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ADAPTATIONS IN MICROBIOLOGICAL POPULATIONS EXPOSED TO DINITROPHENOL AND OTHER CHEMICAL STRESSORS

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Abstract—Microbiological populations in natural and engineered systems may experience multiple exposures to chemical stressors, which may affect system functions. The impact of such exposures on the metabolism of a population of *Pseudomonas aeruginosa* was studied using respirometry. Two serial exposures to low concentrations of 2,4-dinitrophenol (DNP), pentachlorophenol (PCP), or *N*-ethyl maleimide (NEM) did not affect metabolism beyond that expected for a single exposure. However, at higher concentrations, three exposures to DNP led to a combination of metabolic stress and resilience in the population. At a low DNP concentration of 400 mg/L, multiple exposures led to increased stress but indicated no development of resilience. At a high DNP concentration of 1,200 mg/L, no biological activity was observed, indicating that the population did not survive the exposure. At intermediate concentrations of 800 and 900 mg/L DNP, stress was observed, but it was found to decrease after multiple exposures. This, combined with the observation that the size of the population decreased, indicated that resilience in the population had developed because of elimination of the weaker organisms in the population. In contrast, the lack of resilience at the lower DNP concentration was attributed to the survival of the strong as well as weak members, lowering the resilience of the population as a whole. The development of resilience within a window of stressor concentrations is an important finding with implications for predicting the performance of biotreatment processes and biosensor technologies and for interpreting ecotoxicity risk assessments. Environ. Toxicol. Chem. 2010;29:2161–2168. © 2010 SETAC

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INTRODUCTION

Both natural systems such as soils and sediments and engineered systems such as hazardous waste sites and wastewater treatment plants may be exposed to chemical pollutants that stress resident microbiological communities. Microbiological stress responses have been observed on exposure to many environmentally relevant chemicals, including phenols [1], chlorophenols [2,3], dinitrophenols [4,5], toluene [6], methyl-*tert*-butyl ether [7], explosive 2,4,6-Trinitrotoluene [8], explosive cyclotrimethylenetrinitramine [9], and dibenzo-*p*-dioxin [10]. Microorganisms that are effective at degrading pollutants can be stressed by exposure to these chemicals, as has been observed for *Methylocystis* sp. M and trichloroethylene [11], *Acinetobacter calcoaceticus* and phenol [12], *Pseudomonas* sp. B4 and polychlorinated biphenyls [13], and *Pseudomonas putida* KT2440 and toluene [14]. In experiments with *P. putida* F1, the organisms were found to use chemotaxis to accumulate near a trichloroethylene interface, but maintained a measurable distance, presumably because of toxicity of the high trichloroethylene concentrations near the interface [15]. Similarly, enhanced dissolution of toluene attributable to in situ biodegradation was found to be diminished in a groundwater experiment when toluene concentration reached toxic levels [16]. Stress responses have also been studied for common biodegradation metabolites such as catechol and chlorobenzoates [17,18]. Also, in wastewater treatment, defloculation has been attributed to a protective stress response of activated sludge microorganisms [19]. Stress responses can be exploited in biosensor technologies for detection of pollution and indication of suboptimal biotreatment performance [20]. These examples, which demonstrate the profound effect of microbiological stress responses, suggest that understanding the role that chemical stressors play in microbiological community structure and function is essential for monitoring and management of natural and engineered systems

In a previous study, we used respirometry to study the impact of stressors 2,4-dinitrophenol, (DNP), pentachlorophenol (PCP), and *N*-ethyl maleimide (NEM) on the metabolism of *Pseudomonas aeruginosa* [21]. We measured biological activity simultaneously in stressed and nonstressed systems to infer the effect of the chemical stressors and found that at low concentrations these chemical stressors affected metabolism by reducing biomass yields but not diminishing substrate utilization. This suggests that a portion of carbon and energy resources were diverted from growth and used in stress management and protection. At higher concentrations, reductions in biomass yield were accompanied by inhibition of substrate utilization rates and, in some cases, death to a portion of the population. These findings are consistent with other studies that showed that DNP reduced the biomass yield of microorganisms in activated sludge [22].

How these short-term metabolic effects on a population are affected by long-term exposure is little known. Repeated intermittent exposure to chemical stressors may gradually weaken a population through diminished metabolic activity or may strengthen a population by selecting for stronger members. Although all the members in a bacterial population are genetically identical, evidence and understanding of phenotypic

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heterogeneity within populations have increased. This is known as epigenetic variation, which is caused by differences in gene expression alone [23]. These differences in gene expression are attributed to the inherent stochasticity of biochemical reactions [24]. Phenotypic heterogeneity is believed to act as a risk-spreading strategy [25,26], because some individuals within a population are more capable of adapting and surviving in a changing environment than others [24,27–30]. For example, a heterogeneous population of yeast was found to outcompete a homogeneous one when subjected to cadmium stress [31]. Variations in a population may be dynamic, in which a portion of the cells are capable of switching to the fit state by sensing environmental changes, or static, with no capability of switching from one state to another [32]. Collectively, the literature on phenotypic heterogeneity suggests the possibility that under chemical stress some members of a population may be metabolically more resilient than others.

Here, we report on a study of the impact of ongoing intermittent exposure to sublethal concentrations of chemical stressors on *Pseudomonas aeruginosa*. The objective of the current study was to determine whether *P. aeruginosa* populations are strengthened or weakened by repeated exposures, and whether different stressor concentration windows produce different effects. *Pseudomonas aeruginosa* was chosen for the study because of its abundance both in activated sludge and in natural soils and waters. It has been used as a model organism in other studies of microbiological stress [3]. Its capability of surviving in a wide variety of environments, including many different contaminated environments, makes it ideal for study of sublethal chemical stressors. Its metabolic diversity and broad adaptability has been attributed, in part, to the complexity of its genome with respect to the large number of regulatory genes and genes for catabolism, transport, and efflux [33].

The model chemical stressors used in the current study were DNP, PCP, and NEM. The effects of multiple exposures were first tested in a set of experiments involving two exposures to a chemical stressor. The second set of experiments, involving only DNP, investigated the effects of multiple exposures, up to a maximum of three exposures. Biological activity was measured in batch systems in the presence and absence of stressor, and biomass yields, rates of substrate utilization, and the initial biomass concentration were inferred.

THEORY AND HYPOTHESIS

Population-level effects of chemical stressors are determined by microbiological stress responses at the cellular level, which include a wide range of mechanisms. For example, changes in the cell membrane occur in response to the accumulation of hydrophobic chemicals (as are most organic environmental pollutants) in the lipid bilayer of the membrane. The permeability of the membrane is increased, jeopardizing key functions such as maintaining the proton motive force needed for energy storage [2]. This phenomenon is known as uncoupling of oxidative phosphorylation. Strains of *P. aeruginosa* and *P. putida* have been found to adapt to such stresses via *cis-* to *trans*-isomerization of unsaturated fatty acids, which modifies membrane fluidity, as well as through activation of efflux pumps that export toxic solvents [34,35]. Often, multiple stress responses are induced simultaneously. Toluene, for example, caused upregulation of heat shock protein production, activation of efflux pumps and responses to membrane damage in *P. putida* [14].

The chemicals used in the current study are reported to activate more than one stress response. Stressor DNP induces heat shock proteins [36] and is well known for its ability to uncouple oxidative phosphorylation [5,37]. Stressor PCP induces heat shock proteins [36], is an uncoupler of oxidative phosphorylation, and is also reported to upregulate genes coding for efflux pumps that transport chemicals from the cell [3]. Stressor NEM activates an ion transport mechanism known as glutathione-gated potassium efflux [38], which is believed to protect microorganisms by causing changes in the cytoplasmic chemistry.

Upregulation of molecular machinery usually has an associated cost in terms of carbon and energy (with few exceptions such as the *cis-trans* isomerization of unsaturated fatty acids [35]). In fact, in response to stress, a means of saving energy includes the suppression of temporarily unnecessary functions, such as cell motility, to activate mechanisms that are compulsory for survival [14]. Mathematically, the diversion of carbon and energy resources can be represented through a modification of the kinetic growth model. Here we briefly present a version of the model used in the current study, which is identical to that used previously [21]. The biomass growth rate is the product of the biomass concentration, X, and the specific growth rate, μ, which is related to the concentration of growth substrate, S, through the Monod equation.

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{1}$$

where $\mu_{\max}$ is the maximum specific growth rate and K_s is the half saturation constant. A common way to account for endogenous degradation is to offset biomass growth.

$$\frac{dX}{dt} = \mu X - bX \tag{2}$$

where b is the coefficient of endogenous decay. Mathematically, this term also captures the effect that not all the substrate is available for growth; some of it is needed for normal cellular functions such as motility and maintenance of required redox potential.

The yield coefficient of growth, Y_G, relates the biomass growth rate to the rate at which substrate is consumed to support biomass growth. Maintenance energy requirements cause the observed yield Y_{obs}, which is the ratio of the amount of biomass produced to the amount of growth substrate utilized, $(\frac{dX}{dt} / \frac{dS}{dt})$ to be lower than the true yield Y_{G} as follows.

$$Y_{obs} = Y_G\left(1 - \frac{b}{\mu}\right) \tag{3}$$

A change in the metabolic demand in a stressed system translates to a decrease in observed yield. This derives, in part, from an increase in the observed endogenous decay coefficient, b. The increase in b relative to a nonstressed system thus quantifies the excess carbon and energy requirements of the stress responses, which are beyond the carbon and energy requirements needed for biomass growth and normal cell maintenance.

In the previous experiments involving single exposures of DNP or PCP [21], the observed decrease in biomass yield without inhibition of substrate utilization rates provided evidence of a diversion of carbon and energy from growth to support stress responses. The diversion of carbon and energy from growth for adaptive responses to stressors also has been suggested as the cause of the reduction in observed yield in activated sludge exposed to cytostatic drugs [39].

At higher DNP and PCP concentrations, the observed inhibition of substrate utilization rates was interpreted as inhibition

of both anabolism and catabolism, and the observed decrease in initial biomass concentration was interpreted as toxicity to a portion of the population [21]. Exposure to NEM, at and above certain concentrations, decreased biomass yields and inhibited substrate utilization rates, but its effect on initial biomass concentration could not be determined.

Those findings lead to new hypotheses regarding the effects of multiple exposures. In the case of repeated exposures, the reduction of biomass yields and the inhibition of substrate utilization rates are likely to weaken the population and may ultimately lead to its death. The reduction in initial biomass, however, has the potential to strengthen the population if it eliminates those organisms that are less resilient to the stressor. This may leave behind a higher fraction of organisms that are more resilient to the stressor. At each subsequent exposure, elimination of weaker organisms may occur, and the population may continue to develop resilience. The experiments described here were designed to determine which of these competing effects is dominant at which stressor concentration.

MATERIALS AND METHODS

Chemicals

The following chemicals were used in the current study: glucose (Sigma, 99.5%), PCP (Aldrich, 98%), NEM (Fluka chemical, >99%) and DNP (Aldrich, 97%).

Bacterial culture and media

The *P. aeruginosa* strain (PA01 WT) had been originally isolated from the consortium of an activated sludge sequencing batch reactor [40]. The methods for freezing and incubating the culture were previously reported [21]. In incubation, care was taken that the glucose substrate was not completely depleted, to prevent a physiological change associated with starvation. Approximately 20 ml of this bacterial solution was used to inoculate the experimental vessels.

Reaction vessels and chemical concentrations. The reaction vessels, 500 ml in volume, were filled with solutions of approximately 300 ml containing biomass, glucose substrate, and other nutrients. Stressors DNP and PCP were added to the reaction vessels as solids, and NEM was diluted from a concentrated solution. The target initial glucose concentration of 100 mg/L was designed to be two orders of magnitude higher than the target initial biomass concentration so that growth would be substantial. (Both glucose and biomass were measured or estimated in units of oxygen demand.) Glucose concentration was measured by a total carbohydrate test using spectrophotometry, DNP concentration with a spectrophotometer, and PCP and NEM concentration by HPLC. Further details on the methods for measurement are reported in Ray and Peters [21]. Unlike DNP and PCP, the concentrations of which remained constant, stressor NEM was depleted throughout the time scale of the experiments because of both hydrolysis and biotic transformations. The biotic transformation did not yield growth.

A threshold NEM concentration existed, above which biological activity did not occur. When the degradation processes caused NEM concentration to fall below the threshold, biological activity began and showed an increased oxygen uptake. We report the initial NEM concentration in each experiment.

Measurement of biological activity. Cumulative oxygen uptake by the bacteria was measured by respirometry, using a Computox respirometer (N-Con Systems). Each experiment was allowed to continue until shortly after the glucose was depleted. The time for glucose depletion depended on the concentration of the chemical stressor and took approximately 36 h. The methodology is identical to that previously reported [21].

Design of two-exposure experiments

The goal of the two-exposure experiments was to determine the impact of pre-exposure to stressor and the variation of this impact with the pre-exposure stressor concentration. Each experiment, for a given stressor and its concentration, was conducted in two phases (Fig. 1a). In phase 1, bacteria were grown in two replicate vessels that contained the stressor as well as in two replicate control vessels that did not. In phase 2, four identical vessels were prepared, all containing stressor. Two of these were inoculated with bacterial solution that had been pre-exposed to the stressor, and two were inoculated with bacterial solution without pre-exposure (control).

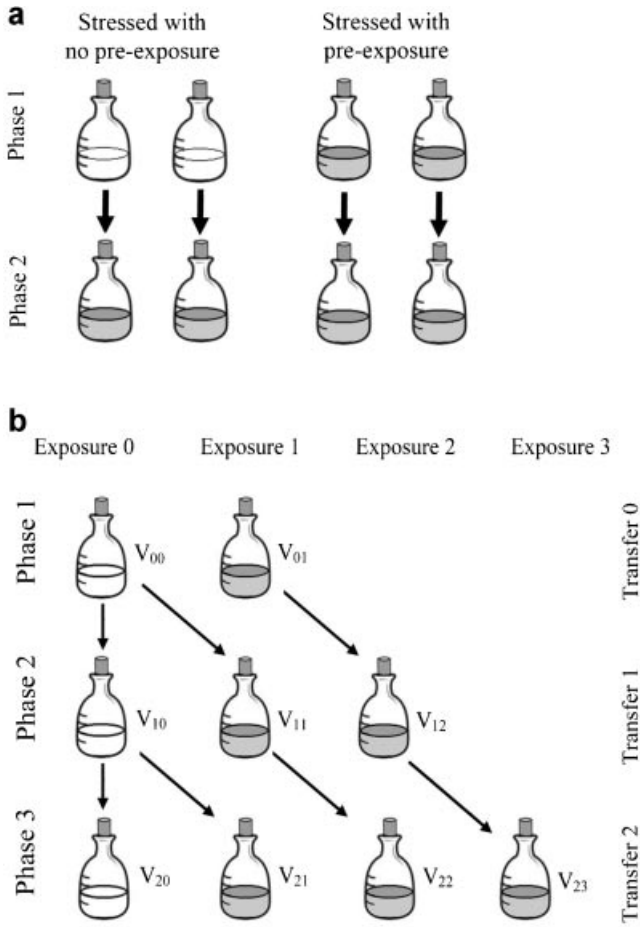

Fig. 1. Design of the two-exposure experiments (**a**). Reaction vessels without stressor are clear, whereas those vessels containing stressor are shaded. The arrows indicate the transfer of bacterial populations from one vessel to another. Design of the multiple-exposure experiments (**b**), showing the sequences of transfer from one vessel to the next and consequent exposure to stressor DNP. Vessels in the extreme left column (unshaded) do not contain stressor, whereas all other vessels do. Each vessel is labeled V_{ij}, in which subscript i refers to the number of times the population has been transferred, and j refers to the number of times the population is exposed to DNP. The sequences of transfer and stress exposure, shown by arrows, are as follows: (1) $V_{00}\rightarrow V_{10}\rightarrow V_{20}$, (2) $V_{00}\rightarrow V_{10}\rightarrow V_{21}$, (3) $V_{00}\rightarrow V_{11}\rightarrow V_{22}$, and (4) $V_{01}\rightarrow V_{12}\rightarrow V_{23}$.

Design of multiple-exposure DNP experiments

The goal of the multiple-exposure experiments was to determine the effects of different histories of successive DNP exposures, up to a maximum of three successive exposures. Throughout one multiple-exposure experiment, the DNP concentration was the same in each of the exposed vessels. Each experiment was conducted in three phases. In phase 1, there was a stressed system and a nonstressed control (Fig. 1b). After glucose depletion in the first phase, solution from each of these vessels was then transferred to fresh media, creating now in the second phase, three vessels in which one system had a history of no exposures ($V_{00}\rightarrow V_{10}$), one received a single exposure ($V_{00}\rightarrow V_{11}$), and the other had two exposures ($V_{01}\rightarrow V_{12}$), In the third phase, inoculations were made to fresh solutions with the end result of four parallel vessels, each with a different series of previous exposures. In the series that was never exposed to DNP ($V_{00} \rightarrow V_{10} \rightarrow V_{20}$), the inoculation from one vessel to the next allowed for quantification of the stress attributable solely to the transfer of organisms. Accounting for this in the data interpretation allowed for the stress effect from DNP exposure to be isolated. The other series involved one ($V_{00} \rightarrow V_{10} \rightarrow V_{21}$), two ($V_{00} \rightarrow V_{11} \rightarrow V_{22}$), and three ($V_{01} \rightarrow V_{12} \rightarrow V_{23}$) exposures.

DATA INTERPRETATION

For each respirometric data set from a reactor vessel, biological growth parameters were inferred by relating the rate of change of oxygen uptake to the rates of change of substrate utilization and biomass growth. Using the methods previously presented [21], regression of the data for oxygen uptake over time produced estimates of the growth kinetic parameters, μ_{max} and $\log(K_s)$, the endogenous decay coefficient b, and the initial biomass concentration, X^0.

In the multiple-exposure DNP experiments, one of the observations of interest is the change in b for a sequential pair of vessels within a series. This change in b may be attributed to two effects: the stress attributable to transfer from one vessel to the next, and the stress attributable to exposure to the chemical. Accounting for the stress attributable to the transfer was particularly important in the multiple-exposure experiments because it was a part of the variable stress history of each series. The stress caused by chemical exposure was quantified by subtracting from the total change in b that which is attributed to the transfer effect, as determined from the corresponding sequential pair of nonexposed controls. Using the sequence $V_{01}\rightarrow V_{12}$ as an example, the total change in b is

$$\Delta b_{12} = b_{12} - b_{01} \tag{4}$$

where the notational subscripts for b follow the vessel notation depicted in Figure 1b. For this sequential pair of vessels, the transfer effect is determined from the $V_{00} \rightarrow V_{10}$ sequence. Assuming the stressor and transfer effects are additive, the change in b attributable solely to the effect of the chemical stressor, Δb_s, is

$$\Delta b_{s\_12} = \Delta b_{12} - (b_{10} - b_{00}) \tag{5}$$

Equation 5 can alternatively be written as

$$\Delta b_{s\_12} = (b_{12} - b_{10}) - (b_{01} - b_{00}) \tag{6}$$

Thus, for a sequential pair of vessels, the effect of chemical stress is the difference between the change in b in the stressed vessel and the change in b in corresponding nonexposed controls. For each exposed vessel (for which there are six per experiment), we denote this latter difference as an adjusted value of the endogenous decay coefficient, b^*_{ij}:

$$b^*_{ij} = b_{ij} - b_{i0} \tag{7}$$

where i denotes the number of transfers in the history of the vessel (0, 1, or 2), and j denotes the number of times it has been exposed to DNP (1, 2, or 3). In general, the change in b attributable solely to the chemical stressor is

$$\Delta b_{s\_ij} = b^*_{ij} - b^*_{i-1,j-1} \tag{8}$$

which is a general form of Equation 6. In each experiment, five such values occur for the exposed vessels in phases 2 and 3.

The other important measure of stress is the reduction in biomass concentration immediately after exposure to the stressor. Consistent with findings in the previously reported single-exposure studies [21], in the current study, when bacteria were transferred from one vessel to the next, the initial biomass concentrations were found to be reduced (relative to what is predicted based on the ending biomass concentration in the previous vessel, adjusted for dilution). Here, the initial biomass concentration is that which is inferred to be the biomass concentration after the transfer and initial exposure to chemical stressor. Unlike the additive manner in which chemical and transfer stress effects were accounted for in the endogenous decay coefficient (Eqn. 8), here the effects are modeled as multiplicative, or fractional. For example, the initial biomass concentration in V_{12}, X^0_{12}, is related to the dilution-adjusted biomass concentration from the end of the run in V_{01}, X^E_{01}.

$$X^0_{12} = (1 - f_{s\_12}) * (1 - f_{t\_12}) * X^E_{01} \tag{9}$$

where f_s is the fraction of biomass lost because of chemical stress, and f_t is the fraction of biomass lost because of transfer. The reduction in biomass attributable to transfer alone is equated with the biomass reduction in the control vessels for the same phase in the experiment. For V_{12}, the value of f_{t_12} is thus equated with f_{t_10}, which is determined from biomass reduction observed in the control vessels $V_{00}\rightarrow V_{10}$.

$$f_{t\_12} = f_{t\_10} = \frac{X^E_{00} - X^0_{10}}{X^E_{00}} \tag{10}$$

Combining Equations 9 and 10, generalizing, and solving for f_s results in an expression for the fractional reduction in biomass attributable to chemical stress:

$$f_{s\_ij} = 1 - \left(\frac{X^E_{i-1,0}}{X^E_{i-1,j-1}}\right)\left(\frac{X^0_{ij}}{X^0_{i,0}}\right) \tag{11}$$

In the current study, Equation 11 was used to interpret the values of biomass concentration in the multiple-exposure experiments.

In the phase 1 vessels (transfer 0), no dilution or transfer effects occur. For the vessel V_{01}, the fractional change in X^0 due to chemical stress is determined by comparison with V_{00}. That is,

$$f_{s\_01} = \frac{X^0_{0,0} - X^0_{0,1}}{X^0_{0,0}} \tag{12}$$

Inferences about significant differences in b^*_{ij} as well as differences attributable to first exposure in the initial biomass concentration were based on a t test that compared the parameters describing biological activity in the stressed and non-

stressed systems. In most cases, two replicate data sets existed, from which the parameter values were averaged and used for comparison of the stressed and the nonstressed systems. To estimate standard deviation, we used data sets obtained before the current study. A set of experiments had been conducted in which stressed (with a single exposure) and nonstressed systems each yielded two replicate data sets of oxygen uptake, each set consisting of approximately 150 data points. The replicate data sets were each fitted to the model, resulting in two replicate values of the parameter set. The large number of data points used in the regressions led to high precision in parameter estimation. The replicate values were used to calculate the standard deviation of each parameter. Using data sets of the experiments conducted over two years, we obtained approximately 20 such values of the standard deviation of each parameter. These standard deviation values were pooled and averaged. This average standard deviation was used as a measure of variability of each parameter in the t test. The variance in each parameter was estimated separately for the stressed and non-stressed systems and are documented in Ray [41]. The variance in b was estimated to be 0.0021 and 0.002 in the stressed and non-stressed cases, respectively. The variance in the initial biomass concentration at the first exposure was estimated to be 0.24 and 0.22 in the stressed and non-stressed cases, respectively. Differences were considered significant at the 90% confidence level. The uncertainty analysis for the parameter $f_{s\_i,j}$, which is multiplicative rather than additive, is described in the Supplemental Data.

RESULTS

Two-exposure experiments

The stressor concentrations used in the two-exposure experiments are included in Table S1 in the Supplemental Data. The concentrations in the first phase varied from a low dose, which was likely to have a small effect on metabolism, to a high dose, likely to affect it strongly and possibly have a toxicity effect. The concentrations in the second phase were designed to all be equal for the experiments for a particular stressor, but experimental variability led to small differences, as shown in Table S1. For each chemical, the concentration selected for the second phase of exposure was known from previous findings to affect metabolism by increasing the coefficient of endogenous decay [21]. Table S1 also shows the complete experimental results for the two-exposure experiments, including the endogenous decay coefficients, the biomass growth parameters, and inferred initial biomass concentrations.

The parameter most significantly affected is the coefficient of endogenous decay, b. In Figure 2, these values are plotted against pre-exposure stressor concentration for each of the stressors, in which each data point is the average of two values from replicate vessels. One comparison that can be made is the difference between the exposed and nonexposed controls in phase 1, which is analogous to the single-exposure experiments reported previously [21]. All three stressors cause a larger b compared with the nonstressed control. This effect is greatest for DNP and is found to increase with DNP concentrations. At higher concentrations, all stressors, in addition to affecting b, cause other effects such as reducing the specific growth rate μ_{max} and the initial biomass concentration X^0 (see Table S1). For example, DNP at 270 mg/L reduced X^0, which, as explained in our previous work [21], is attributed to the death of a portion of the population because of initial exposure. Stressor PCP, at and above 70 mg/L, reduced μ_{max} as well as X^0. Stressor NEM at 0.5 mg/L reduced X^0. These results of the first exposure are consistent with those of our previous study [21].

In the second phase, for all three stressors, the values of b in the pre-exposed populations are slightly but consistently larger than for the non–pre-exposed control (Fig. 2). This implies a larger metabolic stress response for populations that have a recent history of pre-exposure, even though the stressor concentration in phase 2 was in many cases smaller than that in phase 1. Some of these differences in b were small, even though statistically significant at the 90% confidence level. For DNP and PCP, these differences did not show a specific trend with increasing pre-exposure concentrations. Also, no statistically significant changes occurred in the growth parameters or in X^0 (with the exception of a minor effect for DNP at 100 mg/L) (Table S1). The absence of changes in μ_{max}, log (K_s), and X^0 implies that the stress effects in phase 2, albeit small, are attributable to shifts in metabolism and not growth inhibition or toxicity.

Another way to examine the effect of pre-exposure is to compare the values of b between phase 2, pre-exposed (exposed twice), and phase 1 (exposed once). In Figure 2, we see that stressor DNP, for which the largest concentration range was tested, induced a higher b in the same population at the second exposure compared with the first. This indicates that a population may be more stressed at a second exposure to DNP compared with the first. This was true at all first-exposure

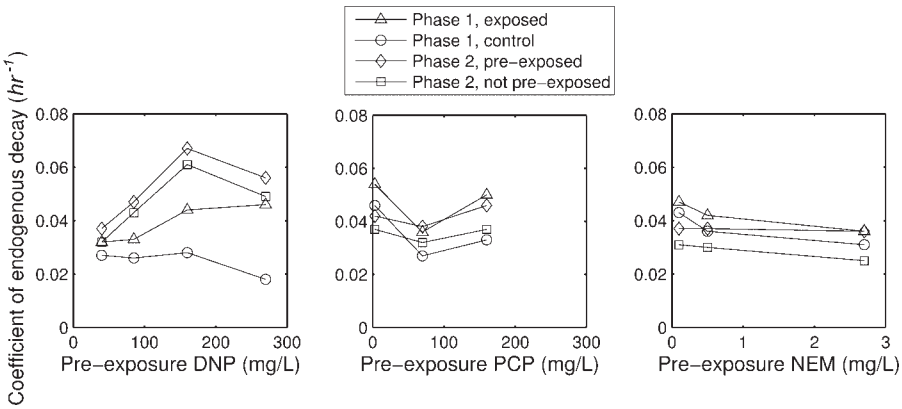

Fig. 2. Coefficient of endogenous decay measured in phase 1 (exposed to stressor and control) and phase 2 (pre-exposed in phase 1 and control) at various pre-exposure concentrations of 2,4-dinitrophenol (DNP), pentachlorophenol (PCP), and *N*-ethyl maleimide (NEM). The phase 2 concentrations were, on average, 95 mg/L DNP, 77 mg/L PCP, and 0.9 mg/L NEM. Each data point is the average of two replicates. Standard deviations were calculated according to the method described in the Data Interpretation section and have values of 0.0021 and 0.002 in the stressed and nonstressed cases, respectively.

concentrations and even when the first exposure concentration (at DNP 170 and 270 mg/L) was higher than the second. This is consistent with the conclusion drawn by comparing the two phase 2 outcomes, described previously.

Multiple-exposure experiments

Five multiple-exposure experiments were conducted at DNP concentrations ranging from 400 mg/L to 1,200 mg/L. These concentrations represent averages of the actual achieved DNP concentrations in each vessel; the exact concentrations are shown in Table S2 (Supplemental Data). These concentrations were selected so that they were higher than those in the two-exposure experiments, which had resulted in small effects. The higher the concentration of DNP, the longer was the duration of the experiment. The approximate duration of each experiment was 4 to 5 d. This duration was considerably greater than the average doubling time of 0.5 h. 2,4-dinitrophenol alone was selected for multiple-exposure study because its high solubility in water compared with PCP and high toxicity threshold compared with NEM enabled the exploration of a wide range of concentrations. At concentration 900 mg/L, two replicate experiments (labeled A and B) were conducted at different times. At the highest DNP concentration tested, 1,200 mg/L, all biological activity ceased, indicating that at this high stressor concentration the population did not survive [41].

Table S3 (Supplemental Data) shows the growth parameters μ_{max} and log (K_s) inferred from the multiple-exposure experimental data. The impact of DNP on these growth parameters was examined; however, changes in these parameters were not found to be correlated with varying DNP concentrations.

Table S4 (Supplemental Data) lists the coefficients of endogenous decay b, which provide the raw data for calculation of values of the parameter b^*_{ij}. The values of b^*_{ij} are arranged in Figure 3 in subplots according to sequence. In the sequence $V_{00}\rightarrow V_{10}\rightarrow V_{21}$, b^*_{ij} is defined only for V_{21} because the other two vessels are non-exposed controls. Hence, in Figure 3a values of b^*_{00} and b^*_{10} are represented as zero. Similarly, in the sequence $V_{00}\rightarrow V_{11}\rightarrow V_{22}$, values of b^*_{00} are represented as zero (Fig. 3b).

An increase in b^*_{ij} (positive value of Δb_s) implies that the population is more stressed compared with the previous exposure, and a decrease (negative value of Δb_s) indicates that stress is lowered. In the sequence $V_{00}\rightarrow V_{10}\rightarrow V_{21}$, for each of the three experiments, the value of b^*_{21} is positive, leading to a positive value of Δb_{s_21} (Fig. 3a). This positive value implies that the first exposure to DNP leads to an increase in stress in the population. This effect increases with DNP concentration, as shown by the value of Δb_{s_21} being smaller at DNP 400 mg/L than for the other two results at 900 mg/L. This increase in stress attributable to a single DNP exposure is consistent with our previous findings [21], and with the DNP results in the two-exposure experiments.

In the sequence $V_{00}\rightarrow V_{11}\rightarrow V_{22}$, the value of b^*_{ij} increases for both the first and second exposures to DNP at all DNP concentrations (Fig. 3b). An interesting observation is the dissimilar behavior of Δb_{s_22} at different DNP concentrations. At DNP 400 mg/L, Δb_{s_22} is greater than Δb_{s_11}, indicating that the population is more stressed at the second exposure than at the first. However, at DNP 800 and 900 mg/L, Δb_{s_22} is less than Δb_{s_11}, indicating that the population is less stressed at the second exposure compared with the first.

In the sequence $V_{01}\rightarrow V_{12}\rightarrow V_{23}$, the response of the population varies with each DNP concentration (Fig. 3c). At the lowest concentration of 400 mg/L, the stress remains constant at the first two exposures (similar values of Δb_{s_01} and Δb_{s_12}) but increases after the third exposure (positive value of Δb_{s_23}). At the medium concentration of 800 mg/L, the stress remains constant in the first two exposures but decreases at the third exposure (negative value of Δb_{s_23}). At the high concentration of 900 mg/L, both experiments show that the stress decreases after the second exposure (Δb_{s_12} is negative) and decreases still further after the third exposure (Δb_{s_23} is still more negative).

Figure 4 shows observed reductions in the initial biomass concentration for the sequence with the most exposures, $V_{01}\rightarrow V_{12}\rightarrow V_{23}$, expressed as the percentage of biomass lost, 100^*f_s, at each exposure. (Table S5 in Supplemental Data reports the full list of error values for all three sequences. The Supplemental Data includes the statistical analysis for the error calculation. The raw biomass concentration data for calculating $100{\cdot}f_s$ are reported in Ray [41]). Although the errors associated with each data point are large, some general conclusions may be made. At the low DNP concentration of 400 mg/L, no reduction in initial biomass concentration

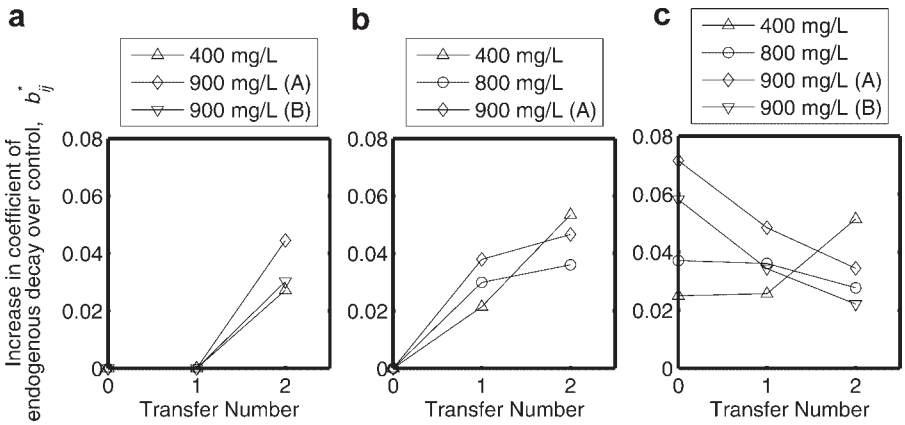

Fig. 3. Increase in b over control (b^*_{ij}) in the sequence $V_{00}\rightarrow V_{10}\rightarrow V_{21}$ at 2,4-dinitrophenol (DNP) concentrations 400 mg/L, 900 mg/L (A), and 900 mg/L (B) (**a**); in the sequence $V_{00}\rightarrow V_{11}\rightarrow V_{22}$ at DNP concentrations 400 mg/L, 800 mg/L, and 900 mg/L (**b**); and in sequence $V_{01}\rightarrow V_{12}\rightarrow V_{23}$ at stressor concentrations 400 mg/L, 800 mg/L, 900 mg/L (A), and 900 mg/L (B) (**c**). In sequences $V_{00}\rightarrow V_{10}\rightarrow V_{21}$ (a) and $V_{00}\rightarrow V_{11}\rightarrow V_{22}$ (b), the b^*_{ij} values are missing at DNP concentrations 800 mg/L and 900 mg/L, respectively, because of leaks in the experimental system. The number of replicates is shown in Figure 1b. Standard deviations were calculated according to the method described in the Data Interpretation section and have values of 0.0021 and 0.002 in the stressed and nonstressed cases, respectively.

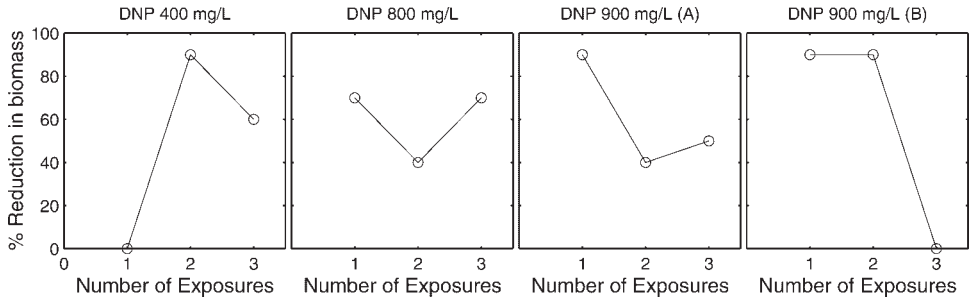

Fig. 4. Percent reduction in biomass at each exposure in vessels $V_{01} \rightarrow V_{12} \rightarrow V_{23}$ at stressor concentrations 400 mg/L, 800 mg/L, 900 mg/L (A), and 900 mg/L (B). The number of replicates is shown in the corresponding sequence in Figure 1b. Uncertainties associated with each data point at exposures 1, 2, and 3, respectively, are as follows: Dinitrophenol (DNP) 400 mg/L: $\pm 0, \pm 30, \pm 20$, DNP 800 mg/L: $\pm 20, \pm 40, \pm 70$, DNP 900 mg/L A: $\pm 6, \pm 9, \pm 12$, DNP 900 mg/L B: $\pm 40, \pm 20, \pm 30$.

occurred at the first exposure, but a substantial reduction occurred for the subsequent exposures. This population also sustained an increase in stress at the subsequent exposures, as indicated by the increase in endogenous decay (Fig. 3a). At DNP 800 mg/L, each exposure reduced the initial biomass concentration (Fig. 4), the reductions being statistically similar. This population sustained a decrease in stress at the third exposure (Fig. 3b). In both experiments at the high DNP concentration of 900 mg/L, the first exposure caused large reductions in initial biomass concentration, which are statistically similar to each other. The reduction in initial biomass was substantially less for subsequent exposures. Both populations experience a decrease in stress at the second and third exposures (Fig. 3c).

DISCUSSION

The stress observation results show that multiple exposures to a low DNP of 400 mg/L as well as two exposures to a range of low concentrations of DNP, PCP, and NEM cause an increase in stress. At intermediate concentrations of 800 and 900 mg/L, multiple exposures to DNP lead to a decrease in stress. At a high concentration of 1,200 mg/L, a single exposure to DNP stops biological activity. To summarize, for DNP, a concentration window exists, within which multiple exposures to a chemical stressor eventually lead to a decrease in stress in the population, below which multiple exposures cause an increase in stress, and above which a single exposure stops biological activity.

Previous studies have found that exposure to chemical stressors cause inhibition of growth or reduced survival in microbiological populations [5,8,42], but that the inhibitory or lethal impact is mitigated by pre-adaptation to either the same stressors or different ones that can cross-protect [43–45]. For example, increasing pre-exposure dose of stressor toluene ensured increased survival of *P. putida* in the presence of various antibiotics [43].

In general, the studies reporting microbiological adaptation to chemical stressors implicitly assume the homogeneity of the population in terms of their biochemistry and responsiveness [27]. Our findings of a concentration window within which multiple exposures eventually reduce stress may be explained by acknowledging that the population is heterogeneous. We speculate that the chemical stressor exerts a selection force on this heterogeneous population. Below a particular stressor concentration, the selection force is not strong enough to eliminate the weaker members of the population, which, as a result, is stressed. Above a certain concentration, the toxic effect of the stressor eliminates the entire population. Within the intermediate concentration range, the selective force eliminates the weaker members, leaving behind a higher fraction of the organisms that are capable of adapting.

Support of this hypothesis lies in the observed reductions of initial biomass concentrations, particularly those obtained from the sequence with the maximum exposures. These results showed that for DNP concentrations that caused a large reduction in biomass, a subsequent reduction in stress occurred. The reductions in biomass concentration are different in each 900-mg/L experiment at the second and third exposures, but the sum of the reductions in the second and third exposures are similar in both experiments.

These results suggest that DNP concentrations of 800 and 900 mg/L eliminate some organisms in the population that, perhaps because of epigenetic differences, are less capable of adapting to the environmental change. This would leave behind a higher fraction of the more adaptable organisms, rendering the remaining population more resilient to the stressor. At the lower stressor concentration of 400 mg/L, the population has less of a metabolic response but may retain its weak and its strong members. In fact, the subpopulation that survives an environmental perturbation is believed to retain the capability of generating all phenotypes present within the original population [30], which may explain the reduction in biomass concentration at each subsequent exposure.

The reduction of stress within a particular concentration range suggests that multiple exposures to a chemical stressor may enable a bacterial population to develop resilience to the stressor. This finding may facilitate better risk assessment of natural and engineered systems such as wastewater treatment plants and contaminated sites when exposed to chemical stress.

SUPPLEMENTAL DATA

Table S1. Parameter estimates from two-exposure experiments.

Table S2. DNP concentrations from the multiple-exposure experiments.

Table S3. Values of μ_{max} and log (K_s) from the multiple-exposure experiments.

Table S4. Values of b from the multiple-exposure experiments

Table S5. Percentage reduction attributable to chemical stress from the multiple-exposure experiments. (197 KB DOC)

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