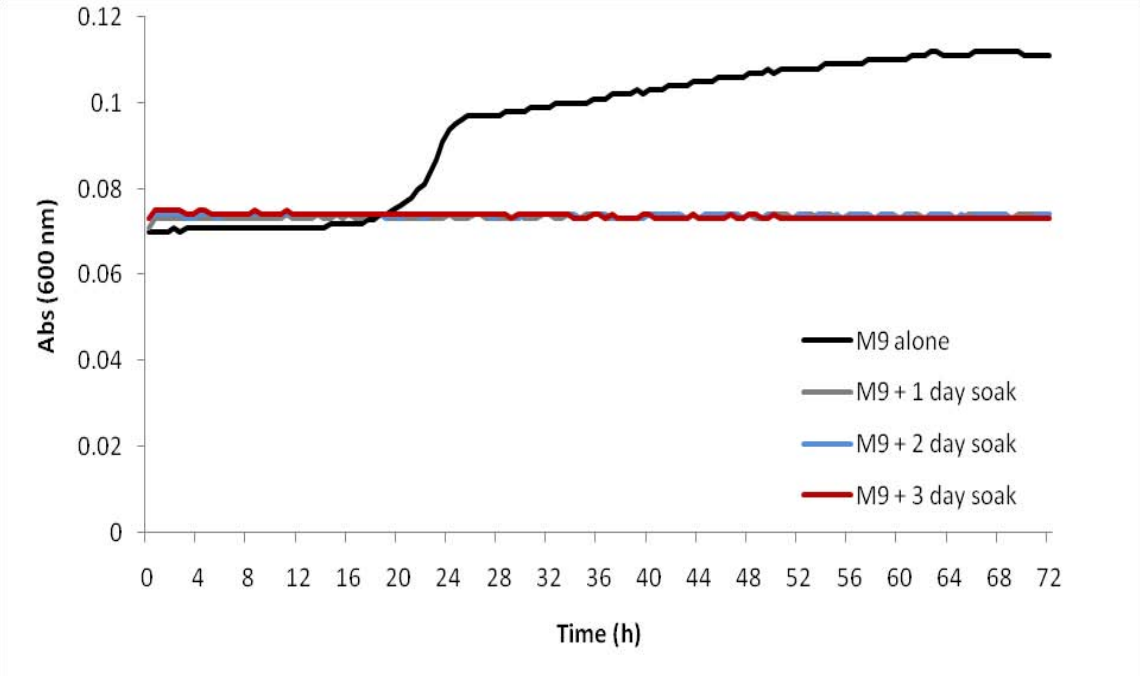

Figure 11: Growth of *Pseudomonas aeruginosa* in M9 minimal media containing water-soluble components of tungsten-nickel-iron alloy shavings. Tungsten-nickel-iron (W-Ni-Fe) alloy shavings were suspended in water at room temperature for 1, 2 or 3 days. This water, with the undefined components, was used to dilute 2x nutrient broth to the proper concentration of media. *Pseudomonas aeruginosa* was aerobically grown at 30°C for 72 h in M9 minimal media alone (black line) or in M9 minimal media with the undefined components following 1, 2 or 3 days of suspension in water (gray, blue and red lines, respectively). Bacterial growth was measured by absorbance at 600 nm. Growth of *P. aeruginosa* was completely inhibited by the presence of the W-Ni-Fe components. Data are representative of at least three independent experiments.