AN ABSTRACT OF THE THESIS OF

Caslin Anne Gilroy for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 29, 2009. Title: Correlation of NE 1545 expression and cell size in *Nitrosomonas europaea* exposed to a suite of aromatic hydrocarbons.

Abstract Approved: _____
Lewis Semprini

Ammonia oxidizing bacteria, including *Nitrosomonas europaea*, are inhibited by aromatic hydrocarbons which can be found in wastewater treatment plants. In recent studies, changes have been observed in *N. europaea* cell size upon exposure to benzene, but not toluene. Additionally, NE 1545, a gene proposed to be involved in fatty acid metabolism, was up-regulated in response to benzene, but not toluene. This work presents the results of a series of short-term experiments where *N. europaea* was exposed to a variety of aromatic hydrocarbons, and cell size and NE 1545 expression were measured. It was found that exposure to compounds with a dipole moment of 1.5 D and greater (*e.g.* aniline, phenol, and p-cresol) resulted in the greatest decreases in cell size (5 to 6%), as well as the greatest up-regulation of NE 1545 (17- to 19-fold). Compounds with dipole moments less than 1.5 D (*e.g.* m-cresol, o-cresol, toluene, and p-hydroquinone) caused negligible changes in size and NE 1545 expression. It is hypothesized that the more polar aromatic hydrocarbons were accumulating in the membrane to which the cells responded by increasing the expression of NE 1545 and this resulted in changes in the cell membrane which resulted in decreases in cell size.

Key Words: ammonia-oxidizing bacteria, *Nitrosomonas europaea*, aromatic hydrocarbons, gene expression, phospholipid membrane

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Correlation of NE 1545 expression and cell size in *Nitrosomonas europaea* exposed to a suite of aromatic hydrocarbons

by

Caslin Anne Gilroy

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Chair, Department of Chemical, Biological, and Environmental Engineering

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Caslin Anne Gilroy, Author

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This thesis is dedicated to my father,

Dr. Duncan James Gilroy:

I hope that being a great scientist runs in the family.

Correlation of NE 1545 expression and cell size in *Nitrosomonas europaea* exposed to a suite of aromatic hydrocarbons

Introduction

Ammonia-oxidizing bacteria (AOB), including *Nitrosomonas europaea* – a model AOB, play an essential role in the global nitrogen cycle by oxidizing ammonia to nitrite. Additionally, AOB are critically important in maintaining proper nitrogen removal from wastewater treatment plants (WWTP). The removal of nitrogen from WWTP is essential to prevent the eutrophication of the receiving body of water, which can result in hypoxic zones and may result in fish and wildlife death. Unfortunately, AOB are highly sensitive microorganisms [1] and their capacity for ammonia removal is greatly affected by many compounds commonly found in WWTP influent, including organic solvents [2].

Organic solvents, particularly aromatic hydrocarbons, can enter the water system as a result of industrial applications and fuel leakages [3,4]. *N. europaea*, as well as many other species of bacteria, exhibits inhibited activity in the presence of these aromatics [4,5]. Several inhibition mechanisms have been proposed including energy drains due to co-metabolic processes [5,6] as well as their tendency to partition into bacterial membranes, resulting in increased membrane permeability and loss of cellular metabolites [4,7]. Cells may combat the disruption in homeostasis by altering the cell envelope. Observed alterations have included changes in the lipid-to-protein ratio of the bilayer, as observed in *Escherichia coli*

upon exposure to phenol [7], as well as changes in overall cell size, as observed in *Pseudomonas putida* upon exposure to phenolic compounds [6].

When exposed to non-lethal concentrations of benzene in previous studies, *N. europaea* exhibited a decrease in cell size and up-regulated a seven-gene cluster that appears to be involved in fatty-acid metabolism [5]. NE 1545, a putative pirin protein likely involved in directing pyruvate metabolism between the Citric Acid cycle and fermentation pathways in prokaryotes [8], displayed the highest up-regulation of the gene cell size cluster and was chosen for further study to represent the gene cluster [5]. In contrast to benzene, exposure to toluene caused no significant changes in *N. europaea* cell size nor in the transcriptional level of NE 1545 [5]. This suggests that there is an aromatic-specific correlation between changes in *N. europaea* cell size and the expression of NE 1545. To explore this correlation further, the effects of exposure to a variety of aromatic hydrocarbons (phenol, toluene, aniline, p-hydroquinone, and cresols) on cell size and NE 1545 gene expression were investigated in this study.

Materials and Methods

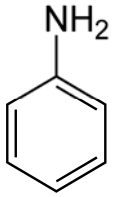
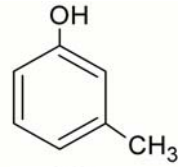
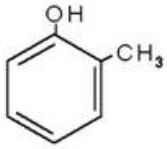
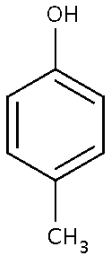
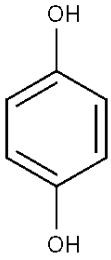
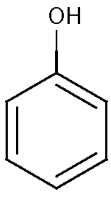
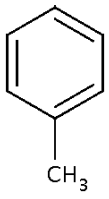
Nitrosomonas europaea culturing protocol

N. europaea cells have a reported doubling time of 8-12 hours. *N. europaea* cells were grown in a minimal growth medium consisting of 25 mM $(NH_4)_2SO_4$, 43 mM KH_2PO_4, 3.92 mM NaH_2PO, 3.77 mM Na_2CO_3, 750 μM $MgSO_4$, 270 μM $CaCl_2$, 18 μM $FeSO_4$, 17 μM EDTA, and 1 μM $CuSO_4$. Cells were inoculated into fresh media and shook in the dark at 100 rpm and 30ºC. After 3 days, cells were in mid-exponential growth (OD600 ~ 0.070) and harvested via centrifugation. Cells were spun at 9000 rpm for 30 min, decanted and suspended in 250 mL of fresh 40 mM KH_2PO_4 buffer pH 7.8. Cells were spun again at 9000 rpm for 20 min, decanted and suspended in 30 mL of 30 mM HEPES buffer (pH 7.8).

Aromatic hydrocarbon inhibition studies

Studies were performed in 155 mL bottles with 50 mL of fresh medium containing 2.5 mM $(NH_4)_2SO_4$ in 10 mM HEPES buffer to maintain a pH of 7.8. Water-saturated stock solutions of various aromatic hydrocarbons (see Table 1 for compounds and associated structures) were prepared and added to the treatment bottles at 2 - 250 μM concentrations and shaken at 250 rpm for 1 hour to allow for equilibration. *N. europaea* cells were grown in batch and harvested as described above. Cells were placed into the 155 mL bottles to achieve OD600 ~ 0.070. Bottles containing cells without the addition of an aromatic hydrocarbon served as controls. Cells were shaken at 250 rpm in the dark at 25ºC. NO_2^- production was monitored via colorimetric assay [9] at 1 hour intervals for 3 hours.

Table 1. Aromatic hydrocarbons used in inhibition experiments with associated compound structure.

Compound	Structure
Aniline	
m-Cresol	
o-Cresol	
p-Cresol	
p-Hydroquinone	
Phenol	
Toluene	

Reversibility experiments

Following 3 hours of aromatic hydrocarbon exposure, cells were harvested, washed 5 times in 30 mM HEPES buffer (pH 7.8), and placed into 155 mL bottles containing fresh media with 2.5 mM $(NH_4)_2SO_4$. Cells were shaken at 250 rpm in the dark at 25ºC, and NO_2^- production was monitored at 1 hour intervals for 3 hours.

Cell harvesting and Total RNA extraction

After 3 hours of exposure to an aromatic hydrocarbon, 40 mL of cells were extracted from control and treatment bottles for qRT-PCR analysis. The extracted cells were spun at 9000 rpm for 15 min, decanted and suspended in 1 mL HEPES buffer, pH 7.8. The cells were spun at 14,000 rpm for 1 min, decanted, suspended in 0.5 mL Trizol (Invitrogen Co., Carlsbad, CA) and stored at -80ºC for further processing.

Cells were thawed for Total RNA extraction and an additional 0.5 mL of Trizol was added to bring the total Trizol volume up to 1 mL. Cells were lysed via shearing by passing the cell suspension through a 25 gauge needle 20 times. Total RNA was extracted from the lysed cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions.

qRT-PCR analysis

qRT-PCR was conducted to track the expression of NE 1545 in cells exposed to various aromatic hydrocarbons. Gene Runner v. 3.00 (Hastings Software, Inc.) was used to generate qRT-PCR primers from 16S, NE 1545 DNA sequences. The qRT-PCR primer sequences are

presented in Table 2. The qRT-PCR primers were optimized for concentration and annealing temperature via PCR to achieve high efficiency with only one product detected. Details on the primer selection and optimization are provided by Radniecki, et al. [5].

Table 2. qRT-PCR primer sequences.

Primer	Sequence
16S For	5'-GGCTTCACACGTAATACAATGG-3'
16S Rev	5'-CCTCACCCCAGTCATGACC-3'
NE 1545 For	5'-GGATGATCTGACGCAAGTGA-3'
NE 1545 Rev	5'-CTGCGACAAAGTCGAAAGTG-3'

cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) per manufacturer's instruction and diluted 100-fold in TE buffer at pH 8. qRT-PCR was carried out in triplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA). The 50 μL reactions contained 1X SYBR Green Supermix, 1X ROX reference dye (Invitrogen, Carlsbad, CA), 500 nM Forward and Reverse primers and 10 ng cDNA. The following cycle conditions were used for qRT-PCR: 2 min at 95ºC, then 50 cycles at 95ºC for 30 s, 55ºC for 45 s and 72ºC for 45 s. At the end of the reaction, dissociation curves of the products were generated by bringing the temperature to 60ºC and then raising the temperature by 0.5ºC every 20 s until a final temperature of 95ºC was attained.

The relative expression values for the reactions were determined using DART-PCR analysis [10] taking into account the efficiency of the reaction and normalizing the data to the amount of 16S mRNA quantified in each reaction. 16S rRNA expression was assumed to be constant

throughout the experiment and is used to normalize differences between starting quantities of cDNA template. Fold changes > 1 = up-regulation of the treatment gene. Fold changes < 1 = down-regulation of the treatment gene. The t-test was used to determine if the observed gene regulation was significant ($p < 0.05$).

Coulter counter analysis

N. europaea cells were extracted from control and treatment bottles after 3 hours of exposure to an aromatic hydrocarbon and their cell diameter was measured using a Multisizer 3 coulter counter (Beckman Coulter, High Wycombe, U.K.). 40 µL of cells were diluted into 20 mL of Isoton 2 (Beckman Coulter, High Wycombe, U.K.) before being measured with a 30 µm aperture tube (size range 0.6 – 12 µm). MS-Multisizer 3 software was used to calculate cell diameters.

Results

Inhibition and cell size

N. europaea activity was measured by the rate of ammonia removal, which is directly proportional to the rate of nitrite production. A comparison of nitrite production during chemical exposure and control conditions was used to determine a percent decrease in ammonia oxidation activity. Similarly, a comparison of cell size during chemical exposure and control conditions was used to determine a percent decrease in cell size resulting from chemical exposure.

Exposure to phenol caused a decrease in cell size that leveled out at -6% (Figure 1), while toluene caused minimal cell size change, leveling out at -1% (Figure 2). Cells exposed to aniline behaved similarly to those exposed to phenol, causing a maximum cell size change of -5% (Figure 3). P-hydroquinone caused minimal cell size change, leveling out at between -1 and -2% (Figure 4). P-cresol caused no cell size changes until an apparent threshold was reached at 40 μM; increasing concentrations caused cell size changes that leveled off at -5% (Figure 5). Both m- and o-cresol caused a decrease in cell size that leveled out between -1 and -2%, respectively (Figures 6 and 7). Maximum observed size changes during phenol, aniline, and p-cresol exposure occurred at concentrations that caused approximately a 50% decrease in activity.

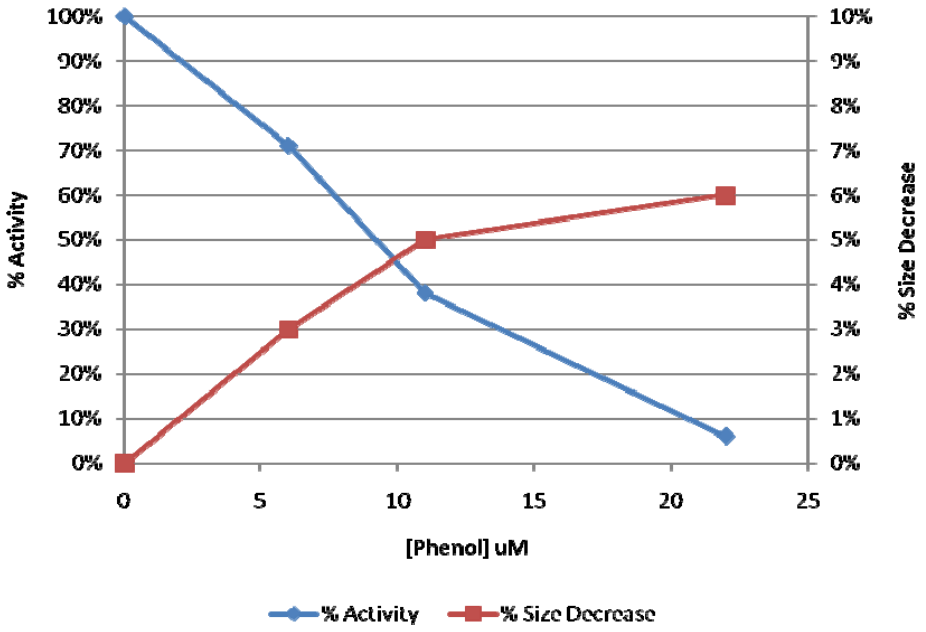


Figure 1. The effect of increasing phenol concentration on % activity and % size decrease of *N. europaea* cells. An increase in % size decrease corresponds to a decrease in cell size. Increasing phenol concentrations resulted in a decrease in activity and a simultaneous steady decrease in cell size. The data suggests an asymptotic approach to just above 6% decrease in cell size as activity decreases to zero.

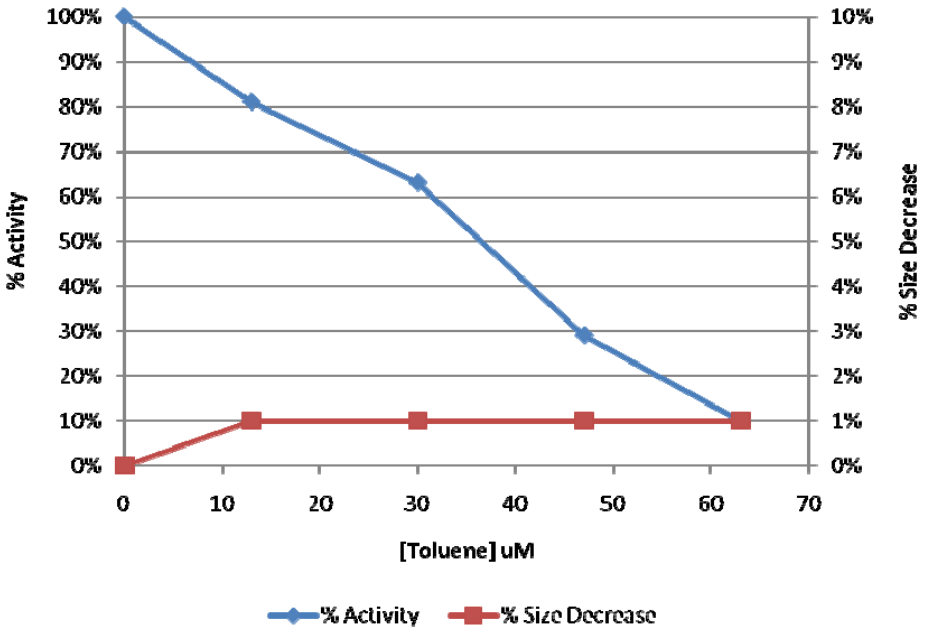


Figure 2. Increasing toluene concentrations caused a decrease in activity and a slight decrease in cell size followed by a no change in cell size with toluene concentrations above 12 µM.

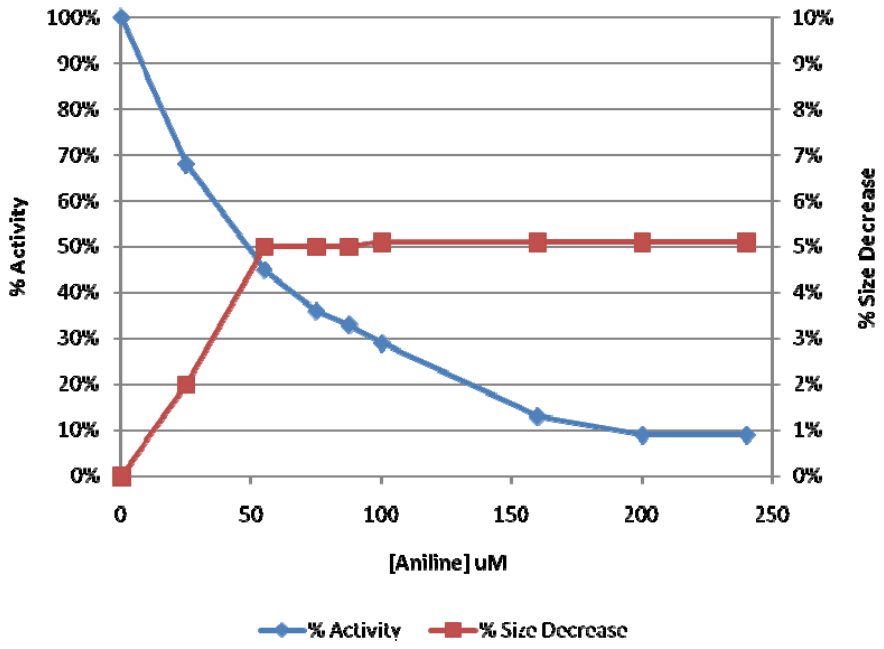


Figure 3. Increasing aniline concentrations caused a decrease in activity and a 5% decrease in cell size at concentrations above 50 µM.

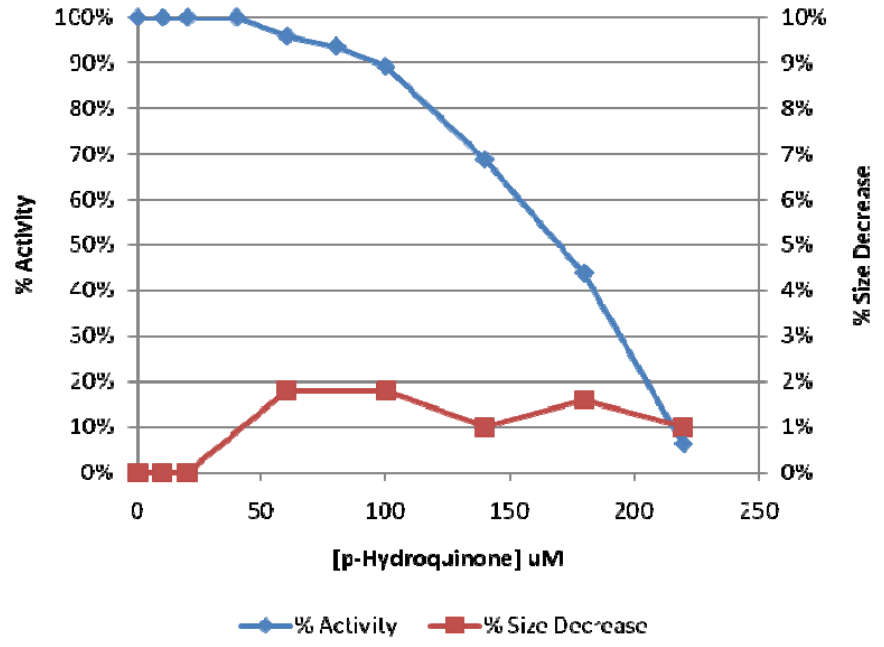


Figure 4. Increasing p-hydroquinone concentrations caused a decrease in activity and a 1-2% decrease in cell size at concentrations above 60 µM.

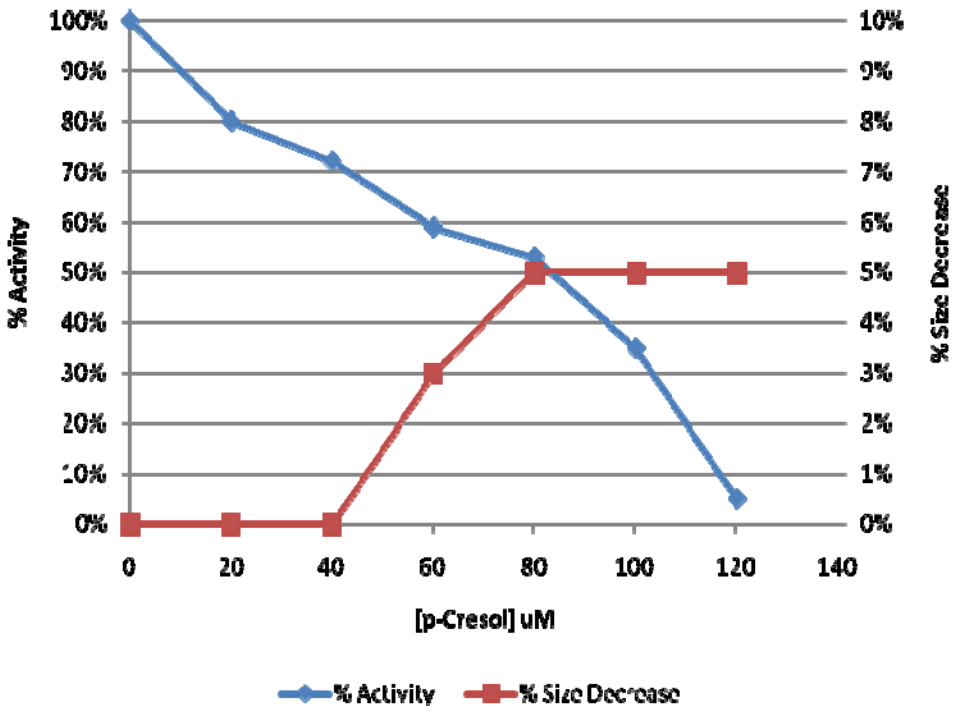


Figure 5. Increasing p-cresol concentrations caused a decrease in activity and a steady decrease in cell size at concentrations above 40 μM, leveling off at a 5% decrease in cell size at concentrations above 80 μM.

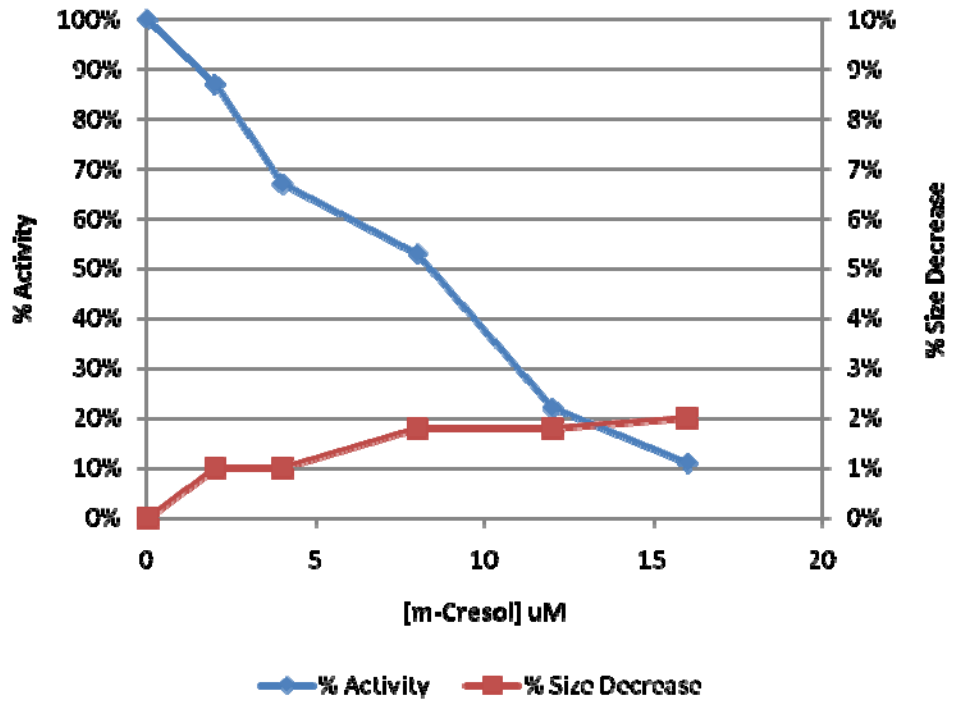


Figure 6. Increasing concentrations of m-cresol caused a decrease in activity and a 1-2% decrease in cell size.

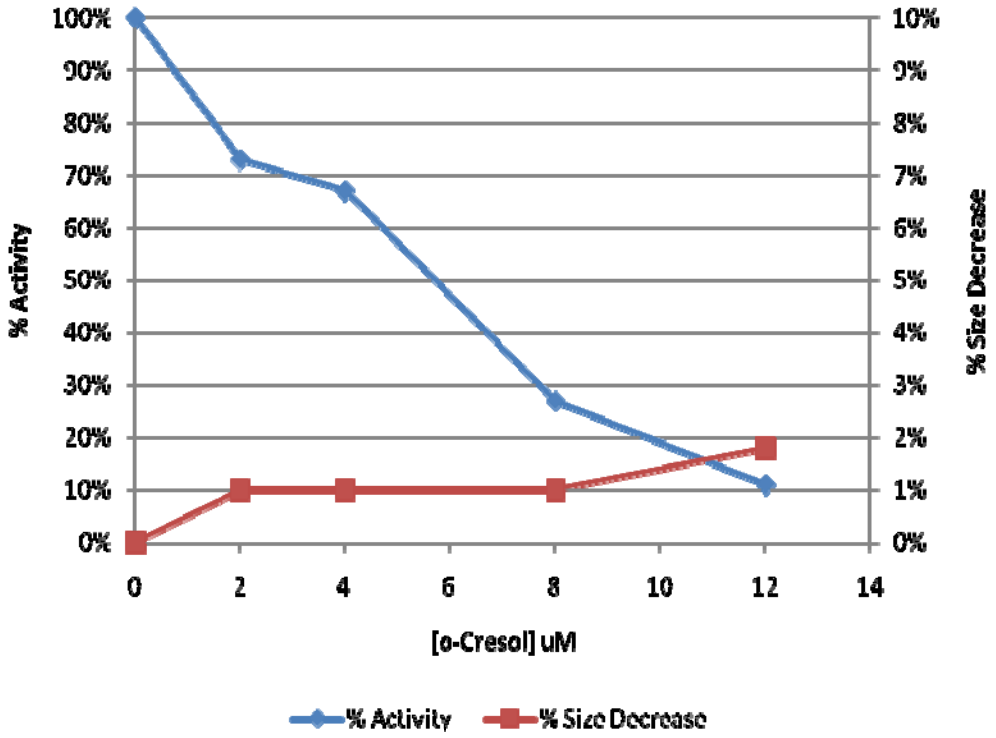


Figure 7. Increasing concentrations of o-cresol caused a decrease in activity and a 1-2% decrease in cell size.

Cell size and activity reversibility

Nitrite and cell size assays were repeated after the removal of the aromatic hydrocarbons to determine if the observed inhibition and cell size changes were reversible. Nearly a complete recovery was observed in activity, but cells retained their decreased size, suggesting a permanent change in the cell membranes (Figure 8).

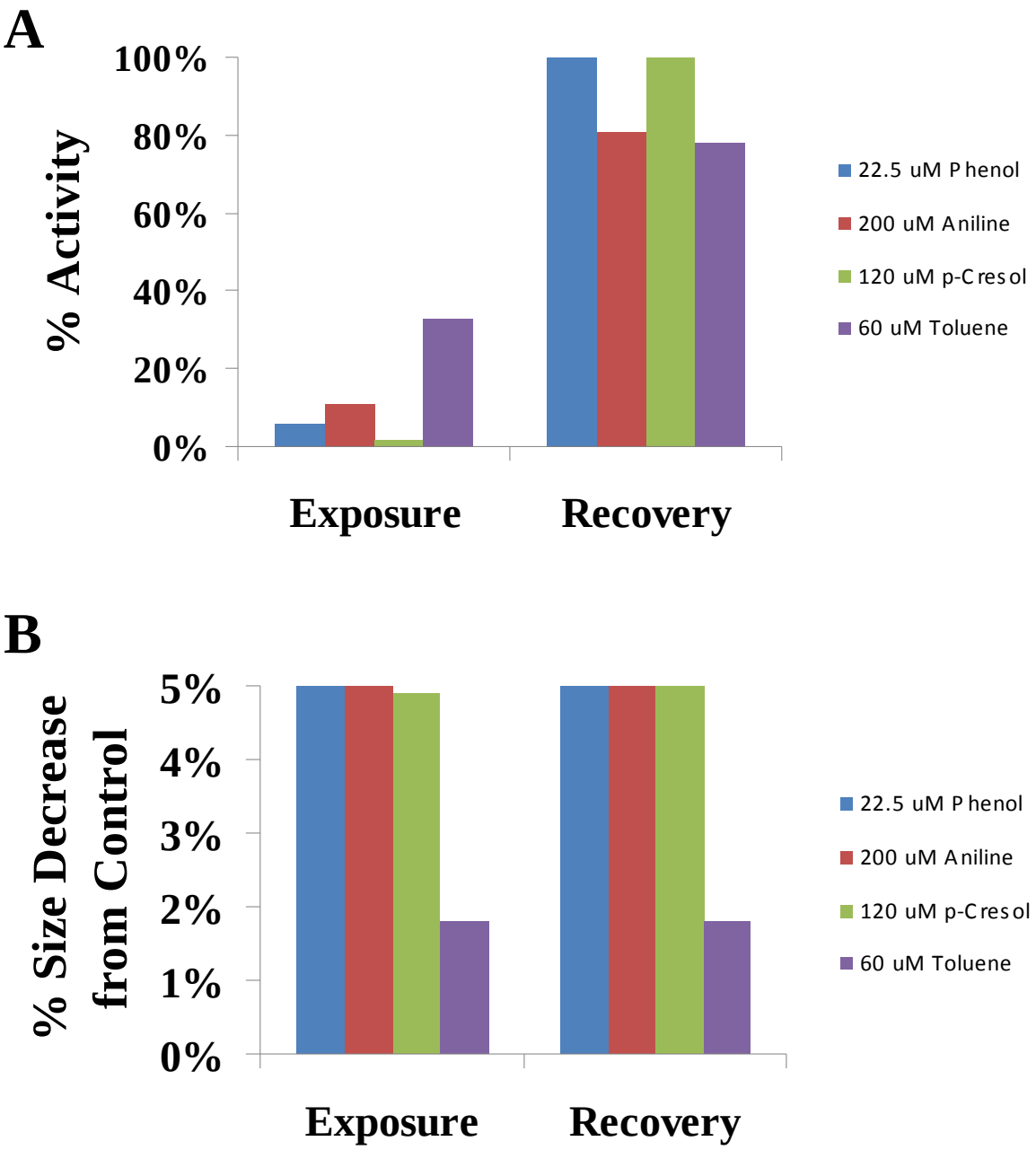


Figure 8. The % activity (A) and % size decrease (B) were measured following 3 hours of aromatic hydrocarbon exposure (Exposure) and 3 hours after removal of the aromatic hydrocarbons (Recovery). *N. europaea* cells exhibited an almost complete recovery in activity after aromatic hydrocarbon removal. Cells did not recover from the size change induced by aromatic exposure.

NE 1545 gene expression

Expression of NE 1545 was investigated in cells exposed to aromatic hydrocarbon concentrations that caused 50 (or 30) and 80% inhibition in activity (Figure 9). Little change was seen in NE 1545 expression in cells exposed to toluene, o-cresol, m-cresol and the low p-cresol concentration, which mirrors the minimal size changes observed in these conditions. Increasing p-cresol to 80% inhibitive concentration caused a 17-fold increase in NE 1545, which correlated with the observed 5% decrease in cell size at that concentration. NE 1545 expression increased 6- and 10-fold in cells exposed to 50% inhibitive concentrations of phenol and aniline, respectively, and increased to a 15- and 19-fold change at 80% inhibitive concentrations. This corresponds closely to the significant cell size decreases (5%) observed after exposure to 50% inhibitive concentrations of phenol and aniline.

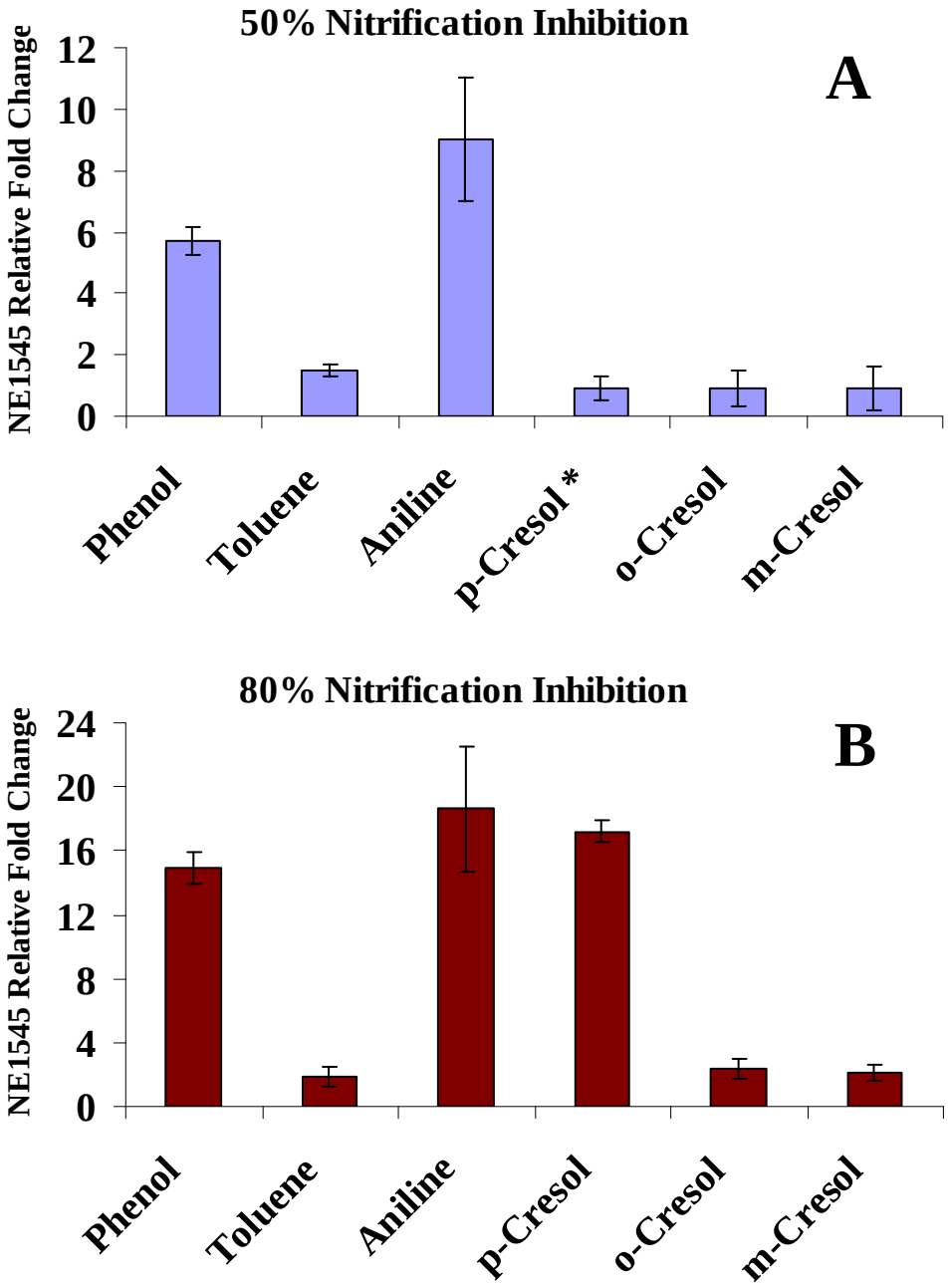


Figure 9. The relative fold increase of NE 1545 following 1 hour of exposure to aromatic hydrocarbons causing 50% (A) and 80% (B) inhibition in *N. europaea* activity. Little change was seen in NE 1545 expression upon exposure to toluene, o-cresol, m-cresol, and low concentrations of p-cresol. Increased concentration of p-cresol caused a 17-fold increase in NE 1545 expression. Phenol and aniline caused 6- and 10-fold increases, respectively, at 50% inhibitive concentrations, and 15- and 19-fold increases in NE 1545 expression at 80% inhibitive concentrations.
* = 30% nitrification inhibition

Summary of data

Results from inhibition, cell size, and NE 1545 gene expression studies are compiled in Table 3, along with physical properties of the aromatic hydrocarbons tested. The data indicates a strong correlation between cell size decrease and NE 1545 up-regulation. A link between *N. europaea*'s responses and aromatic hydrocarbon dipole moments is also indicated.

Table 3. Summary of physical properties of the aromatic hydrocarbons and the changes induced in *N. europaea* during exposure. EC_{50} is the exposure concentration that causes 50% inhibition in cell activity. The data suggests a link between cell size decrease, NE 1545 up-regulation, and aromatic hydrocarbon dipole moment.

Compound	Dipole Moment	pKa	Water Solubility	Log K_{ow}	EC_{50}	Max Size Change	Conc.	Max NE 1545 Change
Aniline	2.30 D	4.87	387 mM	0.90	55 μM	-5%	55 μM	19-fold
Phenol	1.70 D	9.95	882 mM	1.50	10 μM	-6%	22 μM	15-fold
p-Cresol	1.50 D	10.26	176 mM	1.98	80 μM	-5%	60 μM	17-fold
m-Cresol	1.45 D	10.99	222 mM	1.98	4 μM	-2%	8 μM	2-fold
o-Cresol	1.45 D	10.26	231 mM	1.98	7 μM	-2%	8 μM	2-fold
Toluene	0.36 D	28.30	5 mM	2.70	20 μM	-1%	63 μM	2-fold
p-Hydroquinone	0.00 D	10.35	536 mM	0.60	175 μM	-2%	60 μM	N.D.

N.D. = not determined.

Discussion

Table 2 displays a clear trend between cell size change and NE 1545 expression: aromatic hydrocarbons that caused the largest decrease in *N. europaea* cell size (5 to 6%) also caused the highest expression of NE 1545 (15- to 19-fold). In contrast, aromatic hydrocarbons that caused minimal decrease in cell size (1 to 2%) caused minimal increase in NE 1545 expression (2-fold). This trend is displayed best by cells exposed to p-cresol. Low concentrations of p-cresol caused no cell size change, but once a threshold concentration was reached, cell size decreased sharply to -5% (Figure 5). Similarly, concentrations of p-cresol causing 30% inhibition caused only a 1-fold increase in NE 1545 expression, but concentrations causing 80% inhibition caused NE 1545 expression to leap to a 17-fold increase (Figure 9). This makes a strong case for a correlation between specific aromatic exposure, cell size change, and NE 1545 expression.

All of the compounds tested in this study caused inhibition in *N. europaea* activity, but only select aromatic hydrocarbons caused decreases in cell size, suggesting that the change in cell size was not a result of chemical inhibition, but rather from another process. Observations from studies with *Pseudomonas putida* have shown that *P. putida* cells increased in size when exposed to increasing phenol, thereby reducing their surface-to-volume ratio [6]. *P. putida* cell size changes were only observed in cells exposed to non-lethal phenol concentrations, suggesting that reducing the relative surface of the cells is an adaptive response to phenol, which is likely inhibiting the compound via interaction with the membrane.

Similarly, when Heipieper, et al. [7] exposed *E. coli* to non-lethal concentrations of phenol, membrane permeability increased, which suggests that phenol caused a direct injury to the membrane.

Considering the results of these studies, along with the theory that aromatic hydrocarbons penetrate into bacterial membranes [4], it was hypothesized that the decrease in *N. europaea* cell size was a result of the aromatic hydrocarbons interacting with and partitioning into the lipid bilayer. If this hypothesis is correct, then either specific aromatic hydrocarbons entered the membrane, or all aromatic hydrocarbons entered the membrane and only specific ones induced a decrease in *N. europaea* cell size. Physical properties of the compounds tested were analyzed for trends, and included pKa, water solubility, octanol-water partitioning coefficient, and dipole moment (Table 2).

Of the physical properties investigated, a common link was found in the dipole moment of aromatic hydrocarbons and cell size change. Compounds with a dipole moment of 1.5 D and greater caused a 5 to 6% decrease in cell size, while compounds with dipole moments lower than 1.5 D caused minimal size change. For example, phenol, with a dipole moment of 1.7 D, caused cell size to decrease by 6%, while toluene, with a dipole moment of 0.4 D, caused negligible size change. This suggests that the more polar compounds, which have been shown to be attracted to the hydrophilic phosphate heads of the phospholipid membranes, invoke the observed decreases in cell size as opposed to the more nonpolar compounds, which have been shown to either accumulate in the lipophilic portion of the membrane or pass completely through [4].

p-Cresol, however, is structurally a combination of phenol and toluene, and required a threshold concentration necessary to cause cell size change. p-Cresol has a dipole moment of exactly 1.50 D, and therefore was right on the cusp of the apparent dipole moment cut-off value that caused partitioning between the hydrophobic and hydrophilic portions of the membrane. It follows that the concentration of p-cresol had to be increased in order to favor its partitioning into the hydrophilic portion of the membrane.

m-Cresol and o-cresol both have dipole moments very close to that of p-cresol (1.45 D), but did not induce increased expression of NE 1545 nor cell size change. This could be due to the drastically different concentrations necessary to cause inhibition by these compounds. 80 μM p-cresol caused 50% inhibition in *N. europaea* ammonia oxidation activity, while the same degree of inhibition was induced by 7 μM o-cresol and 4 μM m-cresol. It is possible that if m- and o-cresol concentrations were increased to a high enough value, they would induce increased expression of NE 1545 and cause a decrease in cell size change. However, such concentrations would likely be lethal to the bacteria.

Toluene, however, with a dipole moment of 0.36 D, was administered at a relatively high concentration (63 μM) and caused negligible size change and a negligible increase in NE 1545 expression. This follows the trend that compounds with lower dipole moments did not induce these changes in the bacteria. However, the 1.5 D cut-off cannot be concluded due to the extremely low concentrations of m- and o-cresol used.

Figure 8 demonstrates that upon removal of the aromatic hydrocarbons, *N. europaea* ammonia oxidation activity was regained, suggesting a complete removal of the aromatic hydrocarbons. However, the cells did not return to their normal size, suggesting that it was not the presence of the aromatic hydrocarbons in the membrane that caused the cells to decrease in size, but rather a physiological change in the membrane composition in response to the chemical partitioning.

The proposed defense mechanisms employed by microorganisms exposed to aromatic hydrocarbons include the metabolism of the hydrocarbons, the alteration of membranes to decrease permeability, and the rigidification of cell membranes [11]. Rigidification has been observed in all *Pseudomonas* strains, and is believed to be due to an alteration in the phospholipid composition of the membrane to combat membrane fluidity changes resulting from penetration of hydrocarbons into the membrane [11].

One method of changing membrane phospholipid composition is the *cis*-to-*trans* isomerization of unsaturated fatty acids, as observed in *P. putida* strains upon exposure to phenol and toluene [11]. The isomerization is believed to increase the ordering of the membrane, thereby decreasing fluidity [4]. A second method is the changing of the ratio of saturated-to-unsaturated fatty acids, as observed in *E. coli* in the presence of aromatic hydrocarbons that are lipophilic but still soluble in water, such as benzene and aniline [4,11]. Enrichment of the membrane with saturated fatty acids has the effect of increasing membrane ordering, thereby opposing further partitioning of the compounds into the bilayer [4]. The fatty acid saturation ratio changes generally occur after long-term aromatic hydrocarbon

exposure [11]. Either one of these physiological changes may have occurred in *N. europaea* and resulted in the observed decrease in cell size.

NE 1545 was up-regulated at least 15-fold whenever cells displayed a significant (5 to 6%) decrease in cell size, which suggests that NE 1545 was involved in causing the physiological change in membrane composition. NE 1545, along with the gene cluster it was chosen to represent, is proposed to be involved in fatty acid metabolism [8]. It is possible that *N. europaea* cells responded to the decrease in membrane ordering that resulted from aromatic hydrocarbon partitioning by up-regulating the fatty acid metabolism genes. Expression of these genes potentially caused a change in the fatty acids of the phospholipid bilayer. More specifically, the cells metabolized unsaturated fatty acids, leaving the membrane with a higher ratio of saturated-to-unsaturated fatty acids and thus more ordered in structure and less vulnerable to chemical partitioning.

This research revealed a link between *N. europaea* cell size and NE 1545 gene expression upon exposure to aromatic hydrocarbons. Additionally, the dipole moments of the aromatic compounds appeared to be important in determining whether or not the cells changed in size. Finally, the expression of NE 1545 only indicated the presence of *specific* aromatic hydrocarbons (those with high dipole moments), which suggests that NE 1545 could be an effective sentinel gene in a biosensor designed to detect the presence of certain aromatic hydrocarbons in WWTPs.

All of the experiments conducted in this study were short-term (3 hours). Research is currently being conducted to analyze the effects of long-term exposure of *N. europaea* to aromatics, such as phenol. Future studies should include measuring the changes in fluidity of the cell membrane, and attempting to qualify the changes as *cis*-to-*trans* isomerization, saturated-to-unsaturated fatty acid ratio changes, or something else entirely. It has been determined that the NE 1545 gene is expressed in response to specific aromatic hydrocarbon exposure, and it should be investigated whether expression of the gene is resulting in expression of an associated protein. The compounds tested should be expanded to include aromatic hydrocarbons having a wide range of both dipole moments and inhibitory concentrations, to determine if dipole moment is indeed the property determining NE 1545 expression and cell size change.

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