# Linear Free Energy Relationships for the Biotic and Abiotic Reduction of Nitroaromatic Compounds

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S *Supporting Information*

ABSTRACT: Nitroaromatic compounds (NACs) are ubiquitous environmental contaminants that are susceptible to biological and abiotic reduction. Prior works have found that for the abiotic reduction of NACs, the logarithm of the NACs' rate constants correlate with one-electron reduction potential values of the NACs ($E^1_{H,NAC}$) according to linear free energy relationships (LFERs). Here, we extend the application of LFERs to the bioreduction of NACs and to the abiotic reduction of NACs by bioreduced (and pasteurized) iron-bearing clay minerals. A linear correlation ($R^2 = 0.96$) was found between the NACs' bioreduction rate constants (k_{obs}) and $E^1_{H,NAC}$ values. The LFER slope of log k_{obs} versus $E^1_{H,NAC}/(2.303RT/F)$ was close to one (0.97), which implied that the first electron transfer to the NAC was the rate-limiting step of bioreduction. LFERs were also established between NAC abiotic reduction rate constants by bioreduced iron-bearing clay minerals (montmorillonite SWy-2 and nontronite NAu-2). The second-order NAC reduction rate constants (k) by bioreduced

SWy-2 and NAu-2 were well correlated to $E^1_{H,NAC}$ ($R^2 = 0.97$ for both minerals), consistent with bioreduction results. However, the LFER slopes of log k versus $E^1_{H,NAC}/(2.303RT/F)$ were significantly less than one (0.48–0.50) for both minerals, indicating that the first electron transfer to the NAC was not the rate-limiting step of abiotic reduction. Finally, we demonstrate that the rate of 4-acetylnitrobenzene reduction by bioreduced SWy-2 and NAu-2 correlated to the reduction potential of the clay ($E_{H,clay}$, $R^2 = 0.95$ for both minerals), indicating that the clay reduction potential also influences its reactivity.
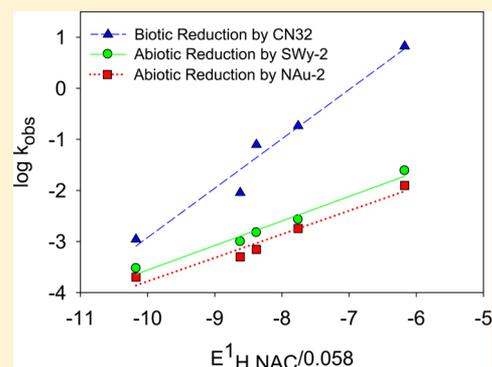
INTRODUCTION

Nitroaromatic compounds (NACs) are widespread environmental contaminants generated from the production of chemical intermediates, dyes, pesticides, and explosives. In anoxic environments, the reduction of NACs to the corresponding aniline products is often thermodynamically favorable.[1,2] NAC reduction yields products that biodegrade faster than the parent compounds but can be more toxic.[3–5] NACs can be reduced abiotically by several commonly occurring natural reductants (e.g., Fe(II) bound to iron oxides, iron(II)-bearing clay minerals, sulfide, and reduced natural organic matter).[2,3,6–11] In these abiotic reduction studies, significant correlations have been found between standard one-electron reduction potential of NACs ($E^1_{H,NAC}$) and the logarithm of the reduction rate constant (k).[2,7–9] This correlation is commonly referred to as a linear free energy relationship (LFER)

$$\log k = a \times E^1_{H,NAC}/(2.303RT/F) + b \tag{1}$$

where a and b are regression constants, R is the universal gas constant, T is the absolute temperature, and F is the Faraday constant. LFERs are extremely useful in predicting contaminant reactivity based on readily attainable thermodynamic data. To date, LFERs have only been reported for the abiotic reduction

of NACs, yet NACs can be biologically reduced by a variety of microorganisms.[6,12]

Iron-bearing clay minerals are ubiquitous in the environment,[13] and the clay-Fe(II)/Fe(III) redox couple plays important roles in biogeochemical cycling and in determining the fate and transport of several classes of environmental contaminants, including pesticides, toxic metals, radionuclides, nitroaromatic explosives, and chlorinated solvents.[14–20] Iron-bearing clay minerals are of great importance for the reductive transformation of NACs in anoxic environments. LFERs have been developed for NAC reduction by fully [i.e., 100% Fe(II)] chemically reduced iron-bearing clay minerals.[3] However, clay-Fe(III)-reducing microbes can rarely reduce 100% of the Fe(III) in iron-rich clay minerals.[21,22] In fact, we found that less than 40% of Fe(III) in iron-rich smectite could be reduced by an Fe(III)-reducing microbe because of thermodynamic constraints.[21] In addition, biological and chemical reduction of iron-rich clay minerals yields spectroscopically different clay mineral products.[23] In a recent study, we found that biologically and chemically reduced iron-bearing clay minerals reduce

Table 1. First-Order Rate Constants (k_{obs}) for Nitroaromatic Compounds (NACs) Bioreduced by *Shewanella putrefaciens* CN32[a]

NAC	abbreviation	$E_{H,NC}^1$ (V)	k_{obs} (h^{-1})	R^2
4-acetylnitrobenzene	4-AC-NB	−0.358	6.67 ± 0.23	0.989
4-chloronitrobenzene	4-Cl-NB	−0.450	0.184 ± 0.0028	0.999
nitrobenzene	NB	−0.486	0.0787 ± 0.0021	0.992
4-methylnitrobenzene	4-CH$_3$-NB	−0.500	0.009 ± 0.00057	0.995
4-aminonitrobenzene	4-NH$_2$-NB	−0.590	0.0011 ± 0.00008	0.995
2-methylnitrobenzene	2-CH$_3$-NB	−0.590	0.0118 ± 0.00014	0.997

[a]All experiments conducted with 1×10^8 cells mL^{-1} CN32, 100 μM NAC, and 5 mM lactate in 10 mM PIPES, pH 6.8 at 20 °C. k_{obs} reported as mean ± standard deviation for three replicate rate estimates.

nitrobenzene at different rates.[24] These results highlight the need to study the reduction of NACs by bioreduced iron-bearing clay minerals.

Prior studies have provided important mechanistic insights into redox reactions that occur between iron-bearing clay minerals and contaminants.[14−20] In some cases, researchers found that contaminant reduction potentials relate to reduction rates.[2,7−9] What remained unclear from these studies was if and how the reduction potential of structural Fe(II) in the clay mineral ($E_{H,clay}$) also influenced contaminant reduction rates. Answering this question has been historically challenging due to difficulties in measuring $E_{H,clay}$. Recently, however, our group developed a mediated electrochemical technique to measure reduction potential values for structural Fe in clay minerals as a function of Fe(II)/Total$_{Fe}$.[25,26] These measurements provided the first redox profile distributions to relate the reduction extent of structural Fe(II) to $E_{H,clay}$, which now makes correlations between $E_{H,clay}$ and contaminant reduction rates possible.

The objectives of this study were to (1) extend the application of LFERs to the biological reduction of NACs, (2) establish LFERs for the abiotic reduction of NACs using bioreduced iron-bearing clay minerals, and (3) investigate if contaminant reduction rates also correlated with $E_{H,clay}$ values of two iron-bearing clay minerals. We bioreduced NACs directly using *Shewanella putrefaciens* CN32. The genus *Shewanella* is an important dissimilatory metal-reducing bacteria (DMRB) and has been studied intensively in terms of the biogeochemistry of Fe(III), U(VI) and NAC reduction.[6,21] The NACs selected have well-characterized $E_{H,NAC}^1$ values that vary as a function of substituent group type and binding location.[27] We selected SWy-2 and NAu-2 as our model clay minerals because (i) we have previously characterized their $E_{H,clay}$ distributions and (ii) each has substantially different structures, $E_{H,clay}$ distributions, and structural Fe contents, allowing for identification of mineral-dependent trends.[25,26]

MATERIALS AND METHODS

Microorganism and Culture Conditions. *Shewanella putrefaciens* strain CN32 was grown aerobically on tryptic soy broth without dextrose (Difco) at 20 °C, and cells were harvested and prepared anaerobically as previously described.[6,28]

Nitroaromatic Compounds. NACs and corresponding reduction products included 4-acetyl-nitrobenzene (4-AC-NB), 4-chloro-nitrobenzene (4-Cl-NB), nitrobenzene (NB), 4-methyl-nitrobenzene (4-CH$_3$-NB), 4-amino-nitrobenzene (4-NH$_2$-NB), 2-methyl-nitrobenzene (2-CH$_3$-NB), 4-acetyl-aniline, 4-chloro-aniline, aniline, 4-methyl-aniline, 1,4-diamino-benzene, and 2-methyl-aniline. All chemicals were reagent grade (Sigma-Aldrich). NACs and corresponding reduction

products were used to prepare 0.16 M stock solutions in methanol. The abbreviations and $E_{H,NAC}^1$ values of the NACs are provided in Table 1.

Minerals. Both nontronite NAu-2 and montmorillonite SWy-2 were purchased from the source clays repository of the Clay Minerals Society (West Lafayette, IN). The solid-phase mineral compositions of NAu-2[29] and SWy-2[17] have previously been reported as

NAu-2

$$M_{0.72}^+ - [Si_{7.55}Al_{0.16}Fe_{0.29}][Al_{0.34}Fe_{3.54}Mg_{0.05}]O_{20}(OH)_4$$

where M may be Ca, Na, or K

SWy-2

$$(Ca_{0.16}Na_{0.24}) - [Si_{6.73}Al_{1.27}][Al_{1.45}Fe_{0.01}^{2+}Fe_{0.12}^{3+}Mg_{0.44}]O_{20}(OH)_4$$

NAu-2 and SWy-2 were suspended in 0.5 M NaCl for 24 h, then separated by centrifugation, yielding the 0.5−2.0 μm clay size fraction. The clay fraction was washed with distilled deionized water (Milli-Q) repeatedly until no Cl$^-$ was detected by silver nitrate and then dried at 60 °C. Mössbauer spectroscopy of the unaltered clay minerals revealed no Fe oxide impurities (mineral phase detection limit ∼2% of Fe mass).[13,22] On the basis of an anoxic HF-H$_2$SO$_4$/phenanthroline digestion,[30] the NAu-2 clay fraction contained 4.1 mmol of Fe/g clay and 99.4% Fe(III), while the SWy-2 clay fraction contained 0.40 mmol of Fe/g clay and 97.3% Fe(III). NAu-2 and SWy-2 clay fraction stock solutions (20 g/L) were prepared in anoxic 10 mM PIPES [piperazine-*N,N*′-bis(2-ethanesulfonic acid), pK_a = 6.8] buffer adjusted to pH 6.8.

Bioreduction of NACs. All experiments were conducted in 30 mL glass serum bottles crimp-sealed with Teflon-faced rubber stoppers and Al caps. All preparations were performed in an anoxic chamber (Coy, Grass Lakes, MI) supplied with a 95:5 N$_2$/H$_2$ gas mix. The anoxic chamber was in a 20 °C constant-temperature room. Reactors were filled with ∼20 mL of deoxygenated 10 mM PIPES buffer (pH 6.8) containing CN32 (1×10^8 cell/mL) and six different NACs (100 μM, Table 1). Sodium lactate (5 mM) was provided as the electron donor. All treatments and no-CN32 controls were run in triplicate. Reactors were incubated at 100 rpm on orbital shakers within the anoxic chamber. After cell inoculation, samples were periodically removed with sterile needles and syringes. Samples were analyzed for NACs and their metabolites as described below.

Abiotic Reduction of NACs by Bioreduced Iron(III)-Bearing Clay Minerals. Bioreduced clay minerals were prepared using CN32 and NAu-2 or SWy-2. Reactors were filled with 20 mL of deoxygenated 50 mM PIPES buffer (pH 6.8) containing CN32 (1×10^8 cell/mL), NAu-2 (1.0 g/L), or

Table 2. Reduction Rate Constants for the Abiotic Reduction of NACs by Clay-Fe(II) in Bioreduced (and Pasteurized) Montmorillonite SWy-2 or Nontronite NAu-2[a]

clay-Fe(II)	clay (g L^{-1})/ clay-Fe(II) (mM)	NAC	first-order model		second-order model	
			k_{obs} (h^{-1})	R^2	k (M^{-1} h^{-1})[b]	R^2
95% bioreduced SWy-2	4.0/1.52	4-acetylnitrobenzene	0.0245 ± 0.00057	0.957	18.30 ± 0.41	0.967
		4-chloronitrobenzene	0.0027 ± 0.00014	0.887	1.94 ± 0.12	0.905
		nitrobenzene	0.0015 ± 0.000062	0.890	1.08 ± 0.043	0.908
		4-methylnitrobenzene	0.0010 ± 0.000013	0.839	0.72 ± 0.012	0.859
		4-aminonitrobenzene	0.0003 ± 0.000011	0.842	0.22 ± 0.0082[c]	0.856[c]
					0.21 ± 0.0082[d]	0.864[d]
					0.20 ± 0.0082[e]	0.871[e]
38% bioreduced NAu-2	1.0/1.56	4-acetylnitrobenzene	0.0125 ± 0.00038	0.950	8.99 ± 0.27	0.960
		4-chloronitrobenzene	0.0018 ± 0.000063	0.987	1.25 ± 0.046	0.992
		nitrobenzene	0.0007 ± 0.000022	0.970	0.46 ± 0.016	0.977
		4-methylnitrobenzene	0.0005 ± 0.000023	0.978	0.32 ± 0.014	0.984
		4-aminonitrobenzene	0.0002 ± 0.0000061	0.887	0.10 ± 0.0032[c]	0.893[c]
					0.09 ± 0.0032[d]	0.897[d]
					0.09 ± 0.0032[e]	0.901[e]

[a]All experiments conducted with [NACs]$_0$ = 60 μM and bioreduced clay minerals in 50 mM PIPES, pH 6.8 at 20 °C. Bioreduced clay minerals were prepared using CN32 (1 × 10^8 cell/mL), NAu-2 (1.0 g/L), or SWy-2 (4.0 g/L). Cell−clay mineral suspensions were pasteurized (75 °C for 60 min, three times over 10 days) to deactivate biological activity. k_{obs} and k reported as mean ± standard deviation for three replicate rate estimates. [b]Regression slope of ln({[NAC]*[clay-Fe(II)]$_0$}/{[NAC]$_0$*[clay-Fe(II)]})/{[clay-Fe(II)]$_0$-6*NAC]$_0$} versus time. [c]n = 6 was used in eq 4 for the reduction of 4-aminonitrobenzene to the corresponding substituted aniline. [d]n = 4 was used in equation 4 for the reduction of 4-aminonitrobenzene to the corresponding hydroxylamino intermediate product. [e]n = 2 was used in eq 4 for the reduction of 4-aminonitrobenzene to the corresponding azo intermediate product.

SWy-2 (4.0 g/L). Differing clay suspension concentrations were used to account for the higher Fe content in NAu-2 and the greater bioreducibility of structural Fe(III) in SWy-2. Sodium lactate (5 mM) was provided as the electron donor. Reactors were incubated in anoxic chamber (20 °C) at 100 rpm for 50 days, at which point the clay-Fe(II) concentration remained near-constant. Cell-clay mineral suspensions were pasteurized (75 °C for 60 min, three times over 10 days) to deactivate biological activity. No further attempt was made to remove spent biomass because reported removal procedures (3-day digestion in 10% NaOH[31]) are severe enough to influence the solubility, redox status, and reactivity of iron-bearing clay minerals.

Several control reactors were prepared to account for NAC sorption and reactivity with spent biomass. Control reactors were prepared with pasteurized biomass and no clay minerals. No reduction of NACs by the spent biomass was observed over 60 days. Control reactors were prepared with pasteurized biomass added to chemically reduced clay minerals. The reduction rate and extent of the NACs were not influenced by the addition of spent biomass. Control reactors were prepared with no cells and unaltered clay minerals. Minimal sorption of NACs (<3% mass) to the clay minerals was measured over 60 days.

Abiotic reduction of NACs was conducted with biologically reduced (and pasteurized) clay minerals. Clay mineral suspension concentrations (g clay/L) were varied such that experiments began with equal clay-Fe(II) concentrations. For NAu-2, clay suspension concentrations were 1.0 g/L and initial clay-Fe(II) concentrations were 1.56 mM Fe(II). For SWy-2, clay suspension concentrations were 4.0 g/L and initial clay-Fe(II) concentrations were 1.52 mM Fe(II). Exact initial clay-Fe(II) concentrations for each mineral were not attained because of uncertainty regarding the final extents of bioreduction. NACs (4-AC-NB, 4-Cl-NB, NB, 4-CH$_3$-NB, and 4-NH$_2$-NB) were always added at a constant concentration

of 60 μM. Reactors were incubated at 100 rpm on orbital shakers within the anoxic chamber. Samples were periodically removed with sterile needles and syringes in the anoxic chamber for measurement of NACs and their metabolites.

Abiotic Reduction of 4-AC-NB by Partially Bioreduced Iron(III)-Bearing Clay Minerals. Bioreduced clay minerals with different Fe(III) reduction extents were prepared using CN32 and NAu-2 or SWy-2. Reactors were filled with 20 mL of deoxygenated 50 mM PIPES buffer (pH 6.8) containing CN32 (1 × 10^8 cell/mL), NAu-2 (2.0 g/L), or SWy-2 (8.0 g/L). Sodium lactate (5 mM) was provided as the electron donor. Reactors were incubated in anoxic chamber (20 °C) for different times to get different Fe(III) reduction extents. Cell-clay mineral suspensions were pasteurized to deactivate biological activity.

Abiotic reduction of 4-acetylnitrobenzene was conducted with biologically reduced (and pasteurized) clay minerals. For NAu-2, clay suspension concentrations were constant at 2.0 g/L, while the initial clay-Fe(II) concentrations varied from 0.7, 1.7, 2.4 to 2.8 mM Fe(II) (corresponding to Fe(III) reduction extents of 8%, 21%, 29%, and 34%). For SWy-2, clay suspension concentrations were constant at 8.0 g/L while the initial clay-Fe(II) concentrations varied from 0.6, 1.7, 2.3 to 3.0 mM Fe(II) (corresponding to Fe(III) reduction extents of 19%, 53%, 71%, and 95%). 4-AC-NB was added at a constant concentration of 50 μM. 4-AC-NB was selected because it reacted most rapidly with the reduced clay minerals. Reactors were incubated at 100 rpm on orbital shakers within the anoxic chamber. Samples were periodically removed with sterile needles and syringes in the anoxic chamber for measurement of NACs and their metabolites.

Analytical Methods. Dissolved NACs and their metabolites were measured with an HPLC equipped with a C18 column and photodiode array detector using a methanol/water (1/1, v/v) mobile phase. Sample suspensions were centrifuged at 14 100g for 10 min and the supernatant was used for NAC

analysis. Total clay-Fe(II) concentrations were measured using the whole suspension with a modified anoxic HF-H$_2$SO$_4$/phenanthroline digestion method.[30]

Kinetic Analyses. The rate of NAC reduction by CN32 and bioreduced (pasteurized) iron-bearing clay minerals was modeled as pseudo-first-order with respect to the NAC concentration according to[6]

$$-d[NAC]/dt = k_{obs} \times [NAC] \quad (2)$$

where k_{obs} is the first-order reduction rate constant (h^{-1}).

The rate of NAC reduction by clay-Fe(II) was also modeled as overall second-order with respect to the NAC concentration and the clay-Fe(II) concentration according to[32]

$$-d[NAC]/dt = k \times [NAC] \times [Fe(II)] \quad (3)$$

where k is the second-order abiotic reduction rate constant (M^{-1} h^{-1}) and [Fe(II)] is the total clay-Fe(II) concentration. In two previous studies on the abiotic reduction of nitrobenzene by clay-Fe(II) and hematite-Fe(II),[6,24] we have demonstrated an excellent electron balance between nitrobenzene transformation to aniline and mineral-Fe(II) consumption. Therefore, in situations where clay-Fe(II) was not measured, we calculated clay-Fe(II) according to

$$[Fe(II)] = [Fe(II)]_0 - n \times ([NAC]_0 - [NAC]) \quad (4)$$

where [Fe(II)] and [Fe(II)]$_0$ are the total clay-Fe(II) concentrations at time t and time 0 (M); [NAC] and [NAC]$_0$ are the NAC concentrations at time t and time 0 (M); and n in eq 4 accounts for the number of e$^-$ per mole for the reduction of substituted nitrobenzenes. In this study, $n = 6$ was used for the reduction of substituted nitrobenzenes to the corresponding substituted anilines.[3,6] An intermediate product was detected in experiments with 4-NH$_2$-NB. We did not identify the intermediate. Therefore, for 4-NH$_2$-NB, $n = 2$ and 4 were also used assuming a sequential transfer of 2 and 4 e$^-$ per mole for hydroxylamino and azo intermediates, respectively. Results showed that $n = 2$ or 4 did not change the model outcome significantly compared to $n = 6$ (Table 2). The persistence and accumulation of the intermediate occurred during both biotic and abiotic reduction experiments (Figures S1 and S2, respectively, in the Supporting Information).

■ RESULTS AND DISCUSSION

LFER for Bioreduction of Nitroaromatic Compounds. *Shewanella putrefaciens* CN32 was used to bioreduce these six NACs (Table 1). The bioreduction kinetics of all the NACs fit well to a pseudo-first-order rate model (eq 2, Figure 1). The first-order rate model yielded R^2 values greater than 0.98 (Table 1). Results for 4-Cl-NB showed that the parent compound was stoichiometrically reduced to the corresponding aniline compound (4-Cl-AN, Figure 1a). We observed similar stoichiometric conversion of substituted nitrobenzenes to their corresponding aniline products for all the NACs except 4-NH$_2$-NB. The bioreduction rate of 4-NH$_2$-NB was very slow compared with other NACs (Table 1) and an intermediate product accumulated in the experiment (Supporting Information Figure S1). CN32 was incapable of further transforming any of the aniline daughter products.

The NACs first-order bioreduction rate constants (k_{obs}) varied by approximately 3 orders of magnitude (Table 1). To probe the relationship between reaction kinetics and NAC one-electron reduction potentials ($E^1_{H,NAC}$), we constructed a linear
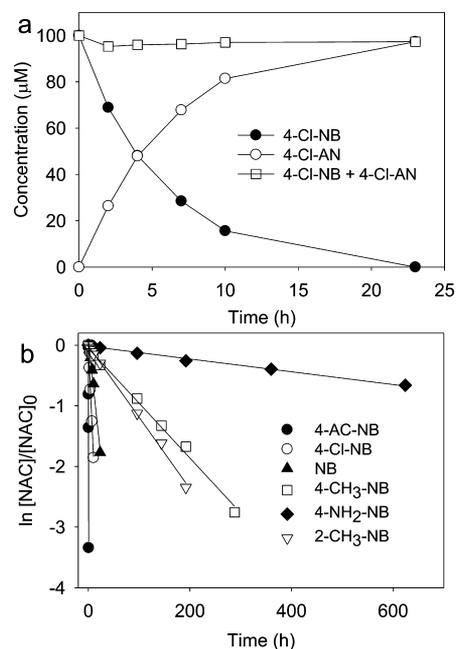

Figure 1. Bioreduction of NACs by *Shewanella putrefaciens* CN32. Experiments conducted with 1×10^8 cells mL^{-1} CN32, 100 μM NAC, and 5 mM lactate in 10 mM PIPES, pH 6.8 at 20 °C. (a) Reduction of 4-chloronitrobenzene (solid circles) to corresponding 4-chloroaniline (open circles). Open squares are the sum of 4-chloronitrobenzene plus 4-chloroaniline. (b) NACs concentrations transformed to ln([NAC]/[NAC$_0$]) versus time to calculate first-order reduction rate constants, k_{obs}.

free energy relationship (LFER), in which we plotted log k_{obs} values versus $E^1_{H,NAC}/(2.303RT/F)$ (Figure 2, where $2.303RT/F$

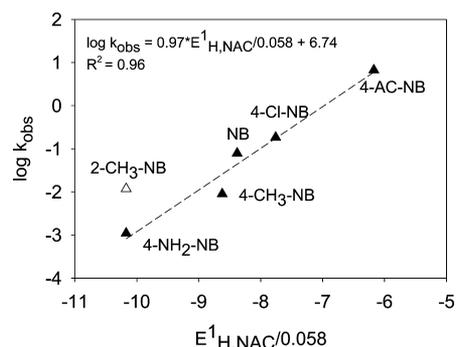**Figure 2.** Linear free energy relationship for first-order log k_{obs} versus $E^1_{H,NAC}/0.058$ (at 20 °C) for the bioreduction of six NACs by *Shewanella putrefaciens* CN32. The regression line was obtained after excluding 2-methylnitrobenzene.

= 0.058 at 20 °C). The LFER yielded an strong linear correlation, with an R^2 value of 0.96. 2-Methyl-nitrobenzene (2-CH$_3$-NB) was excluded from the regression because 2-substituted NBs have been shown to react differently as compared to NB, 3-substituted, and 4-substituted NBs for the abiotic reduction of NACs.[2,33] Specifically, 2-substituted NBs reacted much faster than expected from their $E^1_{H,NAC}$ values, consistent with this study. To our knowledge, these results are the first to demonstrate a LFER between log k_{obs} and $E^1_{H,NAC}$ for the bioreduction of NACs.

The LFER slope has previously been interpreted to determine the relative importance of electron transfer as

compared to other rate-limiting steps in the overall reaction. If the reaction was controlled purely by the first electron transfer step at $E_{H,NAC}^1$, a LFER slope of one would be expected.[2,3,9,33] If, however, the reaction was controlled by any other step, the LFER slope would be less than one. LFERs between log k and $E_{H,NAC}^1$ with slopes close to one have been previously reported for the abiotic reduction of NACs by hydroquinones, reduced natural organic matter, and aqueous Fe(II)-catechol complexes.[7,8,33]

Here, we show that the LFER slope was close to one (0.97, Figure 2) for NAC bioreduction, similar to other aqueous reductants. This implies that the first electron transfer step to the NACs (as the terminal electron acceptor) was the rate-limiting step in the bioreduction of NACs. It is tempting to extend this interpretation. The intracellular microbial reduction of NACs could involve transport of the NAC into the cell, delivery of the NAC to the appropriate respiratory enzyme, electron transfer from the NAC to the enzyme, release of the reduced NAC product, and regeneration of the enzyme's catalytic active site. Considering the summative complexity of these steps, the electron transfer step alone may not be expected to be the rate-limiting process. In comparison, the extracellular reduction of NACs may simply involve contact between the NAC and an extracellular respiratory enzyme (in the outer membrane, oriented toward the bulk solution; or as an exudate). In this context, based on a LFER slope of one, we speculate that NAC reduction by *Shewanella putrefaciens* CN32 is likely an extracellular process.

LFERs for Abiotic Reduction of Nitroaromatic Compounds by Bioreduced Iron-Bearing Clay Minerals. To generate the maximum bioreduction extent of clay-Fe(III), SWy-2, and NAu-2 were incubated with *Shewanella putrefaciens* CN32 for 50 days. After this incubation period, the clay-Fe(II) concentrations plateaued, at which point 95% of the structural Fe(III) in SWy-2 was reduced [0.4 mmol Fe g^{-1}, 1.52 mM Fe(II)] and 38% of the structural Fe(III) in NAu-2 was reduced [4.1 mmol Fe g^{-1}, 1.56 mM Fe(II)]. The observation that the two clay minerals were bioreduced to different extents is consistent with previous observations and attributed to variations in the Fe content and the local Fe binding environment.[20,34] We recently demonstrated that these differences lead to unique distributions of $E_{H,clay}$ values in SWy-2 versus NAu-2. The relationship between the $E_{H,clay}$ distribution and the reduction potential of a specific reductant thermodynamically constrains the extent of clay-Fe(III) reduction.[21,26] In this case, the final extent of bioreduction of these clay minerals was controlled by the reduction potentials of the respiratory enzymes of CN32.[21,35,36] When we examined the relationships of structural Fe(II) contents to $E_{H,clay}$ that we previously measured for native SWy-2 and NAu-2,[26] we found that both clay minerals were bioreduced to an $E_{H,clay}$ value of approximately −0.3 V versus the standard hydrogen electrode (SHE).

We measured reduction rates and product formation for five NACs (nitrobenzene and four para-substituted nitrobenzenes in Table 1) in suspensions of bioreduced SWy-2 and NAu-2 suspensions having similar $E_{H,clay}$ values (−0.29 to −0.32 V vs SHE) and similar Fe(II) concentrations (1.52−1.56 mM) (Figure 3). All the substituted nitrobenzene compounds were stoichiometrically reduced to their corresponding aniline compounds, except for 4-NH$_2$-NB, in which case an intermediate product accumulated (Supporting Information Figure S2). Demonstrative results for 4-Cl-NB reacted with
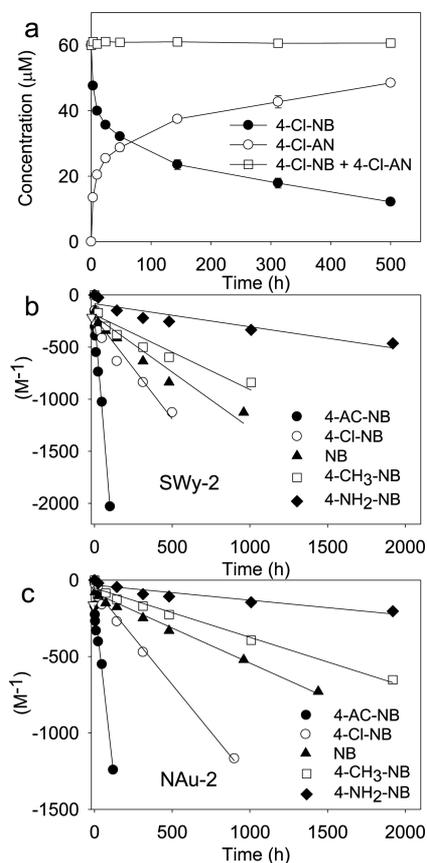

Figure 3. Abiotic reduction of NACs by clay-Fe(II) in bioreduced clay minerals. (a) Reduction of 4-chloronitrobenzene (solid circles) to corresponding 4-chloroaniline (open circles) by bioreduced montmorillonite SWy-2. Open squares are the sum of 4-chloronitrobenzene plus 4-chloroaniline. (b) Results for SWy-2 (95% reduced; 1.52 mM clay-Fe(II); 4.0 g/L). (c) Results for NAu-2 (38% reduced; 1.56 mM clay-Fe(II); 1.0 g/L). y-axis terms in panels b and c transformed to $\ln(\{[NAC]*[clay-Fe(II)]_0\}/\{[NAC]_0*[clay-Fe(II)]\})/\{[clay-Fe(II)]_0-6*[NAC]_0\}$ versus time to calculate second-order reduction rate constants, k.

bioreduced SWy-2 are presented in Figure 3a. Adsorption of any of the NACs by the two clay minerals was very low (<3%). Results from control experiments also showed that pasteurized biomass had no effect on the rate and extent of NAC reduction. A second-order rate model (eq 3) was used to determine rate constants for NAC reduction that accounted for the Fe(II) and NAC concentrations. In general, the second-order model fit the NAu-2 data better than the SWy-2 data, although R^2 values were fairly high for both minerals (0.856−0.992, Table 2). For SWy-2, R^2 values tended to be lower than those for NAu-2, and the kinetics appeared to be biphasic, with rapid initial reduction followed by a slower reduction period (Figure 3b and c).

We suspect that this biphasic behavior can be attributed to the range of $E_{H,clay}$ values of the structural Fe(II) in the SWy-2 changing over the course of the experiment (Figure 4). We propose that the most reduced forms of structural Fe(II) corresponding to the lowest $E_{H,clay}$ values would react first and most rapidly while the less reduced forms of clay-Fe(II) would react slower. An electron balance calculation indicated that the structural Fe(II) content of SWy-2 decreased from 95% to 73% as 60 μM NAC was reduced to the corresponding aniline product. This reaction corresponded to the $E_{H,clay}$ value becoming more positive by approximately 0.20 V[26] for SWy-
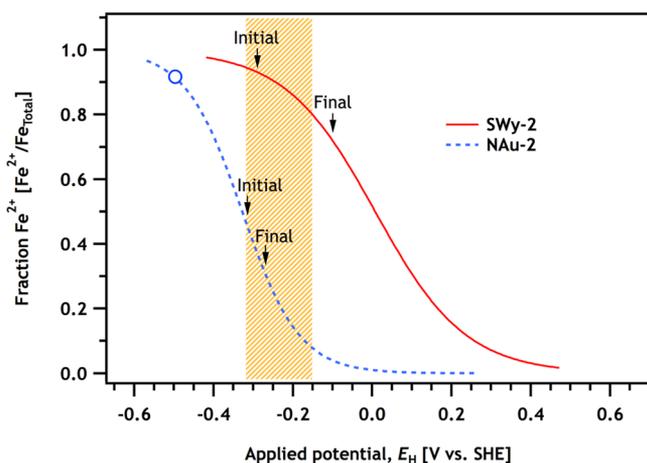
Figure 4. Redox profiles ($E_{H,clay}$) of montmorillonite SWy-2 and nontronite NAu-2 as determined by mediated electrochemical reduction.[26] The "Initial" arrows designate the maximum extent of bioreduction by *Shewanella putrefaciens* CN32 corresponding to the initial condition of the NAC reduction experiments. The "Final" arrows designate the $E_{H,clay}$ values after reduction of 60 μM NAC. The shaded region denotes the range of reduction potentials reported for respiratory enzymes of *Shewanella* species.[35,36] The circle on the NAu-2 redox profile designates the $E_{H,clay}$ value after complete chemical reduction.

2 (denoted with arrows along the SWy-2 redox profile in Figure 4). In comparison, reduction of 60 μM NAC by structural Fe(II) of NAu-2 corresponded to an increase of approximately 0.05 V[26] of the $E_{H,clay}$ for NAu-2 (denoted with arrows along the NAu-2 redox profile in Figure 4).

To probe the relationship between NAC reduction kinetics by SWy-2 and NAu-2 and $E^1_{H,NAC}$, we constructed LFERs by plotting log k versus $E^1_{H,NAC}/(2.303RT/F)$ (Figure 5, where $2.303RT/F = 0.058$ at 20 °C). Both plots yielded strong linear correlations, with R^2 values of 0.97 for both clay minerals. For two minerals, the LFER slopes were less than one (0.48−0.50), which is consistent with other reported LFER slopes for the abiotic reduction of NACs by Fe(II)-bearing mineral suspensions, such as magnetite (slope = 0.08−0.42, 0.34),[2,37] goethite and Fe(II) (slope = 0.6),[9] and clay minerals (slope = 0.67−0.74).[3] As noted above, if the rate-limiting step was purely controlled by the first electron transfer step at $E^1_{H,NAC}$, then the LFER slope would be one.[2,3,9,33] LFER slopes less than one indicate that a process other than the first electron transfer is the rate-limiting step.[2]

Effects of $E_{H,clay}$ on the Reduction Rate of 4-Acetyl-Nitrobenzene. While the $E^1_{H,NAC}$ value of the NAC clearly affects its reduction rate (Figures 2 and 5), we also wanted to test if the reduction potential of the clay mineral affects its reactivity toward NACs. A previous study showed that the abiotic reduction rate of NACs by reduced natural organic matter increased with decreasing E_H of the system.[8] To determine how $E_{H,clay}$ influenced NAC reduction rates, we first bioreduced SWy-2 and NAu-2 to four different extents by limiting the incubation period (Table 3). Bioreduced clay minerals were pasteurized and then reacted with 4-acetyl-nitrobenzene. 4-AC-NB was selected because it has the most positive $E^1_{H,NAC}$ of the NACs we tested and reacted most rapidly with the reduced clay minerals. We conducted these experiments with known but variable clay-Fe(II) concentrations (Table 3). As expected, 4-AC-NB reduction rates were highest
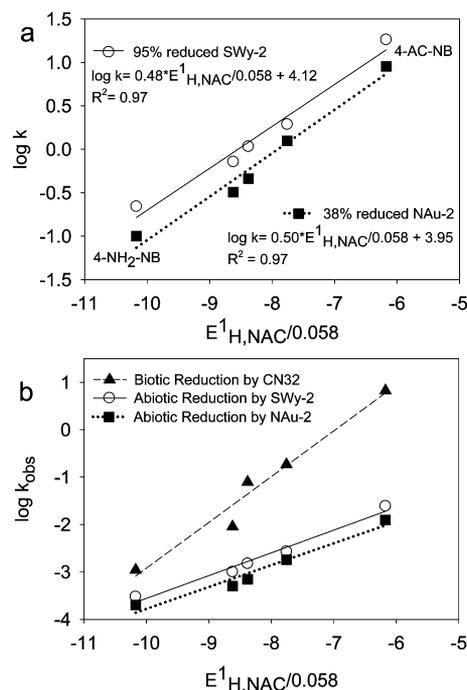

Figure 5. (a) Linear free energy relationships for second-order log k versus $E^1_{H,NAC}/0.058$ (at 20 °C) for the abiotic reduction of five NACs by bioreduced clay minerals. (b) Linear free energy relationships for first-order log k_{obs} versus $E^1_{H,NAC}/0.058$ for the comparison of biotic and abiotic reduction of NACs.

Table 3. Second-Order Rate Constants (k) for the Abiotic Reduction of 4-Acetyl-nitrobenzene by Clay-Fe(II) in Montmorillonite SWy-2 or Nontronite NAu-2 Bioreduced to Varied Extents[a]

clay-fe(II) description	$E_{H,clay}$ (mV)[b]	clay-Fe(II) (mM)	clay (g L^{-1})	k (M^{-1} h^{-1})[c]
8% bioreduced NAu-2	−170	0.66	2	n.d.[d]
21% bioreduced NAu-2	−240	1.73	2	0.87 ± 0.033
29% bioreduced NAu-2	−270	2.38	2	5.32 ± 0.32
34% bioreduced NAu-2	−290	2.79	2	8.10 ± 0.31
19% bioreduced SWy-2	170	0.61	8	n.d.[d]
53% bioreduced SWy-2	0	1.70	8	0.03 ± 0.0012
71% bioreduced SWy-2	−90	2.27	8	1.47 ± 0.075
95% bioreduced SWy-2	−290	3.04	8	56.54 ± 1.87

[a]All experiments conducted with 4-acetyl-nitrobenzene = 50 μM and bioreduced clay minerals in 50 mM PIPES, pH 6.8 at 20 °C. Bioreduced clay minerals were prepared using CN32 (1 × 10^8 cell/mL), NAu-2 (2.0 g/L), or SWy-2 (8.0 g/L). Reactors were incubated for different times to get different Fe(III) reduction extents (19%, 53%, 71%, and 95% for SWy-2; 8%, 21%, 29%, and 34% for NAu-2). Cell-clay mineral suspensions were pasteurized (75 °C for 60 min, three times over 10 days) to deactivate biological activity. k reported as mean ± standard deviation for three replicate rate estimates. [b]$E_{H,clay}$ data are from Figure 4. [c]Regression slope of ln({[NAC]*[clay-Fe(II)]$_0$}/{[NAC]$_0$*[clay-Fe(II)]})/{[clay-Fe(II)]$_0$-6*NAC]$_0$} versus time. [d]n.d. means no reduction of 4-acetyl-nitrobenzene was detected after 50 day reaction period.

for the most extensively bioreduced SWy-2 and NAu-2 samples, with reaction rates being slower for less extensively bioreduced minerals (Figure 6a and b).
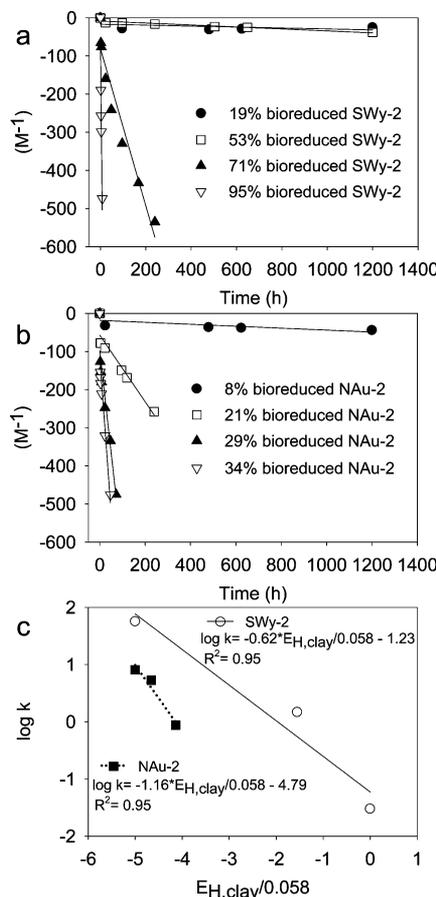

Figure 6. Abiotic reduction of 4-acetyl-nitrobenzene by clay-Fe(II) in clay minerals bioreduced to variable extents. Montmorillonite SWy-2 bioreduced to 19%, 53%, 71%, and 95% Fe(II), and nontronite NAu-2 bioreduced to 8%, 21%, 29%, and 34% Fe(II). (a) Results for SWy-2 and (b) NAu-2. y-axis terms in panels a and b transformed to $\ln(\{[NAC]*[clay\text{-}Fe(II)]_0\}/\{[NAC]_0*[clay\text{-}Fe(II)]\})/\{[clay\text{-}Fe(II)]_0\text{-}6*[NAC]_0\}$ versus time to calculate second-order reduction rate constants, k. (c) Linear free energy relationship for log k versus $E_{H,clay}/0.058$ (at 20 °C) for the abiotic reduction of 4-AC-NB by bioreduced clay minerals.

We fit the 4-AC-NB reduction curves using a second-order rate equation (eq 3). This allowed us to distinguish the effect of $E_{H,clay}$ from the effect of the clay-Fe(II) concentration on 4-AC-NB reduction kinetics. We calculated $E_{H,clay}$ values for each clay mineral using recent data from our group (Table 3), in which we measured $E_{H,clay}$ values for both NAu-2 and SWy-2 using mediated electrochemical techniques.[26] We then constructed an LFER in which we plotted log k for 4-AC-NB reduction versus $E_{H,clay}$ values divided by 2.303RT/F (= 0.058 at 20 °C)

$$\log k = a \times E_{H,clay}/0.058 + b \qquad (5)$$

Note that we only plotted log k values for reactors in which we observed substantial 4-AC-NB reduction over the 50 day period (Figure 6). The second-order reduction rate constants for 4-AC-NB by clay-Fe(II) were correlated to the $E_{H,clay}$ of clay-Fe(II) in NAu-2 and SWy-2 (Figure 6c).

Environmental Significance. LFERs are of great value because they allow one to approximate contaminant reaction rates when limited experimental data exist. An important factor that controls the utility of LFERs is the range of $E^1_{H,contaminant}$ values spanned by the suite of test compounds. Previous studies using polynitro-benzenes included compounds with $E^1_{H,NAC}$ values of −0.300 V to −0.590 V.[9,27] Similarly, the $E^1_{H,NAC}$ values of our selected NACs ranged from −0.358 to −0.590 V. While linear correlations between log k and $E^1_{H,NAC}$ have been reported for the abiotic reduction of NACs by several reductants,[7,8,33] we believe this is the first study to report an analogous correlation for the biological reduction of NACs.

When compared based on first-order rate constants, NACs were reduced more rapidly by *Shewanella putrefaciens* CN32 as compared to clay-Fe(II) in bioreduced clay minerals (Figure 5b). While certain experimental conditions control the magnitude of the rate constants (e.g., concentration of cell and clay-Fe(II), pH, temperature), these results demonstrate that both biotic and abiotic processes will contribute to the reduction of NACs in the environment. Comparisons of these trends show that the relative rate of bioreduction versus abiotic reduction (i.e., $k_{obs\text{-}cells}/k_{obs\text{-}clay}$) increased as the $E^1_{H,NAC}$ value increased (Table S1 in the Supporting Information). Thus, bioreduction could be a more important process for NACs with higher reduction potentials (e.g., more energetic terminal electron acceptors). Conversely, abiotic reduction by clay-Fe(II) could be a more important process for NACs with lower reduction potentials.

NACs were reduced more rapidly by clay-Fe(II) in bioreduced montmorillonite SWy-2 versus nontronite NAu-2 (Figures 5 and 6). This finding contrasts a previous study, where Neumann et al. found that Fe-rich minerals were more reactive than Fe-lean minerals using fully, chemically reduced clay minerals.[3] We believe this contradiction is due to differences in the extents to which the clay minerals were reduced. In this study, 95% of Fe(III) in SWy-2 and 38% of Fe(III) in NAu-2 were bioreduced by CN32, and both clay minerals were bioreduced to an $E_{H,clay}$ value of approximately −0.3 V versus the standard hydrogen electrode (SHE). However, in the Neumann et al. study,[3] the reduction extents of both the iron-rich clay mineral (ferruginous smectite SWa-1) and the iron-lean clay mineral (SWy-2) were around 80% of total Fe. At that reduction extent, the $E_{H,clay}$ value of SWa-1 was approximately −0.53 V versus SHE, which is much lower than the $E_{H,clay}$ value of SWy-2 (−0.20 V versus SHE).[26] In the future, works examining the abiotic reduction of contaminants should consider the means by which Fe(III)-bearing minerals are reduced in analogous environments.

Our results extend the application of LFERs to bioreduced iron-bearing clay minerals, which may be more similar to reduced clay minerals found in naturally reduced environments. This study is the first attempt to describe NAC reduction rates by Fe(II)-bearing clay minerals using reduction potential values of the mineral ($E_{H,clay}$). These results suggest that both the reduction potential of the contaminants (e.g., $E^1_{H,NAC}$) and the reduction potential of the Fe(II)-bearing mineral phase (e.g., $E_{H,clay}$) contribute to contaminant reactivity.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Bioreduction of 4-aminonitrobenzene (4-NH₂-NB) by *Shewanella putrefaciens* CN32, abiotic reduction of 4-aminonitrobenzene (4-NH₂-NB) by clay-Fe(II) in bioreduced nontronite

NAu-2, abiotic reduction of NACs by clay-Fe(II) in bioreduced clay minerals (first-order model), and the ratio of bioreduction rates versus abiotic reduction rates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Linear Free Energy Relationships for the Biotic and

Abiotic Reduction of Nitroaromatic Compounds

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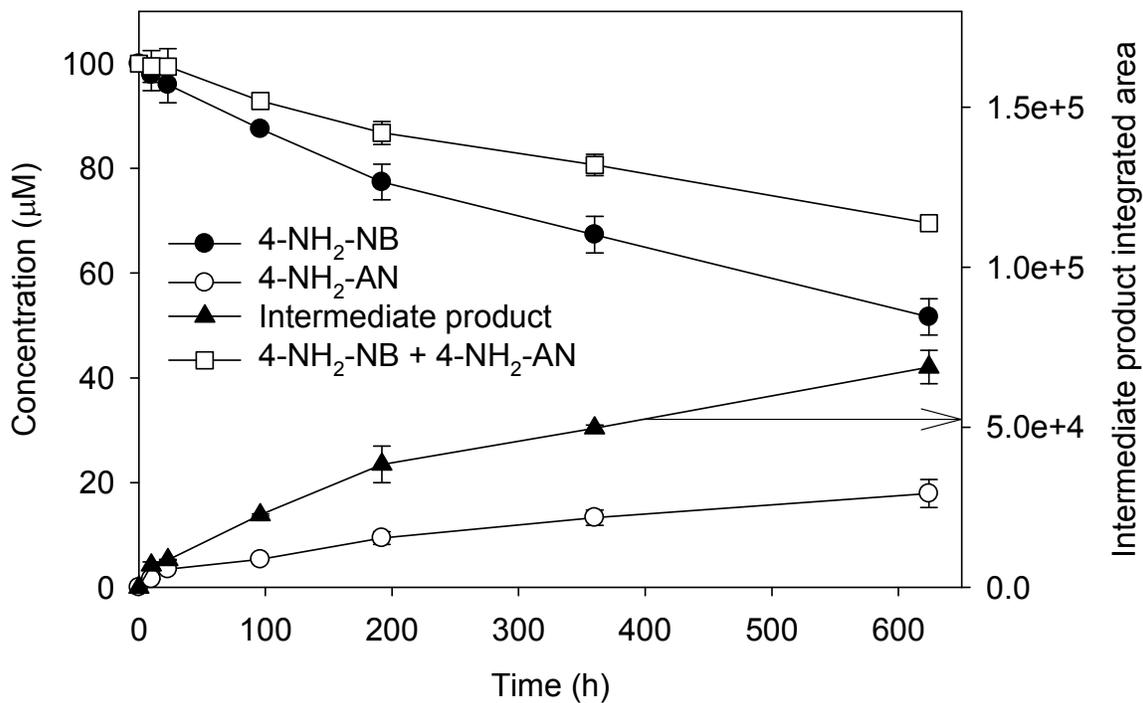phone: 814-863-0578; fax: 814-863-7304. Email: wdb3@psu.edu.

Contents

Figure S1. Bioreduction of 4-aminonitrobenzene (4-NH$_2$-NB) by *Shewanella putrefaciens* CN32. Experiments conducted with $1*10^8$ cells mL^{-1} CN32, 100 μM 4-NH$_2$-NB, and 5 mM lactate in 10 mM PIPES, pH 6.8 at 20°C. Because the intermediate product was not identified, only its relative integrated area was calculated (right axis).
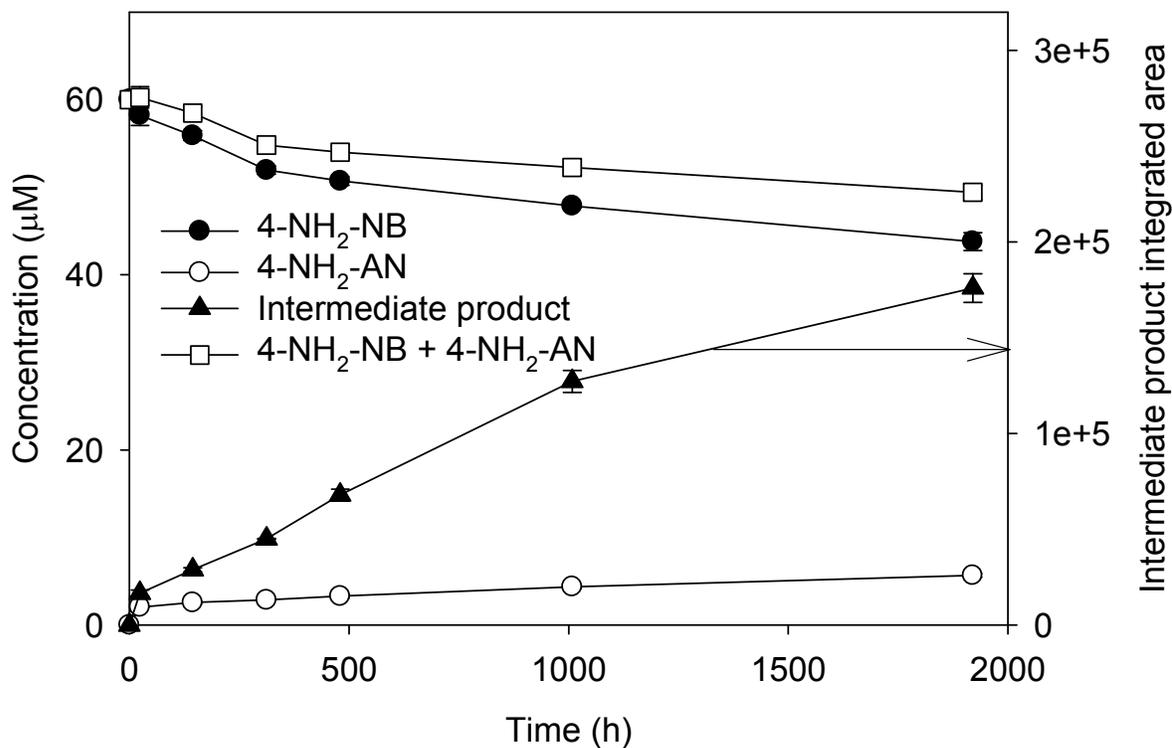
Figure S2. Abiotic reduction of 4-aminonitrobenzene (4-NH$_2$-NB) by clay-Fe(II) in bioreduced nontronite NAu-2. Experiments conducted with with 60 μM 4-NH$_2$-NB and 1.0 g/L bioreduced NAu-2 in 50 mM PIPES, pH 6.8 at 20°C. Bioreduced NAu-2 was prepared using CN32 (1×10^8 cell/mL) and NAu-2 (1.0 g/L). Cell-clay mineral suspensions were pasteurized (75 °C for 60 min, three times over 10 d) to deactivate biological activity. Because the intermediate product was not identified, only its relative integrated area was calculated (right axis).
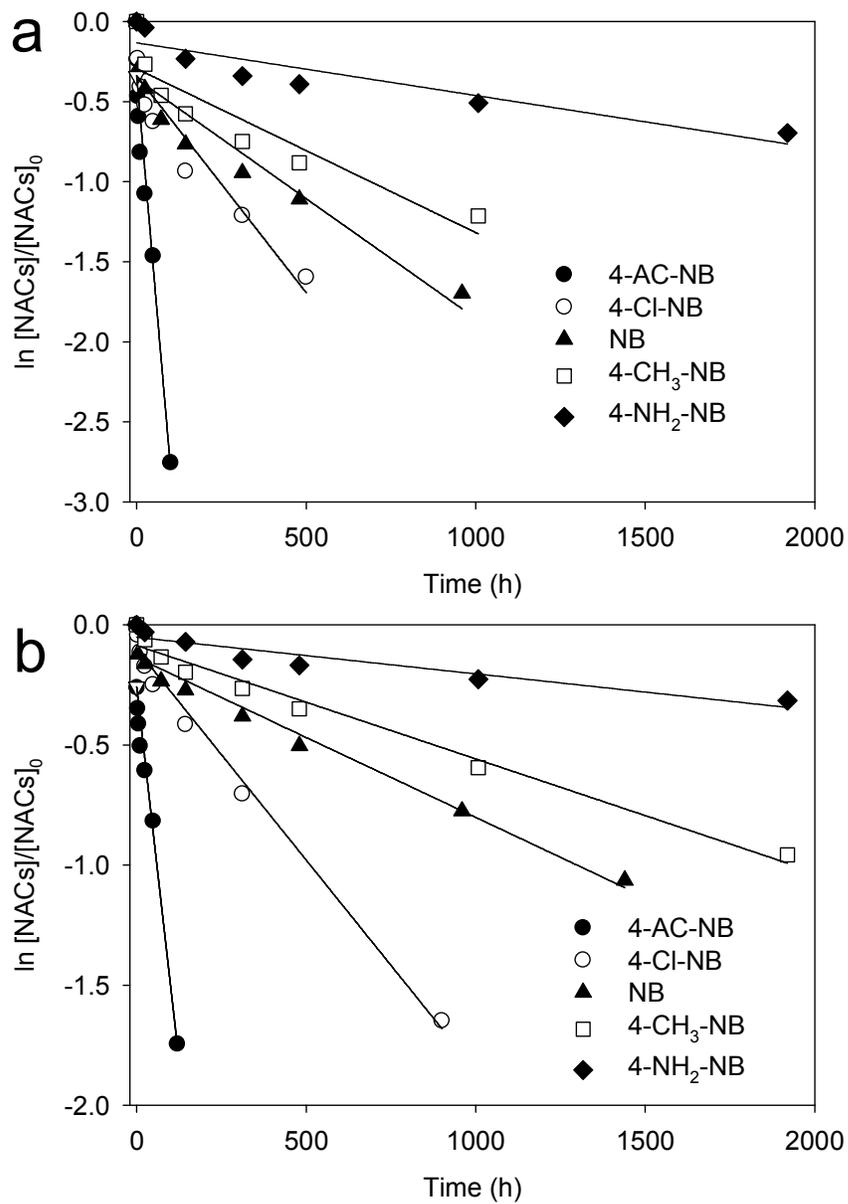
Figure S3. Abiotic reduction of NACs by clay-Fe(II) in bioreduced clay minerals. Kinetics evaluated according to a first-order model. **(**a) Results for SWy-2 (95% reduced). (b) Results for NAu-2 (38% reduced). y-axis terms transformed to $\ln([NAC]/[NAC]_0)$ versus time to calculate first-order reduction rate constants, k_{obs}.

Table S1. The ratio of first-order rate constants for the bioreduction of NACs by *Shewanella putrefaciens* CN32 ($k_{obs-cells}$) and the abiotic reduction of NACs by clay-Fe(II) in bioreduced montmorillonite ($k_{obs-SWy-2}$) or nontronite ($k_{obs-NAu-2}$).

NAC	$E^1_{H,NAC}$ (V)	$k_{obs-cells}$ / $k_{obs-SWy-2}$	$k_{obs-cells}$ / $k_{obs-NAu-2}$
4-acetylnitrobenzene	-0.358	272.2	533.6
4-chloronitrobenzene	-0.450	68.1	102.2
Nitrobenzene	-0.486	52.5	112.4
4-methylnitrobenzene	-0.500	9.0	18.0
4-aminonitrobenzene	-0.590	3.7	5.5