



TETRA TECH NUS, INC.

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C-NAVY-12-05-2026W

December 23, 2005

Project Number N4590

Mr. Brian Helland
Remedial Project Manager
EFA Northeast, Naval Facilities Engineering Command
10 Industrial Highway, Mail Stop 82
Lester, Pennsylvania 19113

Reference: CLEAN Contract No. N62472-03-D-0057
Contract Task Order No. 014

Subject: Microbiological and Biogeochemical Assessment Progress Report
For Denitrification-Based Biodegradation Pilot Test
Navy Exchange Service Station
Naval Air Station, Brunswick, Maine

Dear Mr. Helland:

The Microbiological and Biogeochemical Assessment Letter Report – July 2005, revised October 2005 was recently completed by Geovation Technologies, Inc. (Geovation) that summarizes post-baseline (August 2004) treatment and groundwater sampling results, for the period of October 2004 through June 2005, in support of the in-situ anaerobic biodegradation pilot test at the Navy Exchange Service Station at the Naval Air Station in Brunswick, Maine. The letter report is enclosed with this correspondence in hard copy form. Copies of this letter report will also be provided to the Maine Department of Environmental Protection (MEDEP).

A total of six in-situ treatment events, including an initial N-Blend dispersion test requested by the MEDEP, were completed during this period. A seventh treatment event was in progress in July 2005 at the time Geovation prepared their report.

The N-Blend dispersion test consisted of in-situ delivery of 100 gallons of fully-concentrated N-Blend into four Line 1 application wells (DB-3, -4, -5, -6) followed by a groundwater monitoring event in November 2004 prior to the second round of N-Blend applications. Because field and analytical results indicated a strong westward dispersion component, Geovation biased subsequent applications toward the eastern-most application wells in each line of application wells.

Beginning with the December 2004 and January 2005 treatment events relatively small volumes of less concentrated N-Blend were applied to the Line 1 application wells and to existing upgradient wells (DP-1, -2, -3, -13, -15) that were incorporated into the treatment program. Based on review of the nitrogen species and microbial data, Geovation "ramped-up" N-Blend mass loading in subsequent treatment events and concluded this approach resulted in a large and robust denitrifying consortia and minimized downgradient nitrate migration. Geovation indicated subsequent treatment events, beginning with the July 2005 event, will continue with this "ramped-up" approach.



TETRA TECH NUS, INC.

Mr. Brian Helland
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Based on the laboratory analysis of microbiological and geochemical parameters, Geovation concluded that: the population of microorganisms has increased within the treatment zone, the diversity of the microbial population has decreased (number of species decreased), and the denitrifying population has increased. These results indicate that the applications of N-Blend have indeed stimulated the growth of the denitrifying microbes.

Should you have any questions, please do not hesitate to contact me.

Very truly yours,

Liyang Chu
Project Manager

LC/

Enclosures

c: L. Joy, NASB (w/encl.)
C. Sait, ME DEP (w/2 encl.)
J. Trepanowski/G. Glenn, TtNUS (w/hard copy only)
C. Race, TtNUS (w/hard copy only)
File: N4590-3.2 (w/encl.)



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6 July 2005
Revised 4 October 2004

Liyang Chu
Project Manager
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Wilmington, MA 01887-1020

Re: Microbiological and Biogeochemical Assessment Letter Report – July 2005
Denitrification-Based Bioremediation DBB Pilot Program; NEX Brunswick

Dear Mr. Chu:

As per our recent communications, this letter provides Geovation's evaluation and interpretation of the microbiological and biogeochemical data collected subsequent to the baseline-sampling event conducted in August 2004 and during the course of the ongoing Denitrification-Based Bioremediation (DBB) Pilot Project at the above-referenced site. The data collected and evaluated include:

- Quantitative analyses of microbial population densities (in cells/mL of ground water) via epi-fluorescent light microscopy in representative samples taken from along the plume axis: DP-13 (and/or DP-9); DB-04; DB-10; DB-14; MW-300 (and/or MW-301); and MW-302 (and/or MW-NASB-250).
- Quantitative and qualitative analyses of target sub-populations of microorganisms conducted by Geovation (at no additional charge) via multi-color fluorescence in-situ hybridization ("mFISH") in samples from key treatment locations DB-04 and DB-10.
- Denaturing gradient gel electrophoresis (DGGE) analysis of the microbial consortia in ground water at two locations: SDP-5 (mid-plume) and MW-300 (edge of plume).
- Real-time PCR (rtPCR) analysis of the benzyl succinate synthase (BssA) bacterial gene associated with the anaerobic oxidation of aromatic hydrocarbons;
- Supplemental rtPCR analyses (conducted at no additional charge by Microbial Insights) of samples from SDP-5 and MW-300 targeting the following groups of microorganisms: total ("universal") bacteria; denitrifying bacteria via two key bacterial genes (nirS, nirK) associated with denitrification; total iron- and sulfate-reducing bacteria ("irb/srb"); methanogens; and organisms containing the catechol dioxygenase catabolic-process genes.
- Analyses of fixed-nitrogen species (nitrate, nitrite, ammonium) and phosphate species (orthophosphate, complex phosphate) in site ground water.

Executive Summary

The microbial consortia in site ground water subsequent to DBB treatment were investigated via a combination of genomic and culture-independent methods including denaturing-gradient gel electrophoresis (DGGE), real-time polymerase chain reaction (rtPCR), fluorescence microscopy and multi-color FISH. Direct counts of DAPI-stained cells via epi-fluorescence light microscopy indicate a significant increase in total microbial cells--plots of these data illustrate the development of a "bloom" of microorganisms within the plume in response to DBB treatment, with the highest cell counts observed in the most contaminated areas (which also have been subjected to the most intensive N-Blend additions). Both mFISH and DGGE results indicate a shift towards a beta-proteobacteria dominated bacterial community within the main treatment zone. rtPCR indicated a decrease in delta-proteobacteria, an increase in total eubacteria and increases in the detected copies of the catechol dioxygenase, nirS, nirK and BssA genes in response to treatment.

Biogeochemical DBB-process monitoring has focused on the key nitrogen species nitrate, nitrite and ammonium. Near optimal nitrate dispersion has been achieved both within the main treatment zone (located between Burbank Avenue and Building 27) and upgradient beneath the NEX service station. Moderate to high nitrate concentrations have been established within the treatment areas, as desired, whereas nitrate concentrations at downgradient sentry locations are negligible. Nitrite concentrations have increased markedly within the plume and provide a clear indication of localized and robust denitrification activity in the treatment areas. Ammonium concentrations have decreased within the main treatment zone, (where nitrite concentrations are highest), which suggests that DBB treatment has stimulated ammonium removal via the process of anaerobic ammonium oxidation ("Anammox").

The combined results of the microbiological and biogeochemical monitoring have revealed that a larger, less diverse, more active and primarily denitrifying consortia was stimulated by DBB in comparison to pre-treatment conditions characterized by a smaller, more diverse and less active anaerobic consortia.

Summary of Field Operations

DBB field operations commenced in October 2004. Based on prior communications with the MEDEP, the scope of the first treatment event initiated on 6 October 2004 was modified to be used as a N-Blend injection dispersion test. A limited amount (100 gallons) of fully concentrated N-Blend was injected into a subset of the Line 1 treatment points – DB-3, DB-4, DB-5 and DB-6 – followed by a detailed monitoring event conducted in November immediately prior to the second round of N-Blend injections. The resultant field and analytical monitoring data revealed a strong westward component of dispersion, ostensibly due to the westward component of dip of the underlying unconsolidated strata. Accordingly, subsequent treatment events sought to bias N-Blend additions towards the eastern most treatment wells in

each line of treatment points. Over the course of the next several treatment and monitoring events, it became clear that the field measurements of nitrate obtained with an ion-selective probe were increasingly unreliable due to interferences from dissolved solids in the ground water and from the N-blend additions. Beginning with the ground-water sampling event conducted by TtNUS in late March 2005, Geovation shifted towards the collection and laboratory analysis of ground-water samples for nitrate and other N species by others prior to a given treatment event. This protocol became standardized over the past two months during which time Geovation subcontracted the services of Mark Carver of ECC to obtain a discrete round of ground-water samples for DBB-related biogeochemical and microbiological parameters approximately 2 weeks prior to the next N-blend treatment event. These data are interpreted and plotted as isopleth maps (see attached) and used to modify and prepare the N-Blend addition protocols for the treatment events prior to arriving on site. This advance-sampling protocol has been used for the three most recent treatment events (including the event currently in progress), and it is anticipated that this protocol will be used for the remaining treatment events.

A total of seven (7) treatment events have been completed or initiated, including the initial dispersion-test event and the event currently underway as of this writing. A table summarizing the date and scope of the DBB treatment events conducted so far is included immediately following the text of this report. With the exception of the initial dispersion test conducted in October 2004 and the November 2004 treatment event, Geovation's strategy has been to "ramp-up" the N-Blend additions to allow the microbial consortia to adapt so as to increase the rate and efficiency of denitrification processes. Beginning with the December 2004 and January 2005 treatment events, Geovation began to add smaller volumes of less concentrated N-Blend. In addition, the N-Blend treatments were biased to the Line 1 treatment wells and to the pre-existing upgradient points that were incorporated into the treatment program beginning in November 2004 – i.e., mini wells DP-13, DP-1, DP-2, DP-3 and DP-15. Subsequent to January 2005, both the volume and concentration of N-Blend has been increased over time based on the periodic review of the N-species and microbial population data (see attached). As shown in the attached data plots, this approach has been effective at both (a) establishing a large and robust denitrifying consortia and (b) minimizing downgradient nitrate migration. The July 2005 treatment event currently underway will involve the injection of the largest and most concentrated volume of N-Blend since the ramp-up strategy was initiated in December 2004. The August and September 2005 treatment events are also anticipated to be intensive, large-volume, concentrated N-Blend addition events. The specific protocols for these future treatment events will be based on the review of the process-related DBB sampling events to be conducted by ECC for Geovation approximately two weeks prior to the treatment event.

Monitoring Data, Results and Discussion

Microscopy Data and Observations. Microbial populations in site ground water were quantified via epi-fluorescent light microscopy. This method is superior to plate counting and other indirect or culture-dependent methods of estimating microbial

populations as the samples are preserved in the field and analyzed "as is" without introducing biases from culture media and methods. Ground-water samples were collected directly into sterile vials containing buffered formaldehyde solution as a preservative. In the laboratory, representative aliquots of the preserved samples were doped with a dispersing agent and stained with 4',6-diamidino-2-phenylindole (DAPI). DAPI selectively stains the A-T bridge of double-stranded (i.e., intact) DNA; accordingly, DAPI staining can distinguish the live microorganisms present in the sample at the time of collection. DAPI-stained samples were subjected to epi-fluorescent light microscopy and digital imaging in Geovation's microbiology laboratory. Multiple digital images were obtained from each sample to provide representative and statistically meaningful data. Subsequently, the images for each sample were subjected to semi-automated digital-image processing, analysis and counting using MetaVue™ software (Universal Imaging).

The results of the epi-fluorescence microscopy are illustrated in Figure set 3-1. As shown in Figure 3-1-1b, prior to treatment the highest microbial populations were present along the main axis of the sorbed-phase GRO plume. Subsequent to DBB treatment microbial populations increased, and the isopleth maps illustrate the development of a "bloom" of microorganisms within the plume in response to DBB treatment, with the highest cell counts observed in the most contaminated areas (which also have been subjected to the most intensive N-Blend additions)

mFISH Data and Observations. As part of Geovation's ongoing DBB-related research and development, additional investigations of the microbial consortia growing in response to the DBB treatments have been conducted using multi-color fluorescence in-situ hybridization ("mFISH"). The mFISH analyses have primarily focused on evaluating trends at key locations DB-04 and DB-10 over time with the "MP1" multi-color assay as follows: Cy5-labeled GAM42a (gamma-proteobacteria); Cy3-labeled EUB338 (most eubacteria) and Oregon Green 488 labeled BETA42a (beta-proteobacteria). mFISH assays were counterstained with DAPI for total cell counts and as a reference for the mFISH data. The preliminary results of this work so far indicate that during the initial period of treatment in the early fall of 2004, both beta- and gamma-proteobacteria were numerous in the treatment zone (consistent with the results of the initial DGGE analyses). Over time, beta-proteobacteria appear to have become dominant, whereas gamma-proteobacteria have become less prominent. An example of a mFISH image is attached. Geovation plans to continue conducting mFISH analyses as time and resources permit.

DGGE Data. Denaturing gradient gel electrophoresis (DGGE) analysis was conducted to investigate the microbial consortia in ground water at two key locations relative to the DBB treatment program: SDP-5 (mid-plume) and MW-300 (edge of plume). Microbial biomass was allowed to accumulate on "Biotraps" installed within these wells in late January 2005, which were retrieved in April 2005 and submitted to Microbial Insights, Inc. (MII) of Rockford, Tennessee for analysis of bacterial, archaeal and fungal DNA via DGGE. The DGGE gels and bands that were successfully sequenced are illustrated in the attached figure and summarized in Table DGGE-2. Geovation analyzed the DGGE sequence data by conducting

“BLAST” (Basic Local Alignment Search Tool) analyses in comparison to sequences deposited in the databases maintained by the National Center for Biotechnology Information (NCBI) and the Ribosomal Database Project (RDP) at Michigan State University.

The results of the second round of DGGE analyses documented that a less diverse microbial consortia developed at each location in response to DBB treatment as evidenced by the predominance of fewer phylogenetic groups over time. Prior to treatment, bacteria related to the phylum Bacteroidetes (or “CFB”), and the alpha-, beta-, gamma- and delta- subdivisions of the Proteobacteria were detected via DGGE. After treatment, the eubacterial DGGE profile was largely dominated by beta-proteobacteria (4 of 6 sequences). Noteworthy is that 3 of the 6 eubacterial sequences were related to a single family within the beta-proteobacteria—i.e., the *Comamonadaceae*. Changes were also noted for both the archaeal and fungal domains, with fewer and different microorganisms observed after treatment by comparison to baseline conditions.

Real-Time PCR Data. Microbial Insights, Inc. (MII) conducted several real-time polymerase chain reaction (“rtPCR”) analyses for several target genes and/or groups of microorganisms. In addition to the DGGE analyses described above, Geovation subcontracted MII to conduct PCR analyses targeting the benzyl succinate synthase (BssA) gene in ground-water samples from SDP-5 and MW-300. The BssA gene is found in some anaerobic, aromatic-hydrocarbon degrading bacteria and was recently discovered to mediate the initial step in the anaerobic oxidation of certain aromatic hydrocarbons (e.g., toluene, ethylbenzene). BssA adds a fumarate molecule to a methyl group to form benzyl succinate, which is amenable to further, more rapid biodegradation. MII also conducted several additional analyses on the above-referenced wells at no additional charge: total (“universal”) bacteria; denitrifying bacteria via two key bacterial genes (*nirS*, *nirK*) associated with denitrification; total iron- and sulfate-reducing bacteria (“irb/srb”); methanogens; and organisms containing the catechol dioxygenase catabolic-process genes.

The PCR results are summarized in the attached report from MII. The BssA gene was measured in the range of 2.91×10^3 to 8.3×10^4 copies/mL, a three-to-four order of magnitude increase relative to the baseline event. The rtPCR data for total (“universal”) bacteria correlate very closely with the microbial population data from the epi-fluorescence microscopy and show about a one-order of magnitude increase over baseline conditions. As noted previously for the baseline event, the bacterial populations estimated by rtPCR were consistently higher than those determined by direct microscopic counts.

The denitrification rtPCR assay targeted two genes that encode two nitrite reductases involved in respiratory nitrate reduction (i.e., “true” denitrification)—the *nirS* and *nirK* genes. The April 2005 PCR data indicate that the measured copies of the *nirS* and *nirK* genes increased in both SDP-5 and MW-300. For example, in SDP-5, the total combined copies of the *nirS* and *nirK* genes increased from about 1.1×10^5 copies/mL in August 2004 to more than 5.5×10^5 copies/mL in April 2005.

However, given that the total cell counts are estimated to be in the range of 10^7 cells/mL (DAPI counts) to 10^8 cells/mL (rtPCR), and given that it is likely that the majority of microbes growing in response to DBB treatment are capable of denitrification, the total *nirS* and *nirK* assays are only detecting from about 0.1 to 1% of the denitrifiers present in the ground water. Accordingly, it is clear that different denitrification genes and enzyme systems other than *nirS* and *nirK* are present in the microbes comprising the microbial consortia.

The iron-reducer/sulfate-reducer ("irb/srb") rtPCR assay targeted the 16S rDNA of members of the γ -proteobacteria that are capable of the reduction of Fe^{+3} and sulfate, such as members of the family *Geobacteraceae*. Whereas approximately 10^5 copies of the irb/srb assay were measured in MW-NASB-25 (downgradient of MW-300) during the baseline event, no copies of this assay were measured in either SDP-5 or MW-300 in April 2005. These results would be consistent with a shift from a more diverse bacterial community that included an abundance of the (typically) strictly anaerobic γ -proteobacteria to a less diverse community dominated by denitrifiers. Similarly, whereas γ -proteobacteria (*Geobacter* spp.) were detected by DGGE during the baseline assessment, γ -proteobacteria were not detected via DGGE as of April 2005. The gene marker for methanogens, which are strictly anaerobic archaea, was detected in the range of 10^4 (SDP-5) to 10^6 (MW-300) copies/mL.

The catechol dioxygenase rtPCR assay(s) target a catabolic-process gene that is present in many different bacteria. The catechol rtPCR results for SDP-5 show a slight increase from more than 10^4 copies/mL (August 2004) to more than 10^5 copies/mL (April 2005).

Biogeochemical Data. Geovation has conducted routine sampling and/or analyses for key fixed-nitrogen ("N") species (nitrate, nitrite, ammonium) and phosphate ("P") species (orthophosphate, complex phosphate) during the DBB program. Samples were collected for low-MDL analysis of these parameters using modified SW-846 methods designed to reduce detection limits to within the range of 1-15 ug/L. In addition, some samples have been sent for analysis of N-species by Katahdin Analytical Services ("Katahdin"), a Maine-certified laboratory.

The distribution of nitrate, nitrite, ammonium before and in response to treatment are illustrated in figure sets 1-1, 1-2 and 1-3, respectively. The nitrate maps (Figures 1-1-1 through 1-1-5) indicate that N-Blend additions have achieved nitrate dispersion throughout the treatment areas without causing significant increases in nitrate downgradient of the treatment areas. Field probe readings of nitrate have proven to be unreliable, most likely as a result of interferences from dissolved solids and N-Blend related salts. The DBB-related process monitoring will continue to key on nitrate analyses to be performed by Southampton University and Katahdin rather than field measurements with an ion-selective probe.

The nitrite data are illustrated in Figures 1-2-1 through 1-1-5. Whereas nitrite was essentially absent in most areas prior to treatment (Figure 1-2-1), nitrite levels have

increased by one to several orders of magnitude in the main treatment areas but remain low or absent at downgradient locations. Nitrite is a short-lived intermediate species produced by denitrification processes, and it is also consumed by the process of anaerobic ammonium oxidation ("Anammox"). Nitrite will continue to be a key parameter for the DBB-related process monitoring and will be evaluated via analyses performed by Southampton University and Katahdin.

The ammonium data are illustrated in Figures 1-3-1 through 1-3-3. Prior to DBB treatment, the highest ammonium levels were present both within and downgradient of the plume (Figure 1-3-1). The pattern of pre-treatment ammonium distribution is consistent with dissimilatory (non-respiratory) nitrate reduction within the plume and is typical of baseline-sampling observations from other DBB sites (including the DBB pilot conducted in Groton). Subsequent to DBB treatment, ammonium concentrations have decreased within the main treatment zone, coincident with the pattern of highest nitrite concentrations. Figure 1-3-3 indicates a slight "leakage" of ammonium immediately downgradient of the treatment zone, where nitrite concentrations are lower. The observed patterns of ammonium and nitrite distribution suggest that DBB treatment has stimulated ammonium removal via anammox. Anammox is a chemoautotrophic process facilitated by a specialized lineage of bacteria, the *Planctomycetales*, which derive energy by coupling the reduction of nitrite to the anaerobic oxidation of ammonium.

Discussion. The combined results of the microbial and biogeochemical analyses indicate that the N-Blend additions have increased the microbial populations within the treatment zone and that the microbial community has become less diverse, more active and primarily denitrifying relative to the consortia present prior to treatment. The nitrate, nitrite and ammonium data indicate that the "ramping up" of N-Blend volumes and concentrations over time has been effective at establishing a robust denitrifying microbial community with minimal loss of nutrients downgradient of the treatment zone. Accordingly, this strategy will be continued throughout the course of the remaining warm-weather treatment events and altered, if and when necessary, based on continued monitoring of the key N-species parameters and microbial populations. Given the recent results of the microbial and biogeochemical monitoring, it appears that sufficient progress has been made at establishing robust DBB treatment conditions such that it may be worth considering conducting another round of bioremediation progress soil sampling by the late summer or fall of 2005.

Sincerely,
GEOVATION CONSULTANTS, Inc.



Eric C. Hince, P.G.
CTO, President

Attachments

NEX Brunswick DBB Pilot Program
Summary of DBB Treatment Events Conducted to Date
Biogeochemical - Microbiological Evaluation Letter Report No. 2 - 6 July 2005

Treatment Event	Date	Notes	N-Blend Loading		Treatment Points Utilized			
			Tot. Volume (Gal.)	Concentration	Line 1	Line 2	Line 3	Upgradient
1	6-Oct-04	Dispersion Test	100	Highest	X			
2	2-Nov-04		192	Highest	X	X	X	X
3	14-Dec-04		400	Low	X	X	X	X
4	28-Jan-04		95	Low	X			X
5	12-Apr-05		350	Medium	X			X
6	2-Jun-05		322	Med-High	X	X		X
7	7-Jul-05	In Progress	400	High	X	X		X



Isopleth Maps: Bacteria Count Data

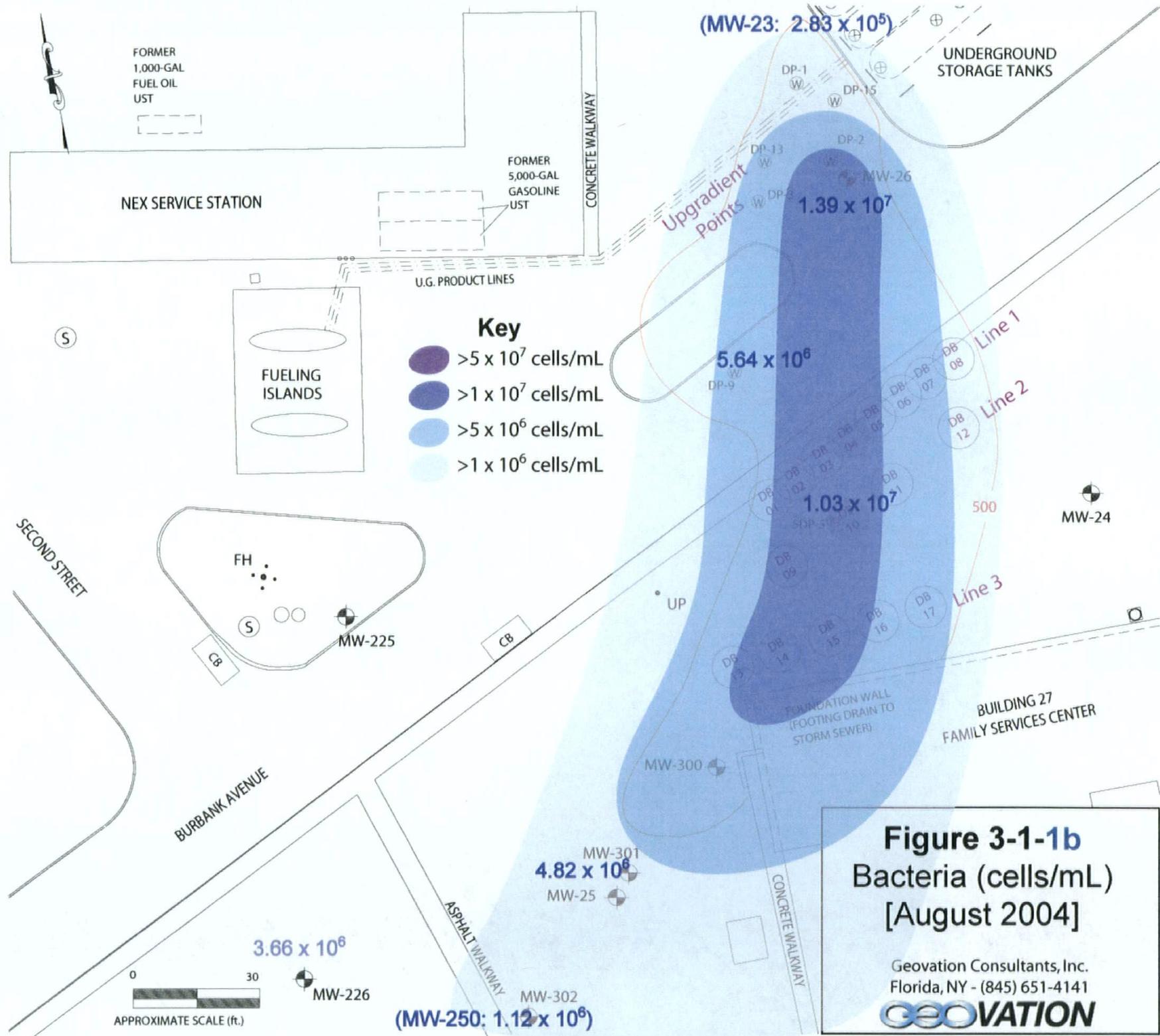
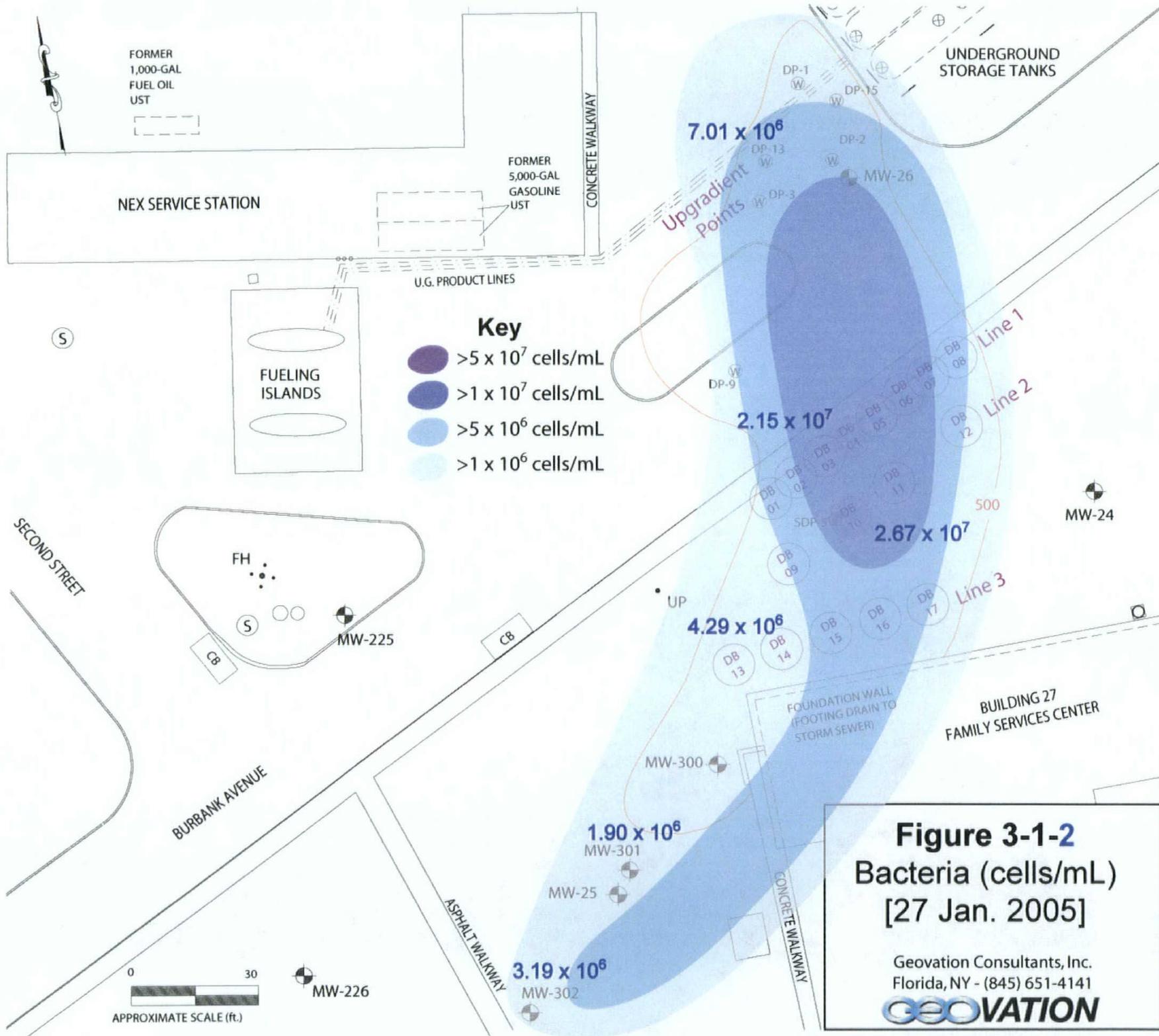


Figure 3-1-1b
Bacteria (cells/mL)
[August 2004]

Geovation Consultants, Inc.
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GEOVATION



FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

CONCRETE WALKWAY

U.G. PRODUCT LINES

FUELING
ISLANDS

Key

- $>5 \times 10^7$ cells/mL
- $>1 \times 10^7$ cells/mL
- $>5 \times 10^6$ cells/mL
- $>1 \times 10^6$ cells/mL

UNDERGROUND
STORAGE TANKS

7.01×10^6

Upgradient
Points

2.15×10^7

2.67×10^7

4.29×10^6

1.90×10^6

3.19×10^6

Line 1

Line 2

Line 3

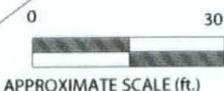
SECOND STREET

BURBANK AVENUE

ASPHALT WALKWAY

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

BUILDING 27
FAMILY SERVICES CENTER



MW-226

FH

MW-225

MW-300

MW-25

MW-302

MW-24

DP-1
DP-15
DP-2
DP-13
DP-3
MW-26

DP-9

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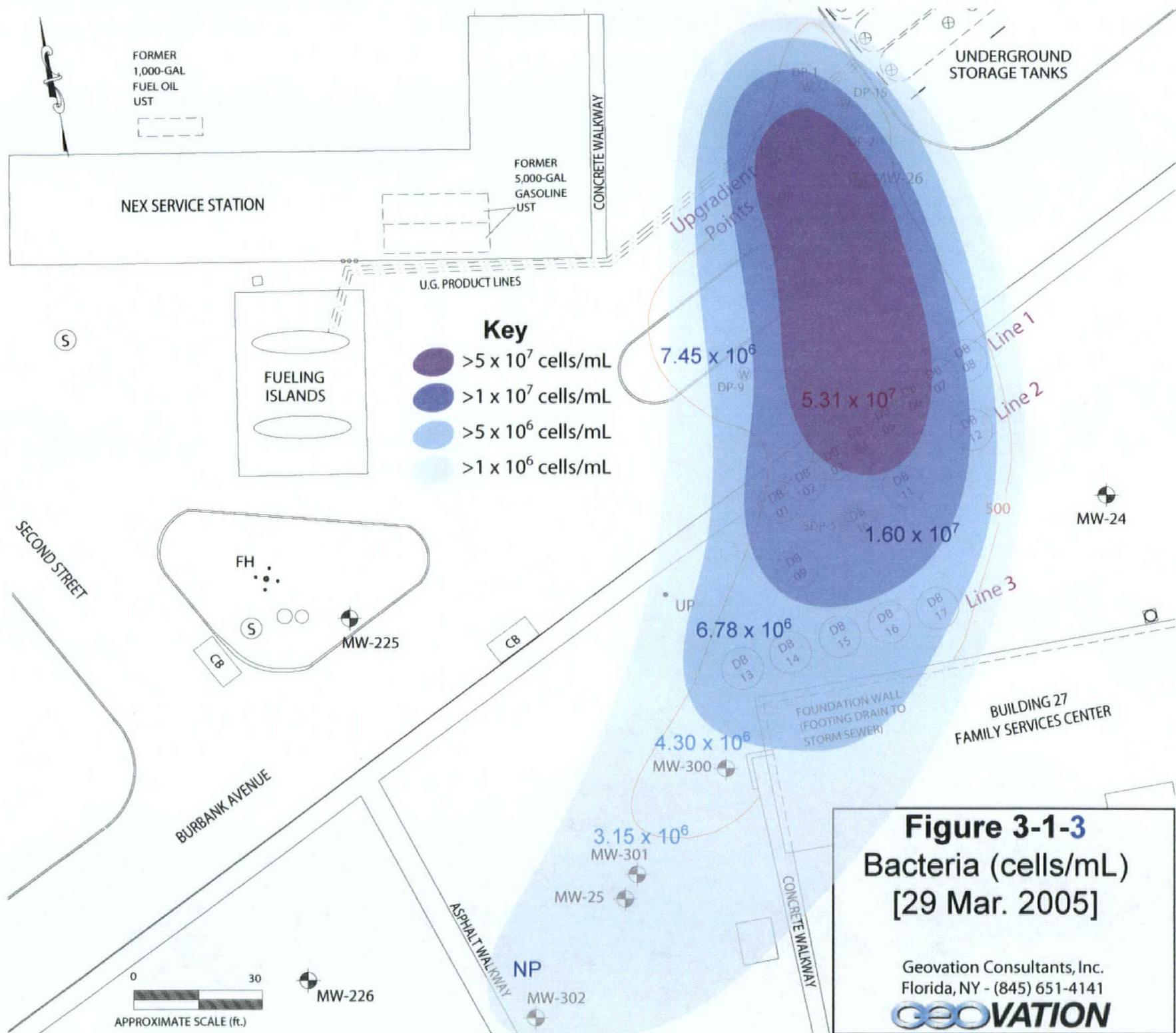
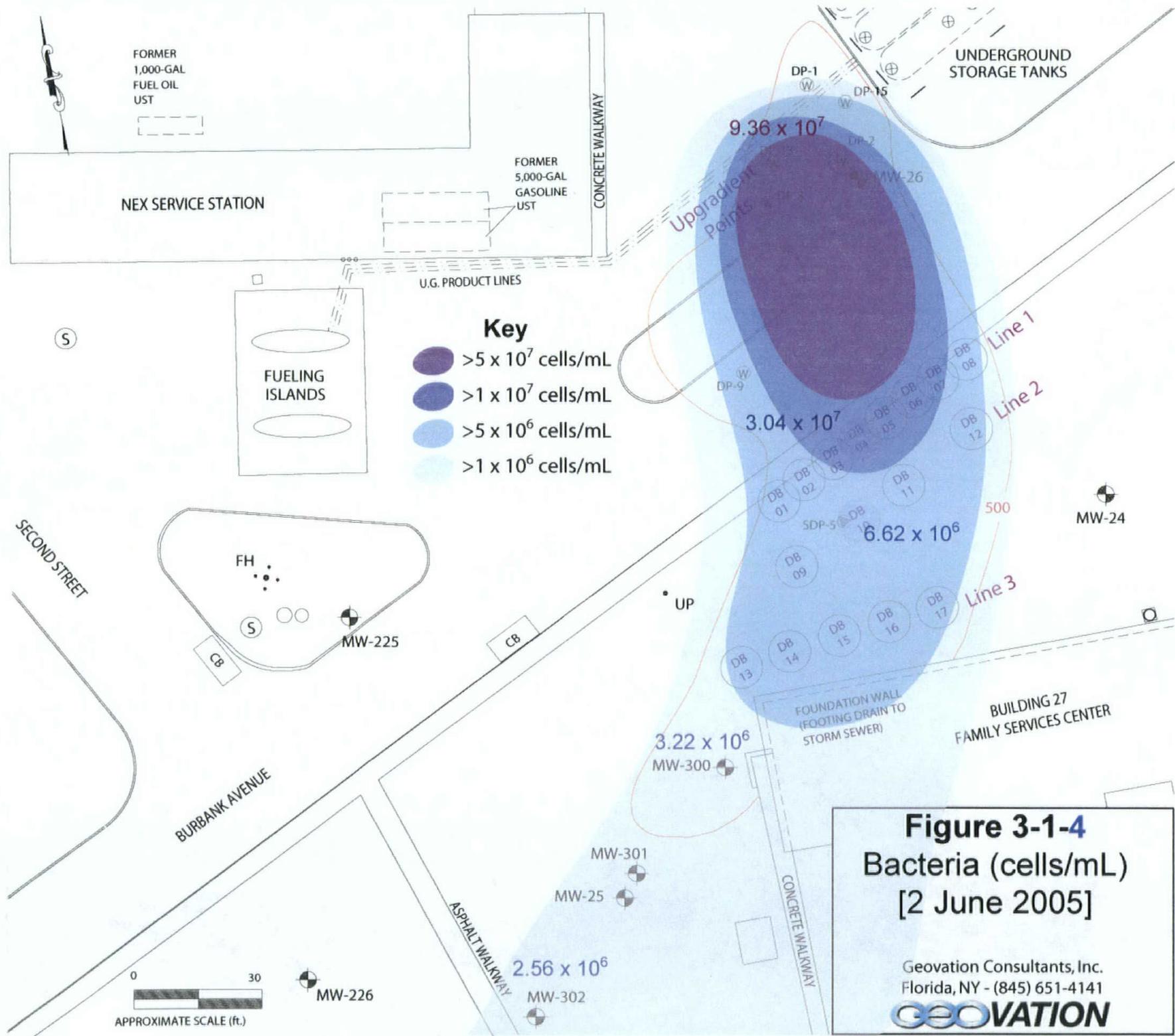


Figure 3-1-3
Bacteria (cells/mL)
[29 Mar. 2005]

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FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

UNDERGROUND
STORAGE TANKS

CONCRETE WALKWAY

U.G. PRODUCT LINES

FUELING
ISLANDS

Key

- $>5 \times 10^7$ cells/mL
- $>1 \times 10^7$ cells/mL
- $>5 \times 10^6$ cells/mL
- $>1 \times 10^6$ cells/mL

9.36×10^7

3.04×10^7

6.62×10^6

3.22×10^6

2.56×10^6

Upgradient
Points

Line 1

Line 2

Line 3

500

SECOND STREET

BURBANK AVENUE

ASPHALT WALKWAY

CONCRETE WALKWAY

BUILDING 27
FAMILY SERVICES CENTER

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

S

FH

MW-225

CB

UP

MW-24

MW-301

MW-25

MW-226

MW-302

DP-9

DP-1

DP-15

DP-2

MW-26

DB 01

DB 02

DB 03

DB 04

DB 05

DB 06

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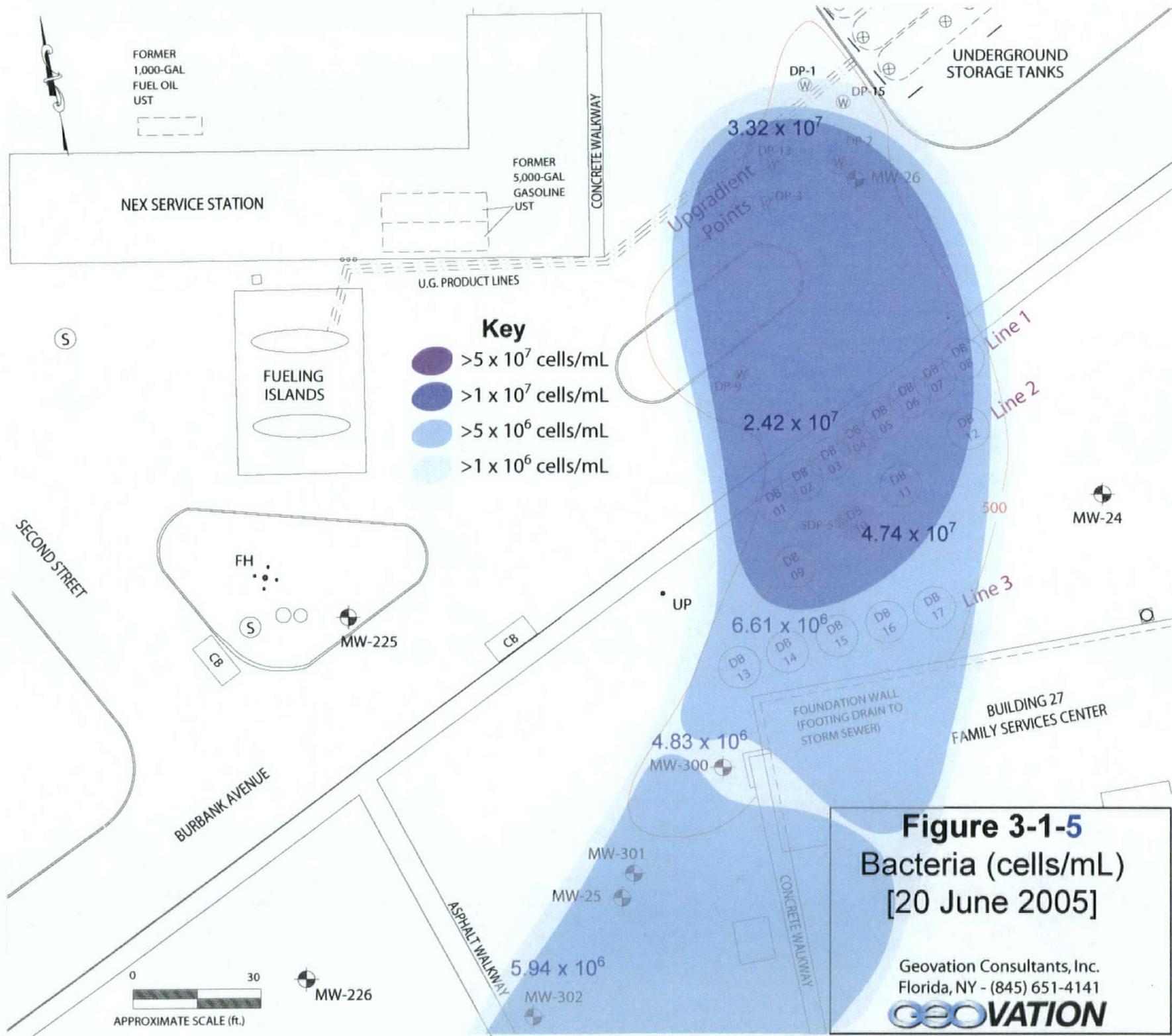
DB 15

DB 16

DB 17

SDP-5





FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

CONCRETE WALKWAY

U.G. PRODUCT LINES

FUELING
ISLANDS

Key

- $>5 \times 10^7$ cells/mL
- $>1 \times 10^7$ cells/mL
- $>5 \times 10^6$ cells/mL
- $>1 \times 10^6$ cells/mL

UNDERGROUND
STORAGE TANKS

DP-1

DP-15

3.32×10^7

DP-13

DP-2

MW-26

Upgradient
Points

DP-3

2.42×10^7

Line 1

Line 2

4.74×10^7

Line 3

6.61×10^6

4.83×10^6

MW-300

BUILDING 27
FAMILY SERVICES CENTER

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

MW-301

MW-25

5.94×10^6

MW-302

ASPHALT WALKWAY

CONCRETE WALKWAY

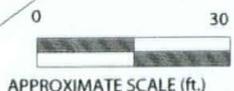
SECOND STREET

BURBANK AVENUE

FH

MW-225

MW-24

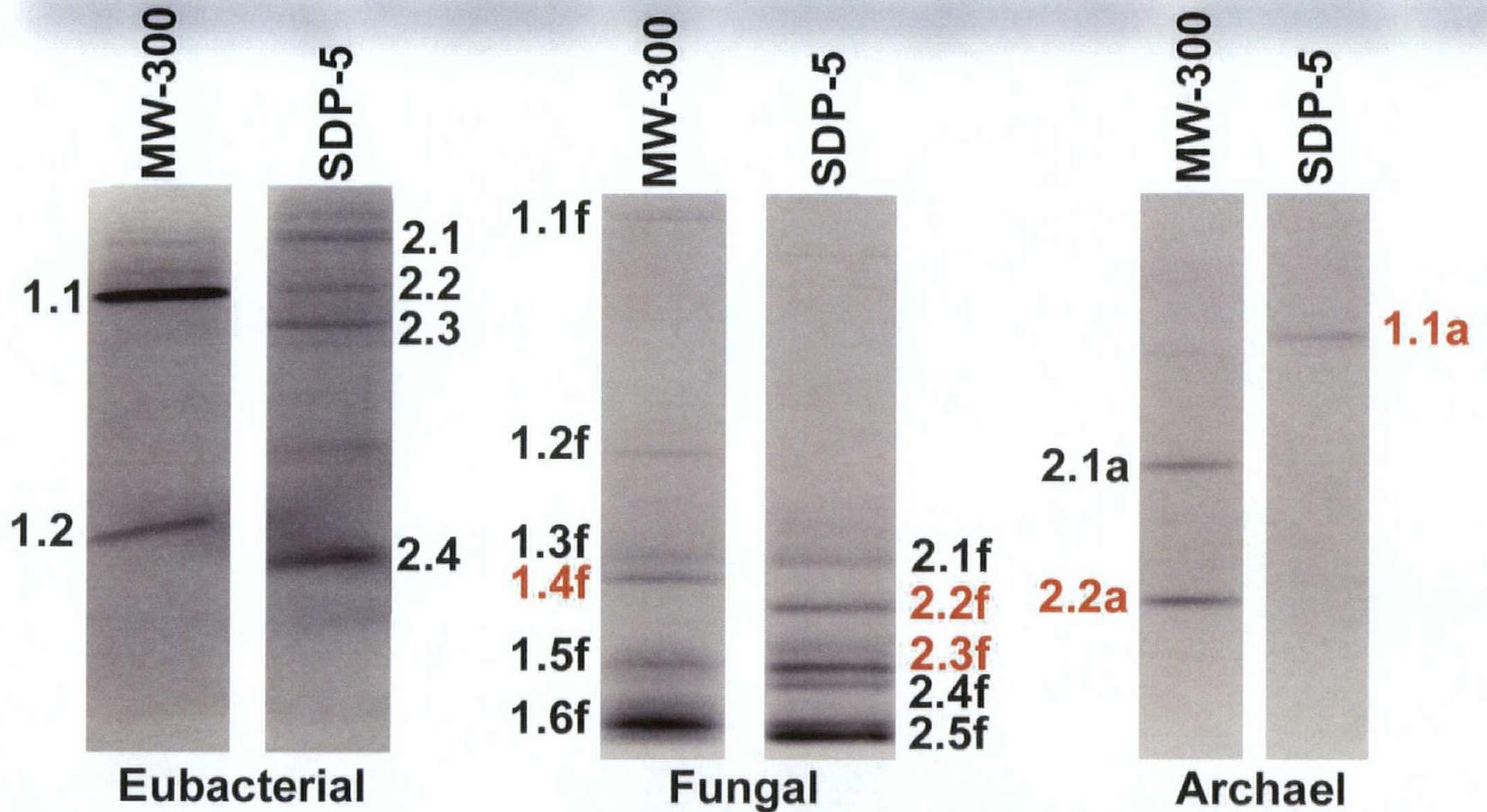


MW-226

Results Summary:

Denaturing Gradient
Gel Electrophoresis
(DGGE) Data

Denaturing Gradient Gel Electrophoresis (DGGE) Gels



Red labels indicate bands which failed to produce readable sequences

Table DGGE-2
Biotrap DGGE Results for Ground Water Samples (April 2005)
DBB Pilot Program - NEX Brunswick, ME
(Data Analysis Completed: April 2005)

Band (Well)	Genus/Species	Phylogeny	GenBank ID No.	Database	Similarity
Bacteria (16s rDNA)					
1.1 (MW-300)	Beta-proteobacterium RG-4 <i>Comamonadaceae</i> bact. PIV-20-1 <i>Variovorax</i> sp. ANRB-Zg	Eubacteria; Proteobacteria; β -Proteobacteria	AY561571	NCBI	100%
		" " " Burkholderiales; Comamonadaceae	AJ505862	" "	100%
		" " " " " <i>Variovorax</i>	AJ276398	" "	99%
1.2 (MW-300)	Uncultured gamma proteobacterium clone LTUG00356 Denitrifying bacterium L(A2)	Eubacteria; Proteobacteria; γ -Proteobacteria	AY144234	NCBI	99%
		Unclassified Eubacteria	AY122310	" "	97%
2.1 (SDP-5)	Uncultured bacterium clone A0/8 <i>Rhodoferrax ferrireducens</i>	Unclassified Eubacteria	AY465127	NCBI	96%
		" " β -Proteobacteria; Burkholderiales; Comamonadaceae; <i>Rhodoferrax</i>	AF435948	" "	95%
2.2 (SDP-5)	Beta proteobacterium TH-H1 Uncultured <i>Variovorax</i> sp. clone CI-1-TB3-I 16S	Eubacteria; Proteobacteria; β -Proteobacteria	AJ785996	NCBI	96%
		" " " Burkholderiales; Comamonadaceae; <i>Variovorax</i>	AY599728	" "	96%
2.3 (SDP-5)	Uncultured <i>Hydrogenothermus</i> sp. clone OPPB049 Uncultured <i>Aquificales</i> bacterium clone FL06G10 16S Uncultured <i>Sulfurihydrogenibium</i> sp.	Bacteria; Aquificae; Aquificales;	AY861784	NCBI	90%
		Hydrogenothermaceae; <i>Hydrogenothermus</i>	AY293478	" "	94%
		Bacteria; Aquificae; Aquificales;	AY882759	RDP	0.645
		Bacteria; Aquificae; Aquificales; Aquificaceae;			
		<i>Sulfurihydrogenibium</i>			

= gaps in sequence match
= significant gaps in sequence match
= reported match is for a small sequence

Table DGGE-2
Biotrap DGGE Results for Ground Water Samples (April 2005)
DBB Pilot Program - NEX Brunswick, ME
(Data Analysis Completed: April 2005)

Band (Well)	Genus/Species	Phylogeny	GenBank ID No.	Database	Similarity
2.4 (SDP-5)	Beta proteobacterium "PB7"	Proteobacteria; β -Proteobacteria	AY686732	NCBI	98%
Archaea (16s rDNA)					
2.1a (MW-300)	Uncultured crenarchaeote clone PET1-26	Archaea; Crenarchaeota	AY278103	NCBI	100%
2.2a (MW-300)	Uncultured archaeon clone APL3	Archaea	AY904332	NCBI	100%
Fungi (28s rDNA)					
1.1f (MW-300)	<i>Phoma</i> sp. 200	Eukaryota; Fungi; Ascomycota; mitosporic Ascomycota; Phoma	AY293786	NCBI	91%
1.2f (MW-300)	<i>Geomyces</i> sp. SW151	Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Onygenales; mitosporic Onygenales; Geomyces	AY234969	NCBI	88%
1.3f (MW-300)	<i>Neofabraea alba</i> strain CBS109875	Eukaryota; Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Helotiales; Dermateaceae; Neofabraea	AY064705	NCBI	84%
1.5f (MW-300)	<i>Penicillium angulare</i> strain NRRL 28140	Eukaryota; Fungi; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiales; Trichocomaceae; mitosporic Trichocomaceae; Penicillium	AY313613	NCBI	97%

= gaps in sequence match
= significant gaps in sequence match
= reported match is for a small sequence

Table DGGE-2
Biotrap DGGE Results for Ground Water Samples (April 2005)
DBB Pilot Program - NEX Brunswick, ME
(Data Analysis Completed: April 2005)

Band (Well)	Genus/Species	Phylogeny	GenBank ID No.	Database	Similarity
1.6f (MW-300)	Fungal sp. CL048 <i>Phoma glomerata</i> strain ATCC 36804	Eukaryota; Fungi Eukaryota; Fungi; Ascomycota; mitosporic Ascomycota; Phoma	AY234962 AY293796	NCBI " "	96% 95%
2.1f (SDP-5)	<i>Cryptococcus victoriae</i> KCTC 17059	Eukaryota; Fungi; Basidiomycota; Hymenomycetes; Heterobasidiomycetes; Tremellomycetidae; Tremellales; mitosporic Tremellales; Cryptococcus	AF459674	NCBI	98%
2.4f (SDP-5)	<i>Aureobasidium pullulans</i> strain HA1556	Eukaryota; Fungi; Ascomycota; Pezizomycotina; Dothideomycetes et Chaetothyriomycetes incertae sedis; Dothioraceae; mitosporic Dothioraceae; Aureobasidium	AJ507454	NCBI	95%
2.5f (SDP-5)	<i>Venturia hanliniana</i>	Eukaryota; Fungi; Ascomycota; Pezizomycotina; Dothideomycetes; Pleosporales; Venturiaceae; Venturia	AB100681	NCBI	99%

= gaps in sequence match

= significant gaps in sequence match

= reported match is for a small sequence

Results Summary: Real Time PCR (rtPCR) Data



2340 Stock Creek Blvd.
Rockford TN 37853-3044
Phone: (865) 573-8188
Fax: (865) 573-8133
Email: info@microbe.com

Analysis Report

Client: Eric C. Hince
Geovation Consultants, Inc.
PO Box 293
468 Rt. 17A
Florida, NY 10921

Phone: (845) 651-4141

Fax: (845) 651-0040

MI Identifier: 031CD

Date Rec: 04/13/2005

Report Date: 04/18/2005

Analysis Requested: CENSUS (final)

Project: NEX Brunswick

Comments:

All samples within this data package were analyzed under U.S. EPA Good Laboratory Practice Standards: Toxic Substances Control Act (40 CFR part 790). All samples were processed according to standard operating procedures. Test results submitted in this data package meet the quality assurance requirements established by Microbial Insights, Inc.

Reported By:

Reviewed By:

NOTICE: This report is intended only for the addressee shown above and may contain confidential or privileged information. If the recipient of this material is not the intended recipient or if you have received this in error, please notify Microbial Insights, Inc. immediately. The data and other information in this report represent only the sample(s) analyzed and are rendered upon condition that it is not to be reproduced without approval from Microbial Insights, Inc. Thank you for your cooperation.

MICROBIAL INSIGHTS, INC.

2340 Stock Creek Blvd. Rockford, TN 37853-3044
 Tel: (865) 573-8188; Fax: (865) 573-8133

CENSUS

Client: Geovation Consultants, Inc.
Project: NEX Brunswick

MI Project Number: 031CD
Date Received: 04/13/2005

Sample Information

Client Sample ID:	SPD-5-Brunswick	MW-300-Brunswick
Sample Date:	04/12/2005	04/12/2005
Units:	cells/bead	cells/bead

Functional Genes

Benzyl Succinate Synthase	bssA	2.91E+03	8.3E+04
Catechol Dioxygenase	Cat	1.2E+05	8.4E+06

Phylogenetic Group

Eubacteria	EBAC	3.3E+08	4.07E+08
Methanogens	MGN	5.3E+04	1.02E+06
Sulfate & Iron Reducing Bacteria	IRB/SRB	<3.75E+01	<3.75E+01
Denitrifying Bacteria (nirK)	nirK	9.4E+04	2.03E+05
Denitrifying Bacteria (nirS)	nirS	4.78E+05	5.55E+05

Legend:

NA = Not Analyzed NS = Not Sampled J = Estimated gene copies below PQL but above LQL I = Inhibited
 < = Result not detected

Notes:

1 Bio-Dechlor Census technology was developed by Dr. Loeffler and colleagues at Georgia Institute of Technology and was licensed for use through Regenesys.

Example Images:

Fluorescence Microscopy;

Fluorescence in-situ

Hybridization (FISH)



False-color RGB composite bitmap image of a "MP1" mFISH assay showing denitrifying bacteria from the gasoline-contaminated aquifer at location DB-10. Aqua cells are beta-proteobacteria (dual hybridizations with "blue" BET42a and "green" EUB338 probes), yellow cells are gamma- proteobacteria (dual hybridizations with "red" GAM42a and "green" EUB338 probes) and green cells are undifferentiated bacteria (single hybridizations with "green" EUB338 probe). Red, purple, and blue objects are noise from cells within biofilms (arrows).

Isopleth Maps:

Nitrate - NO_3^-

Monitoring Data

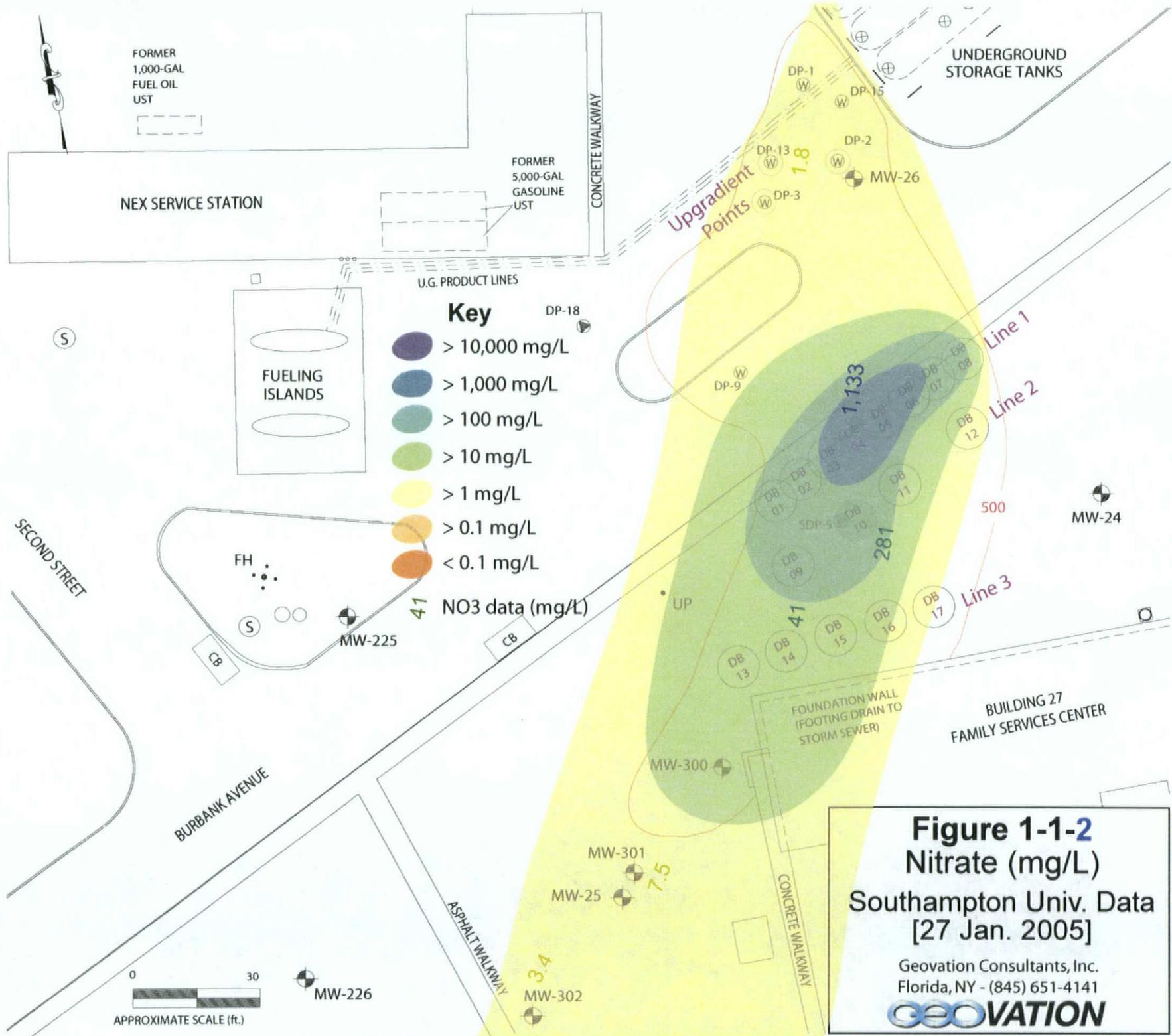


Figure 1-1-2
Nitrate (mg/L)
Southampton Univ. Data
[27 Jan. 2005]

Geovation Consultants, Inc.
 Florida, NY - (845) 651-4141

GEOVATION

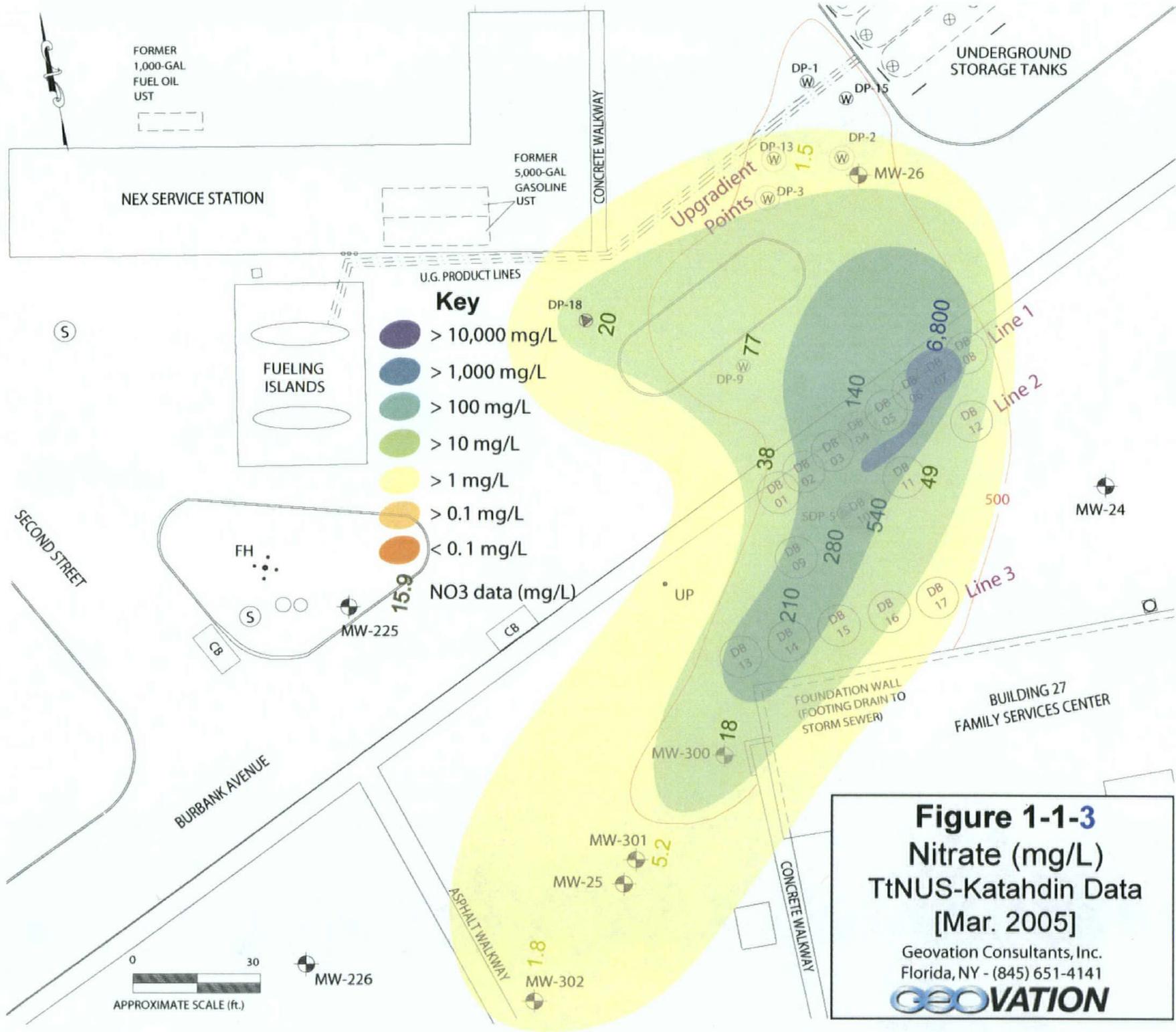


Figure 1-1-3
Nitrate (mg/L)
TtNUS-Katahdin Data
[Mar. 2005]
 Geovation Consultants, Inc.
 Florida, NY - (845) 651-4141
GEOVATION

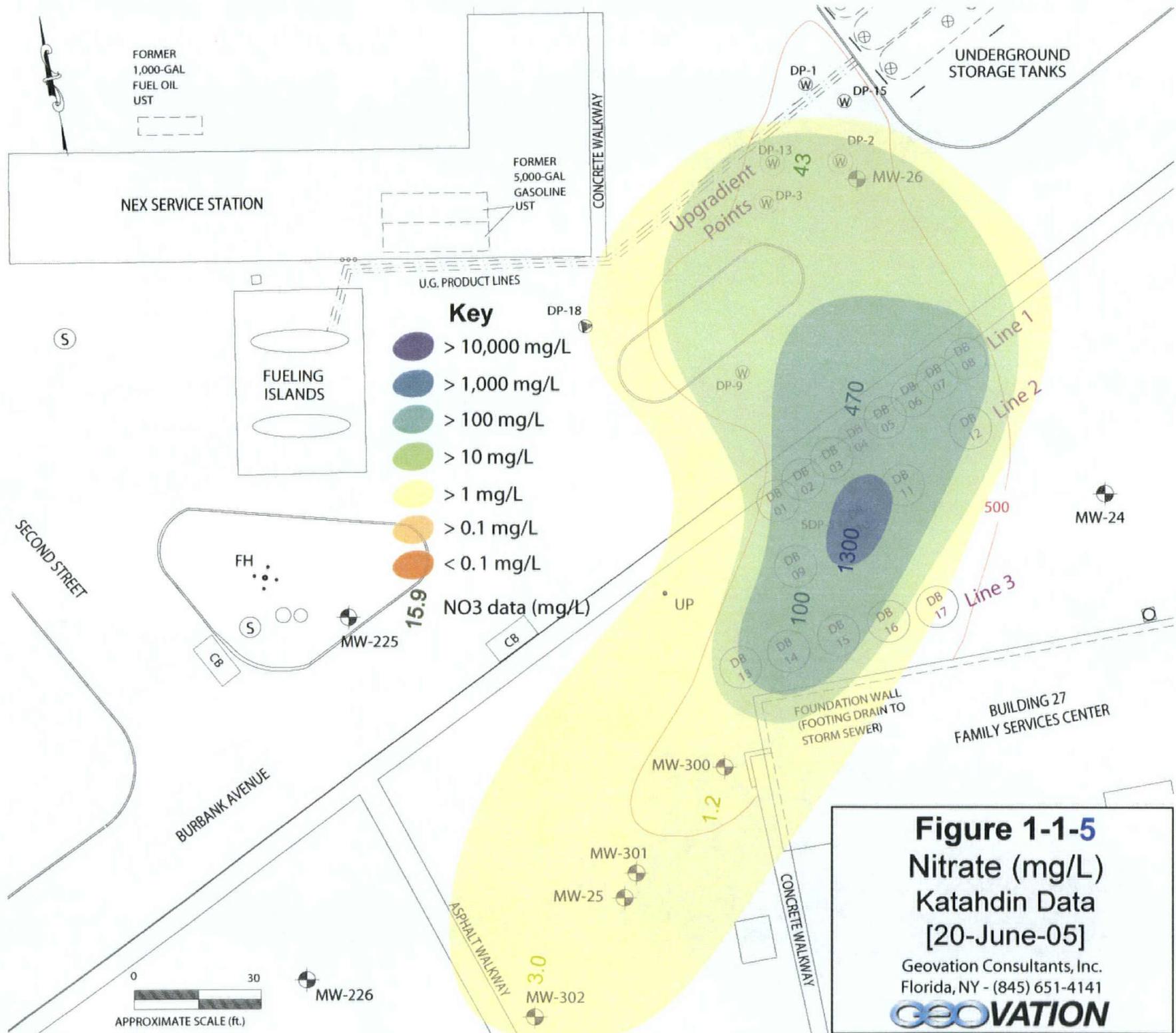
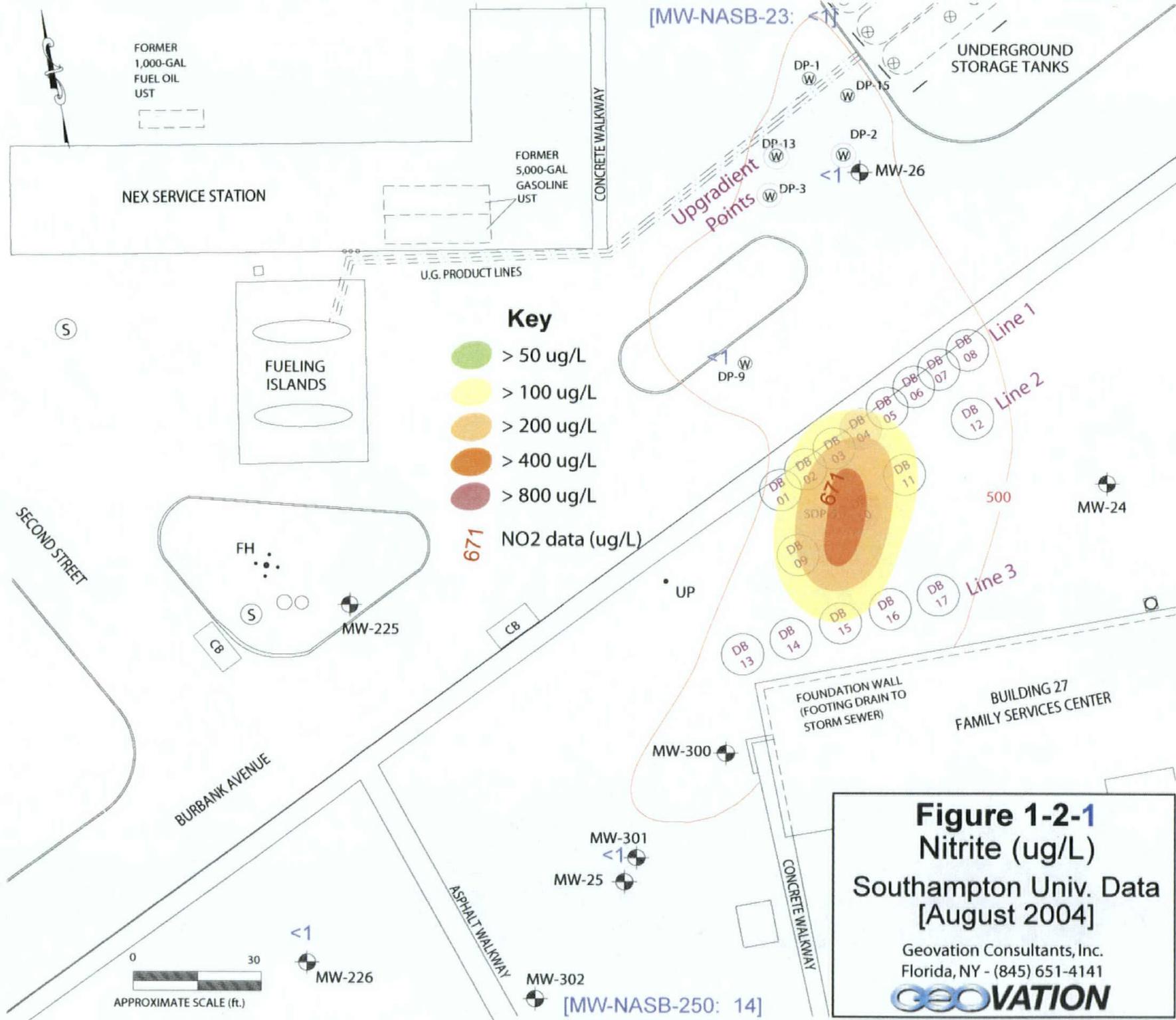


Figure 1-1-5
Nitrate (mg/L)
Katahdin Data
[20-June-05]
 Geovation Consultants, Inc.
 Florida, NY - (845) 651-4141

Isopleth Maps:

Nitrite - NO_2^-

Monitoring Data



[MW-NASB-23: 11]

UNDERGROUND STORAGE TANKS

FORMER 1,000-GAL FUEL OIL UST

FORMER 5,000-GAL GASOLINE UST

NEX SERVICE STATION

U.G. PRODUCT LINES

CONCRETE WALKWAY

FUELING ISLANDS

Key

Upgradient Points

Line 1

Line 2

Line 3

SECOND STREET

BURBANK AVENUE

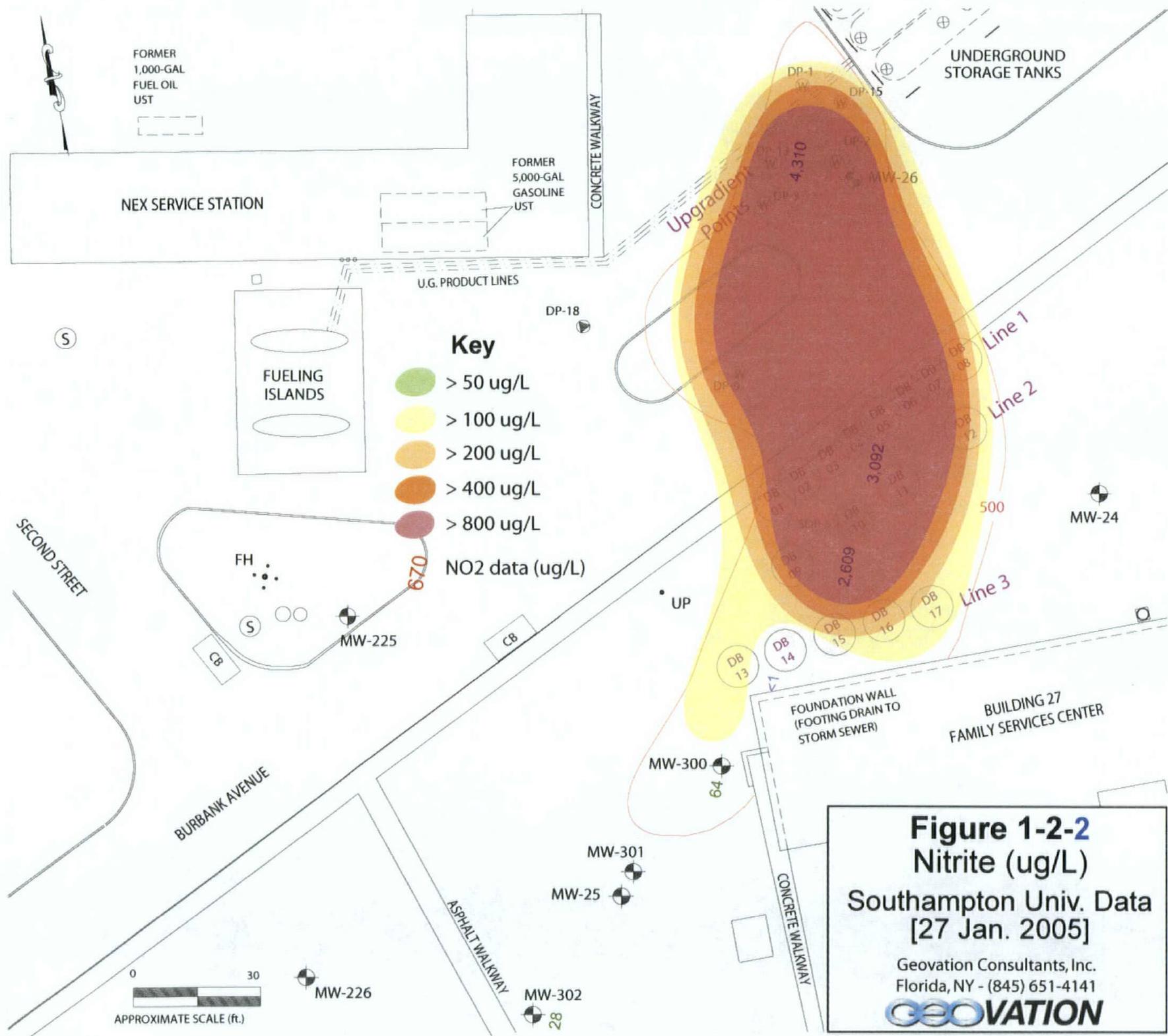
ASPHALT WALKWAY

FOUNDATION WALL (FOOTING DRAIN TO STORM SEWER)

BUILDING 27 FAMILY SERVICES CENTER



[MW-NASB-250: 14]



FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

UNDERGROUND
STORAGE TANKS

CONCRETE WALKWAY

U.G. PRODUCT LINES

Key

- > 50 ug/L
- > 100 ug/L
- > 200 ug/L
- > 400 ug/L
- > 800 ug/L

670 NO2 data (ug/L)

FUELING
ISLANDS

SECOND STREET

BURBANK AVENUE

ASPHALT WALKWAY

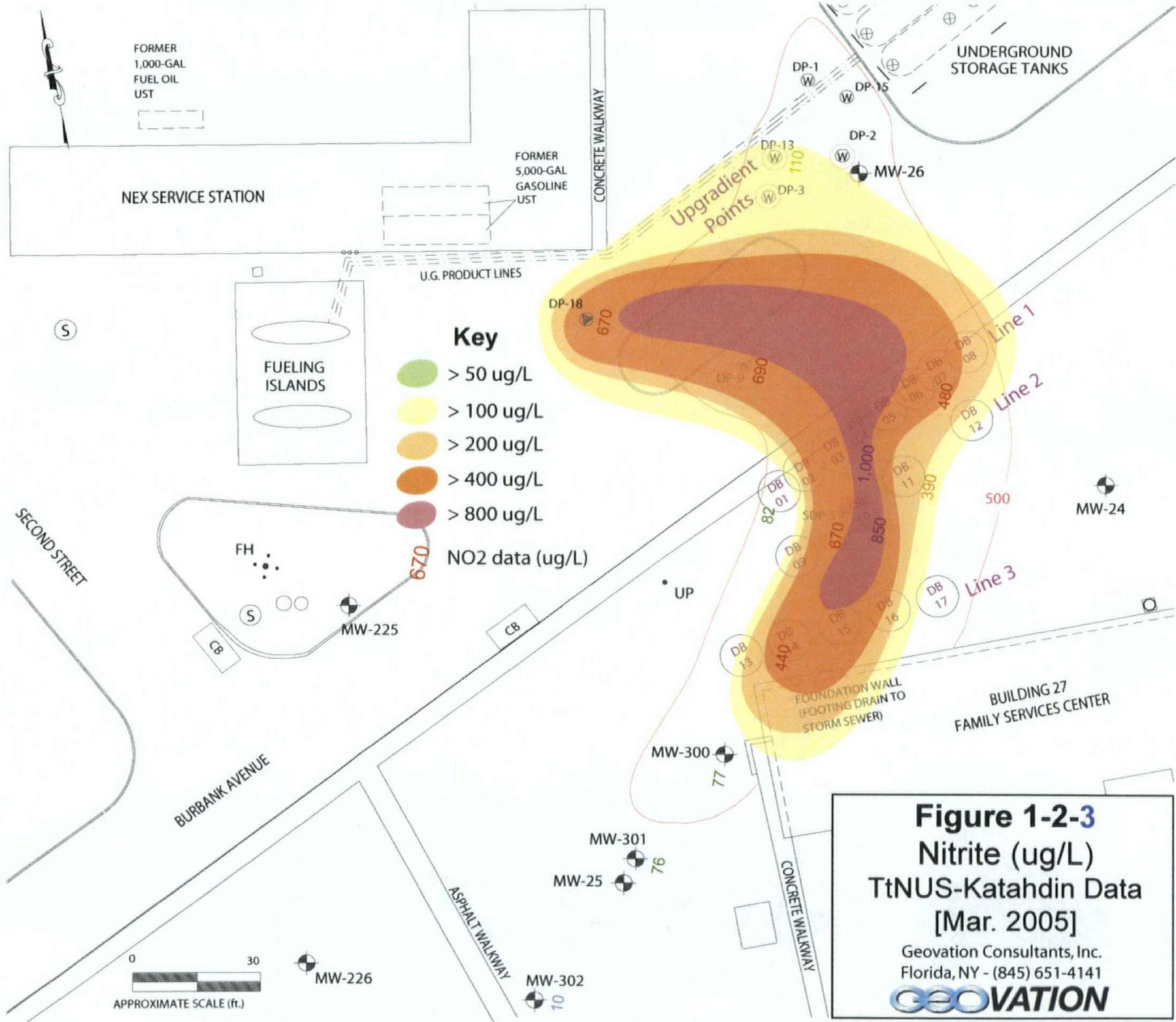
FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

BUILDING 27
FAMILY SERVICES CENTER



Figure 1-2-2
Nitrite (ug/L)
Southampton Univ. Data
[27 Jan. 2005]

Geovation Consultants, Inc.
Florida, NY - (845) 651-4141



FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

CONCRETE WALKWAY

UNDERGROUND
STORAGE TANKS

U.G. PRODUCT LINES

FUELING
ISLANDS

Key

- > 50 ug/L
- > 100 ug/L
- > 200 ug/L
- > 400 ug/L
- > 800 ug/L

NO₂ data (ug/L)

SECOND STREET

BURBANK AVENUE

ASPHALT WALKWAY

CONCRETE WALKWAY

BUILDING 27
FAMILY SERVICES CENTER

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)



Figure 1-2-3
Nitrite (ug/L)
TtNUS-Katahdin Data
[Mar. 2005]
Geovation Consultants, Inc.
Florida, NY - (845) 651-4141
GEOVATION

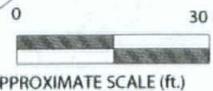
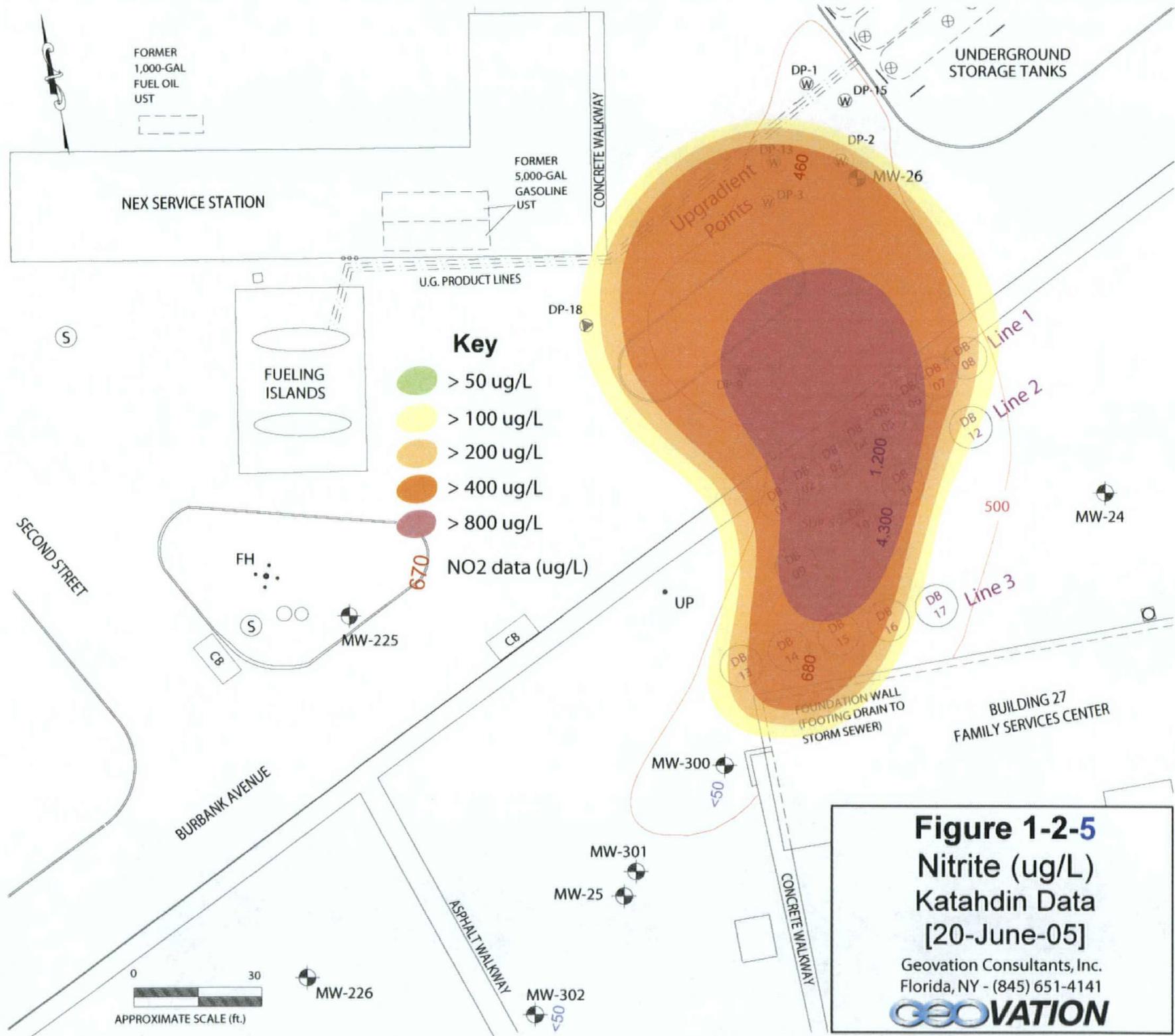


Figure 1-2-5
Nitrite (ug/L)
Katahdin Data
[20-June-05]

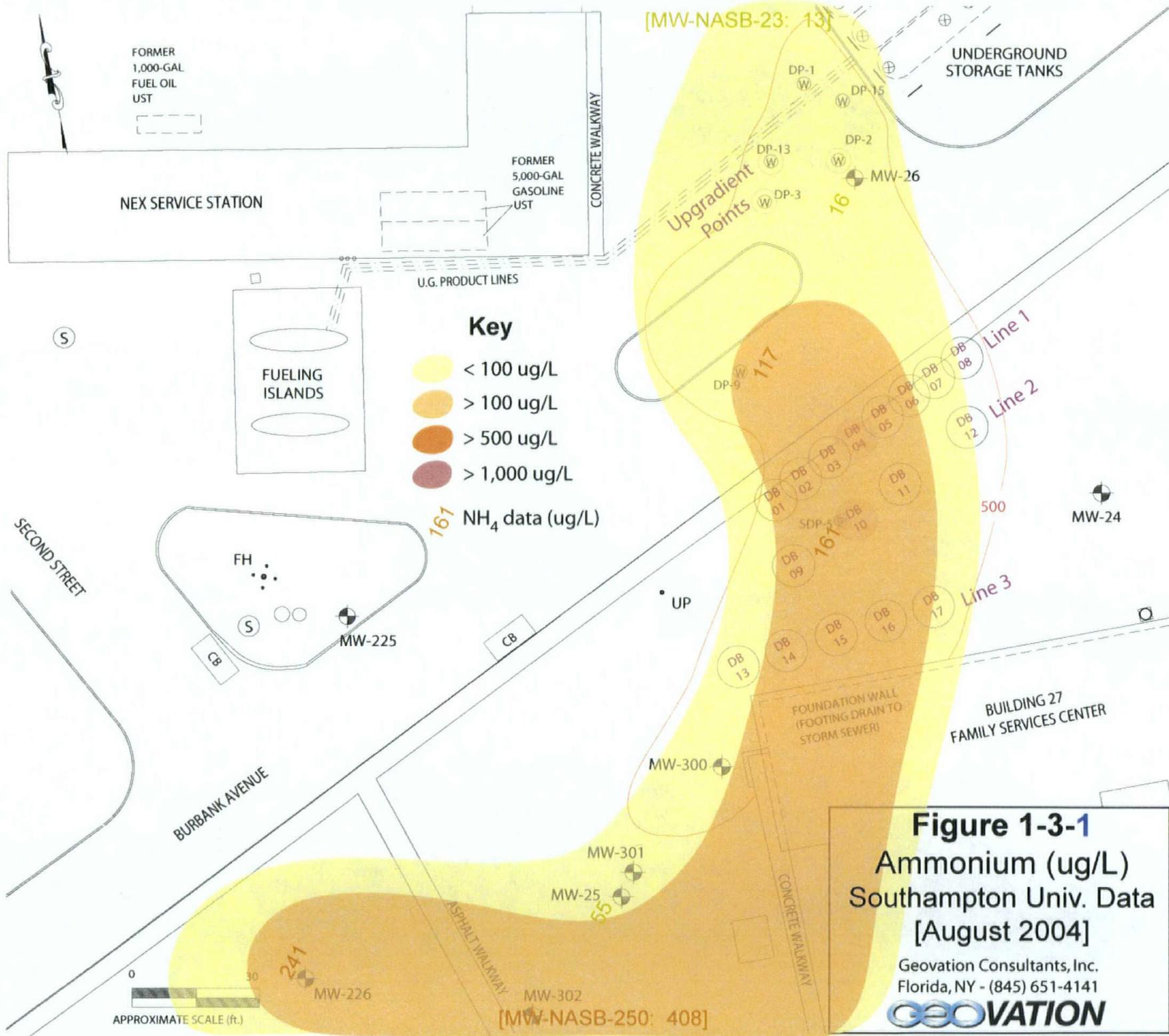
Geovation Consultants, Inc.
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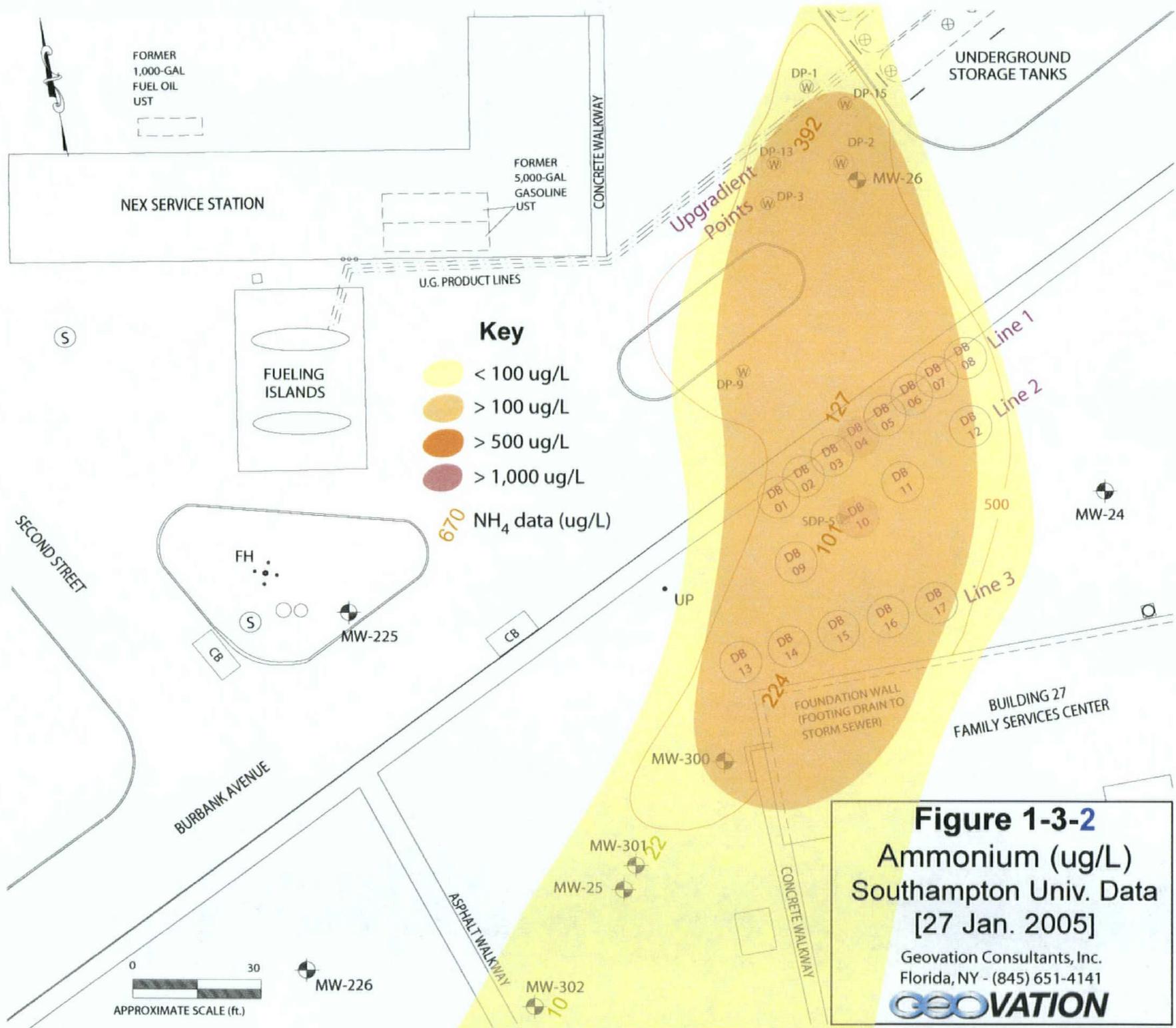
GEOVATION

Isopleth Maps:

Ammonium - NH_4^+

Monitoring Data





FORMER
1,000-GAL
FUEL OIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

CONCRETE WALKWAY

UNDERGROUND
STORAGE TANKS

U.G. PRODUCT LINES

FUELING
ISLANDS

Key

- < 100 ug/L
- > 100 ug/L
- > 500 ug/L
- > 1,000 ug/L

670 NH₄ data (ug/L)

SECOND STREET

FH

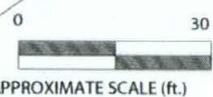
MW-225

BURBANK AVENUE

MW-300

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

BUILDING 27
FAMILY SERVICES CENTER



MW-226

ASPHALT WALKWAY

MW-302

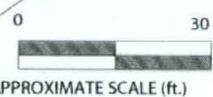
CONCRETE WALKWAY

MW-301

MW-25

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

BUILDING 27
FAMILY SERVICES CENTER



MW-226

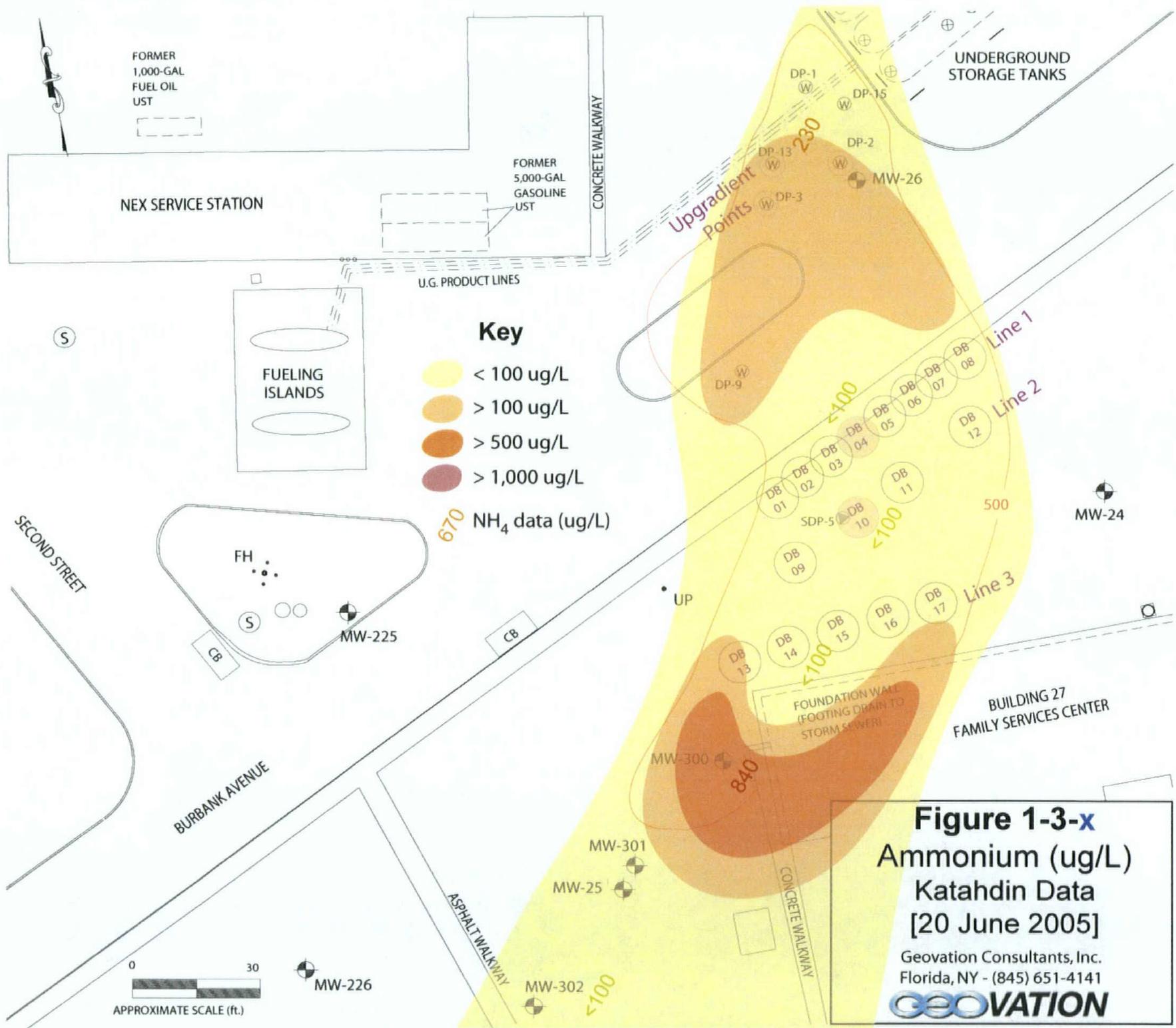
ASPHALT WALKWAY

MW-302

CONCRETE WALKWAY

MW-301

MW-25



FORMER
1,000-GAL
FUELOIL
UST

NEX SERVICE STATION

FORMER
5,000-GAL
GASOLINE
UST

CONCRETE WALKWAY

UNDERGROUND
STORAGE TANKS

U.G. PRODUCT LINES

Key

- < 100 ug/L
- > 100 ug/L
- > 500 ug/L
- > 1,000 ug/L

670 NH₄ data (ug/L)

FUELING
ISLANDS

FH

MW-225

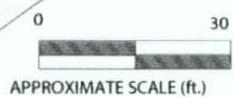
SECOND STREET

BURBANK AVENUE

ASPHALT WALKWAY

FOUNDATION WALL
(FOOTING DRAIN TO
STORM SEWER)

BUILDING 27
FAMILY SERVICES CENTER



MW-226

MW-302

Figure 1-3-x
Ammonium (ug/L)
Katahdin Data
[20 June 2005]

Geovation Consultants, Inc.
Florida, NY - (845) 651-4141



Copies of Abstracts:

University of Massachusetts
Contaminated Soils Conference
(Amherst, MA - Oct. 2005)

12th International Petroleum
Environmental Conference
(Houston, TX - Nov. 2005)

Visualization and Enumeration of Microorganisms Growing Under Denitrifying Conditions with Multi-Color FISH

Eric C. Hince, P.G., Geovation Consultants, Inc.; 468 Route 17A, Florida, NY, 10921; Tel: 845-651-4141, Fax: 845-651-0040; e-mail: echince@geovation.com

Molecular, culture-independent methods have revolutionized our current understanding of the microbial ecology of many natural and anthropogenic environments. Fluorescence in-situ hybridization ("FISH") is emerging as a powerful and quantitative genomic-based method for investigating the relative abundance of microorganisms in environmental samples. Multi-color FISH ("mFISH") involves the simultaneous hybridization of a sample with multiple oligonucleotide probes conjugated to different color fluorochromes so as to provide quantitative information on different sub-populations in the same sample. mFISH also enables the simultaneous visualization of spatial arrangements of microbes in mixed consortia to investigate the potential for mutualistic or syntrophic interactions among different organisms.

mFISH data and images will be presented from an ongoing denitrification-based bioremediation ("DBB") project at a DoD-owned hydrocarbon contamination site. Initial DGGE and PCR screening of ground-water samples were used to identify bacteria, archaea and fungi present within the DBB treatment zone and to help design mFISH assays targeting microorganisms suspected to be numerically important members of the DBB consortia. Multiple probes targeting the 16S or 23S rRNA of various taxonomic classes of microorganisms, from Domain- to Genus-specific probes, are being used to enumerate members of the DBB consortia. Probes being used include Domain-level probes EUB338 (eubacteria), ARC915 (archaea), and PF2 (yeast) and division- or phyla-specific probes BETA42a (β -proteobacteria), GAM42a (γ -proteobacteria), ALF968 (α -proteobacteria), DELTA495 (δ -proteobacteria) and CFB560 (*Cytophaga-Flexibacter-Bacteroides*). Group- and genus-specific probes are being used to target sub-populations of bacteria shown by DGGE to be among those consistently found at DBB treatment sites including members of the *Comamonadaceae* (BONE23a), *Burkholderiales* (SUBU1237) and *Pseudomonas* spp. (PS56a). Probes were labeled with Oregon Green, Cy3, or Cy5 for CCD imaging with a Nikon 90i epi-fluorescent microscope and narrow-band filter sets.

Initial mFISH results have revealed an abundance of β -proteobacteria and dimorphic, *Yarrowia*-like fungi in dense DBB consortia comprised of $\geq 10^7$ total cells/mL. A dual-probe mFISH assay for bacteria (Cy3-labeled EUB338) and yeast (Cy5-labeled PF2) revealed clusters of mixed bacteria and small yeast in contact with one another, raising the possibility of some type of mutualism or opportunistic association.

Preliminary results indicate promising potential for mFISH assays using both the BETA42a (Oregon Green) and GAM42a (Cy5) probes. BETA42a and GAM42a differ by only one base, so they are normally used with a competitor probe, i.e., the labeled probe to be imaged (e.g., BETA42a) together with the unlabelled "competitor" probe (e.g., GAM42a) to minimize erroneous hybridizations. Preliminary results suggest both probes can be imaged simultaneously with different color fluorochromes while minimizing errant hybridizations. mFISH assays that combine multiple probes may enable more rapid and cost effective enumeration and examination of important groups of microorganisms for monitoring *in-situ* bioremediation programs.

Investigation of Microbial Consortia Stimulated by Denitrification-Based Bioremediation in a Gasoline-Contaminated Aquifer via DGGE, real-time PCR and multi-color Fluorescence in-situ Hybridization (“mFISH”)

Eric Hince, P.G., Geovation Consultants, Inc. and
Dora Ogles, Microbial Insights, Inc.

Abstract

A large-scale field demonstration of a proprietary denitrification-based bioremediation (DBB) technology was initiated in October 2004 at a DoD-owned service station in the extreme northeast U.S. More than 4,500 Kg (>99%) of the gasoline-related hydrocarbon mass was present in the sorbed-phase within the aquifer media and ground-water conditions were anaerobic and highly reducing prior to DBB treatment. The microbial consortia present prior and subsequent to DBB treatment were investigated via a combination of denaturing-gradient gel electrophoresis (DGGE), real-time polymerase chain reaction (rtPCR) and multi-color FISH. DGGE profiling was conducted for eubacteria, archaea and fungi. rtPCR assays were conducted on target groups and genes including total eubacteria, iron/sulfate reducing bacteria (delta-proteobacteria), methanogens, nirS and nirK (genes involved in denitrification), benzyl succinate synthase (BssA), and the catechol dioxygenase genes. mFISH analyses primarily focused on the evaluating trends at a few key locations over time with the “MP1” multi-color assay as follows: Cy5-labelled GAM42a (gamma-proteobacteria); Cy3-labelled EUB338 (most eubacteria) and Oregon Green 488 labeled BETA42a (beta-proteobacteria). mFISH assays were counterstained with DAPI for total cell counts and as a reference for the mFISH data. The combined results of these genomic methods, taken together with the results of biogeochemical monitoring, revealed a smaller, more diverse and less active anaerobic consortia was present prior to treatment whereas a larger, less diverse, more active and primarily denitrifying consortia was stimulated by DBB. Both mFISH and DGGE indicated a shift towards a beta-proteobacteria dominated bacterial community within the main plume / treatment zone, whereas rtPCR indicated a decrease in delta-proteobacteria as well as an increase in total eubacteria and the detected copies of the catechol dioxygenase, nirS, nirK and BssA genes in response to treatment.