U.S. Consumer Product Safety Commission Staff
Summary of Contractor's Report "The Use of Epifluorescent Microscopy and Quantitative Polymerase Chain Reaction to Determine the Presence/Absence and Identification of Microorganisms Associated with Domestic and Foreign Wallboard Samples"

Joanna Matheson, Ph.D., Lori E. Saltzman, M.S., Mary Ann Danello, Ph.D.,
Directorate for Health Sciences[1]
September 2011

Background

This study was conducted as part of the U.S. Consumer Product Safety Commission ("CPSC") staff's forensic investigation of health effects and corrosion issues reported by consumers to be associated with the presence in residences of problem drywall, often reported to be connected to Chinese made drywall.[2] In the Environmental Health & Engineering (EH&E) 51-home and 6-home follow-up studies, hydrogen sulfide levels in homes reportedly constructed with imported drywall ("complaint homes") were low but statistically significantly higher than in the noncomplaint homes.[3] Complaint homes had significantly greater rates of copper and silver corrosion than noncomplaint homes and hydrogen sulfide was associated with both copper and silver corrosion.

Various organizations have proposed that sulfur-reducing bacteria may be a source of sulfur emissions from problem drywall. To expand on the earlier microbiological evaluation by EH&E,[4] CPSC staff contracted with the U.S. Geological Survey (USGS) to determine whether sulfur-reducing bacteria are present in samples of imported and domestic drywall and gypsum rock, and if present, could the bacterial source have been from the gypsum rock stock or was the bacteria introduced into the drywall during the manufacturing process.

Methods

The USGS analyzed 12 drywall samples (Appendix A)—supplied by CPSC—for the presence of sulfur-reducing bacteria (SRB). These samples included subsamples of the two colony growth positive samples from the EH&E microbiological assessment. In addition, four gypsum rock samples supplied by CPSC staff (Appendix A) were

---

[1]This document was prepared by CPSC staff and has not been reviewed or approved by, and may not necessarily reflect the views of, the Commission.

[2]CPSC staff cautions that it is not correct to assume that all Chinese or imported drywall is problem drywall; nor is it correct to assume that no domestic drywall is problem drywall.

[3]EH&E 51-home report: http://www.cpsc.gov/library/foia/foia10/os/51homeFinal.pdf, and 6-home follow-up report: http://www.cpsc.gov/info/drywall/ehemay2011.pdf.

[4]Draft Report on Preliminary Microbiological Assessment of Chinese Drywall, http://www.cpsc.gov/info/drywall/microbio.pdf.

evaluated for the presence of SRB. The drywall samples were collected by CPSC staff from manufacturers, drywall suppliers, storage warehouses, and complaint and non-complaint homes. The uninstalled and unfinished North American drywall samples were manufactured in 2009, and the Chinese drywall samples were manufactured in 2005–2006. The drywall samples obtained from individual homes were finished (*i.e.*, paint, plaster, or other modifications had been applied). The Chinese gypsum rock samples were collected by CPSC staff during their 2009 trip to China. Additional subsamples of these same Chinese drywall samples were among those tested in emissions chambers by the Lawrence Berkeley National Laboratory (LBNL), including several which were among the highest hydrogen sulfide emitters in the LBNL testing.[5]

The paper covering both the face and back of each drywall sample ("dark and light paper liners") was removed from the gypsum core ("gypsum filler"), and each component was analyzed separately. One drywall sample also had edging tape present. This tape was separated from the gypsum core and also analyzed for the presence of SRB.

The USGS employed four methods to determine whether viable sulfur-reducing bacteria were present in the Chinese drywall and rock samples compared to the North American samples: (1) epifluorescent microscopy, (2) quantitative polymerase chain reaction, (3) enrichment culture followed by quantitative polymerase chain reaction and, (4) genetic sequencing. Epifluorescent microscopy was performed on all drywall and gypsum rock samples using a modification of the protocol by Noble and Fuhrman, 1997. SYBR Gold stain is a sensitive stain that binds all nucleic acids (*e.g.*, double- and single- stranded DNA and RNA). This stain, however, is not specific for SRB; thus, any microbe with DNA or RNA will take up the stain. Viruses and bacteria stained with SYBR Gold will emit a bright yellow-green fluorescence under epifluorescent microscopy. The epifluorescent microscope enables an investigator to view, in this case, drywall samples and gypsum rock samples, for the presence of a single bacterial colony and larger clusters of bacterial growth. An active bacterial cluster would appear under the microscope as a very large mass of fluorescence, while a single bacterial colony will appear as a small bright dot of fluorescence under high magnification.

Quantitative polymerase chain reaction (PCR) for bacterial species (16S rRNA PCR) and PCR specific for SRB (using DSR1F and DSR4R primers) were performed on DNA isolated from the drywall and gypsum rock samples. PCR is a method that makes a large number of copies of a defined DNA sequence from very little DNA. PCR works by repeatedly copying genetic material using heat cycling and enzymes similar to those used by cells. The 16S rRNA gene (also called Universal 16S) is a commonly used tool for identifying bacteria. This gene, a section of highly conserved prokaryotic DNA found in almost all bacteria and archaea, codes for ribosomal RNA. Thus, PCR for the 16S rRNA gene will identify whether any bacteria are present in a sample but cannot identify to what species they may belong. The DSR1F and DSR4R primers were used to amplify the number of copies of DNA specifically for SRB. These SRB primers will amplify DNA for dissimilatory sulfite reductase (DSR), an essential enzyme for SRB metabolism.

[5] LBNL drywall chamber test report: http://www.cpsc.gov/library/foia/foia11/os/lblreport.pdf.

This enzyme catalyzes the final steps in sulfate and sulfite reduction. PCR is useful for determining whether bacteria and SRB are present, however, it does not provide information about whether the bacteria are viable. Furthermore, real-time PCR, using SYBR Gold binding to amplified DNA, measures the fluorescence increase as the dye binds to the increasing amount of DNA. The intent is that this increase in fluorescence is coming from the DNA that is desired to be measured; however, some fluorescent signal can come from alternate DNA sequences being amplified. A melting curve analysis is performed to determine whether this nonspecific amplification of undesired DNA occurred. All PCR products for a particular primer pair should have the same melting temperature. If there is a difference in melting temperatures, then there is contamination, mispriming, primer-dimer artifacts, or some other problem.

The USGS staff also carried out PCR on DNA isolated from drywall and rock samples that had been cultured in a sulfate-reducing broth base. The purpose of the culture was to enrich the numbers of any viable SRB in the samples. However, although the broth culture is designed to support SRB growth, other microbial species will make use of the nutrients in this broth and grow. Both 16S rRNA PCR and specific SRB PCR were performed on these cultured samples.

In order to address whether any novel (unknown sulfur bacterial species) might be present in the drywall and gypsum rock samples, a subsample of DNA, isolated from the positive cultured samples, was sent by USGS to Northwoods DNA, Inc., to perform full genetic sequencing (GenBank Blast).

Results

The facing paper, edging paper (if available) and the gypsum core of each sample were evaluated separately for the presence of "universal" bacteria (the presence or absence of any bacteria), as well as specifically for sulfur-reducing bacteria. No bacterial colonies were observed under epifluorescent microscopy for any of the drywall or gypsum rock samples. Two drywall samples (1 Chinese, 1 North American) showed evidence of small numbers of bacteria present on the facing paper, edging paper, and gypsum core. No bacterial colonies were seen on the rock samples, although all four gypsum rock samples showed evidence of small amounts of bacteria on the rock particle surfaces, as well as evidence of nonspecific (nonmicrobial) fluorescent particles. General bacterial species were observed in the PCR amplified DNA for 7 drywall samples (5 Chinese, 2 North American) and 3 gypsum rock samples (1 Chinese, 2 North American). When the drywall and gypsum rock samples were culture enriched, nearly all samples (all but 1 North American drywall sample) were positive for "universal" bacterial DNA. Three culture enriched samples (2 drywall, 1 gypsum rock) initially demonstrated the presence of SRB when the DNA was amplified via PCR. However, upon repeat testing, the amplified DNA was no longer detected. The melt curve analysis demonstrated that these positive samples were due to amplification of DNA that was not from SRB. Furthermore, genetic sequencing was also not able to show that novel SRB species were present in any of the culture enriched samples.

Conclusions

- No sulfur-reducing bacteria were observed in imported and domestic drywall collected from manufacturers, suppliers, storage warehouses, and complaint and noncomplaint homes.
- No differences were seen in the presence or absence of sulfur-reducing bacteria between imported Chinese drywall and North American domestic drywall, including Chinese samples found by LBNL to have some of the highest reactive sulfur gas emissions in the chamber tests.
- No SBR were found in rock samples that were collected by CPSC staff during their 2009 trip to China.
- Because the manufacture, transport, and storage of drywall is not sterile, bacteria were found in nearly all drywall and rock samples when the samples were culture enriched and DNA amplified by PCR. This was not an unexpected result.
- The low number of bacteria and the lack of observation of any viable bacteria clusters do not support the contention that sulfur-reducing bacteria were metabolically active in problem drywall and causing the emission of sulfur gases, the reported health effects, and the reported corrosion to metal components in homes.

Appendix A

Sample Table

CPSC Sample ID	Country	Sample Type	Collection Site
09-810-7339	China	Drywall	Supplier
09-810-8357	China	Drywall	Supplier
09-840-9673	China	Drywall	Supplier
09-840-9858	North America	Drywall	Manufacturer
09-840-9962	North America	Drywall	Manufacturer
09-302-1394	China	Drywall	Home
09-302-1395	China	Drywall	Home
09-302-2542	China	Drywall	Home
10-810-5462	North America	Drywall	Home
09-302-2544-02	China	Drywall	Home
09-302-2544-03	North America	Drywall	Home
09-302-2544-04	North America	Drywall	Home
09-302-2624	China	Gypsum Rock	Mine
09-302-2625	China	Gypsum Rock	Manufacturer
09-302-1498	North America	Gypsum Rock	Manufacturer
10-302-1140	North America	Gypsum Rock	Manufacturer

September 14, 2011

THE USE OF EPIFLUORESCENT MICROSCOPY AND QUANTITATIVE POLYMERASE CHAIN REACTION TO DETERMINE THE PRESENCE/ABSENCE AND IDENTIFICATION OF MICROORGANISMS ASSOCIATED WITH DOMESTIC AND FOREIGN WALLBOARD SAMPLES

Dale Warren Griffin, Ph.D., MSPH

Environmental and Public Health Microbiologist

U.S. Geological Survey, 2639 North Monroe Street, Suite A200, Tallahassee, Florida 32303.

Phone 850-553-3675, email dgriffin@usgs.gov

Abstract

Epifluorescent microscopy and quantitative polymerase chain reaction (qPCR) were utilized to determine the presence, concentration and identification of bacteria, and more specifically sulfate reducing bacteria (SRB) in subsamples of Chinese[1] and North American wallboard, and wallboard-mine rock. Bacteria were visible in most subsamples, which included wallboard-lining paper from each side of the wallboard, wallboard filler, wallboard tape and fragments of mined wallboard rock via microscopy. Observed bacteria occurred as single or small clusters of cells and no mass aggregates indicating colonization were noted. Universal 16S qPCR was utilized to directly examine samples and detected bacteria at concentrations ranging from 1.4×10^3 to 6.4×10^4 genomic equivalents per mm^2 of paper or per gram of wallboard filler or mined rock, in 12 of 41 subsamples. Subsamples were incubated in sulfate reducing broth for ~30 to 60 days (enrichment assay) and then analyzed by universal 16S and SRB qPCR. Enrichment universal

[1] This report uses the terms "Chinese" and "foreign" to identify wallboard and rock samples; however, CPSC staff has reported that not all Chinese drywall is problem drywall. Importantly, CPSC staff provided samples, including several that were among the highest hydrogen-sulfide emitters in the Lawrence Berkeley Laboratory report on Small-Chamber Measurements of Chemical-Specific Emission Factors for Drywall (http://www.cpsc.gov/library/foia/foia11/os/lblreport.pdf).

16S qPCR detected bacteria in 32 of 41 subsamples at concentrations ranging from 1.5×10^4 to 4.2×10^7 genomic equivalents per ml of culture broth. Evaluation of enriched subsamples by SRB qPCR demonstrated that SRB were not detectable in most of the samples and if they were detected, detection was not reproducible (an indication of low concentrations, if present). Enrichment universal 16S and SRB qPCR demonstrated that viable bacteria were present in subsamples (as expected given exposure of the samples following manufacture, transport and use) but that SRB were either not present or present at very low numbers. Further, no differences in trends were noted between the various Chinese and North American wallboard samples. In all, the microscopy and qPCR data indicated that the suspected 'sulfur emissions' emanating from suspect wallboard samples is not due to microbial activity.

Introduction

The emissions of sulfurous gasses in homes that have been built using wallboard of Chinese origin have received considerable attention in the press due to the profuse use of this wallboard in domestic construction and its potential economic impact. These 'rotten egg' odor emissions were associated by consumer reports with copper corrosion (copper is a common plumbing component) and adverse health effects. Suspected causes of these emissions have been hypothesized to include natural, non-biologically mediated emissions due to its construction material and natural, non-biological emissions due to the manufacturing process, or microbiological processes (Burdack-Freitag, Mayer et al. 2009; Curtis, Jones et al. 2009; Hooper, Shane et al. 2010). The prime microbiological suspect is SRB, which readily discharge hydrogen sulfide as a waste product and are common environmental micro-fauna. The classic 'rotten egg' odor of swamps, aquatic sediments and in some cases sinks is due to SRB metabolic activity. Due to the association of 'rotten egg' emissions with SRB, comprehensive examination

of suspect and non-suspect wallboard and wallboard mine rock was needed to determine if SRB were present, metabolically active, and responsible for Chinese wallboard emissions.

The objectives of this research project were to: 1) Utilize direct-count assay to determine the presence of bacterial cells associated with various wallboard matrices (front and back paper, filler, and tape) and wallboard mine rock; 2) utilize universal 16S qPCR to determine the concentrations of bacteria in subsamples; and 3) utilize enrichment universal 16S qPCR and SRB qPCR to determine the viability of bacteria in the samples and to determine if SRB are present and active.

Methods

Samples

U.S. Consumer Product Safety Commission (CPSC) samples were received at the U.S. Geological Survey (USGS) Tallahassee Microbiology Laboratory on August 25, 2010, November 18, 2010, and February 22, 2011 (see Tables 1, 2 and 3). Samples were individually wrapped in plastic and shipped in manila shipping envelopes. All samples were placed in a laboratory refrigerator upon receipt and were only taken out of the refrigerator for processing purposes. All remaining samples are currently stored by refrigeration.

Acquisition of sample subsets for experimentation

Sterile technique was utilized at all times including wiping down the level II biological safety (BSL II) cabinet with a solution of 70% ethanol/water prior to use, ethanol/flame sterilization of tweezers and scissors between samples and sample fragments, and use of sterile gloves and sample containers.

Drywall: Drywall samples were taken out of refrigeration and placed in the BSL II cabinet, removed from their wrapping and placed onto a piece of aluminum foil. Three primary types of

sample subsets were collected: clippings from the dark and light paper liners (standard drywall has a lighter-colored front side, and a dark colored back side), gypsum filler, and in one case a piece of drywall tape (from 09-810-7339-09, the only sample that had a piece of drywall tape attached to it). Paper liner fragments used for epifluorescent microscopy analyses were removed from the tip of the wallboard corner using sterilized tweezers. The paper was dissociated from the gypsum filler and sterile scissors used to clip a piece that had a height of approximately 3mm and a base length of approximately 4mm (a right-triangle shape). The drywall sample was then flipped over and a matched piece obtained from the other side. A similar-sized right-triangle fragment was obtained from the tape sample. For culture and molecular analyses, a paper fragment in the shape of a right triangle with an area of $2mm^2$ was taken in duplicate. For the tape sample, an area the size of $1mm^2$ was taken in duplicate. For the paper-liner and edging-tape samples, one fragment of each sample set was used for direct molecular analyses and the other fragment for culture-based work. For the gypsum-filler sample analyses, a sterile spatula was used to break off pieces of the gypsum filler from the corner sections where the paper-liner samples had been obtained. The gypsum filler was then weighed on a Mettler Toledo laboratory balance. Approximately 1.0g of gypsum filler was collected for culture-based work and ~0.3g for non-culture-based work.

Rock samples: Sterile tweezers were used to obtain small fragments from the CPSC samples. These fragments were weighed and then placed in new sterile containers. Approximately 1.0g of gypsum rock was collected for culture-based work and ~0.3g for non-culture-based work.

Epifluorescent microscopy

A modification of the protocol published by Noble and Fuhrman (1997) was used for direct counting. Additional paper and tape samples were taken from the August 25, 2010, samples as

described above. These subsamples were directly stained by placing 100 microliters (µl) of diluted SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR: 97.5µl of 0.02µm-filtered H_2O + 2.5 µl of a 1/10 dilution of SYBR Gold) onto the underside (gypsum-filler side) of the sub-samples and incubated at room temperature in the dark for about 10 to 15 minutes. The subsamples were then placed, stained side up, on a glass microscope slide. Twenty-seven microliters of antifade solution (990.0 µl 50% 1X phosphate buffered saline/50% glycerin + 10.0µl 10% p-phenylenediamine) were placed on a coverslip, and the coverslip placed over the subsample. The coverslip was lightly pressed to expel any trapped air and the slide was then refrigerated in the dark (~<1 to 24 hours) until examined under epifluorescent microscopy. Although antifade solution is not needed when using SYBER Gold, it does assist in keeping the filter at a fixed position on the glass slide when scanning at 1000X and using oil. The entire stained portion of the sub-sample was scanned at 400X and for higher magnification of various subsample fields at 1000X (oil) using a Carl Zeiss Inc. (Jena, Germany), Axioskop 40 epifluorescent microscope. For gypsum filler and rock samples, fine fragments of either were placed on the surface of a Whatman Anodisc 0.02µm pore-sized 25mm-diameter, glass fiber filter (Whatman # 6809-60 02). The sub-samples were stained by placing the filter sample-side up on top of a drop of diluted SYBR Gold nucleic acid stain and incubated at room temperature in the dark for approximately 10 to 15 minutes. The filters were removed from the drop of diluted SYBR Gold, excess stain removed by blotting the back of the filter on tissue paper, and the filters placed on a glass slide. Twenty-seven microliters of antifade solution were placed on a coverslip, and the coverslip placed over the filter. The coverslip was lightly pressed to expel any trapped air and the slide was then refrigerated in the dark until counted by epifluorescent

microscopy. A Sony 1080p HD camcorder that is capable of 10 megapixel still image photography was utilized to capture photographs and movies of the stained samples.

Direct and culture-based analyses using quantitative polymerase chain reaction (qPCR)

Sulfate Reducing Broth Base (FLUKA Analytical, Buchs, Switzerland) was prepared according to the manufacturers' instructions. Twenty-five milliliters (ml) of sterile room-temperature broth were transferred to a sterile 50ml conical tube. Paper, tape, gypsum-filler and rock subsamples were then each added to separate culture tubes. Each subsample and broth mixture was then overlain with 2.5ml of sterile mineral oil. The tubes were tightly capped and incubated at room temperature for approximately 30 days. Negative and positive controls were set up in 50ml tubes as described above by using sterile media (negative control) and media inoculated with a vial of ATCC 13541 *Desulfovibrio desulfuricans*, a known SRB (positive control). After the 30-day incubation period, the culture tubes were opened and a sterile micropipette was used to penetrate the mineral oil layer. The broth layer was mixed by pipetting up and down several times. Using a micropipette, 100µl of the broth were extracted with a micropipette and transferred to a sterile 1.8ml microcentrifuge tube. DNA was extracted from the aliquot using QIAGEN's (Valencia, California) DNeasy Blood and Tissue Kit's gram + positive bacteria extraction protocol. Two µl of the DNeasy kit elluent (total 100µl per sample) were used as the amplification template.

For SRB specific qPCR, a preliminary run using extract from the positive control was conducted to obtain standard template for post culture SRB qPCR analyses. The primer set and amplification profile ultimately used for SRB qPCR were primers DSR1F (5'-ACSCACTGGAAGCACG-3') and DSR4R (5'-GTGTAGCAGTTACCGCA-3') as previously published (Wagner, Roger et al. 1998). The SRB qPCR utilized an Applied Biosystems (Foster City, California) StepOne Real-Time PCR System and SYBR Green PCR Master Mix. The

master mix recipe was 12.5µl of SYBR Green PCR Master Mix, 2µl of each primer (from a 10µM working stock), 6.5µl of PCR grade H_2O, and 2µl of template per reaction. StepOne Real-Time PCR System melt curve analyses were conducted to verify any positive signal.

For universal 16S qPCR, *Bacillus atrophaeus* DNA was utilized as the standard template for direct and post-culture universal 16S qPCR analyses. The primers, probe and amplification profile utilized for universal 16S rRNA analyses were forward primer 5'-TCCTACGGGAGGCAGCAGT-3', reverse primer 5'-GGACTACCAGGGTATCTAATCCTGTT-3', and probe [6~FAM]-5'-CGTATTACCGCGGCTGCTGGCAC-3'-[BHQ1] as previously published (Nadkarni, Martin et al. 2002).

Results

Epifluorescent microscopy

Figure 1 Panel A (an Afghanistan soil sample) illustrates what bacteria and virus-like particles look like when stained with SYBR Gold and are visualized with epifluorescent microscopy. Bacteria produce a bright green fluorescence and virus-like particles produce small to fine 'pin-prick' sized fluorescence. Clusters of bacteria produce much larger and brighter areas of fluorescence as identified by the short arrow in Panel A. Figure 1 Panel B is standard lined school paper stained with SYBR Gold and this illustrates a bacteria and virus-like particle free area depicting the fibrous nature of paper when viewed at 400X magnification. This fibrous nature is observed in some of the images of wallboard paper in the following Figures.

Figure 2 panels are images of stained drywall liner paper (inner and outer facing sides), gypsum filler and edging tape for sample 09-810-7339-09. Bacteria were observed on the outer portions of each paper fragment (inner and outer) but they were widely dispersed and showed no evidence

of colony formation. Widely dispersed bacteria were also noted in the gypsum filler material but again showed no evidence of colony formation. The highest concentration of bacteria observed in any of the wallboard samples was within the glue matrix on the underside of the 09-810-7339-09-tape sample (Figure 2, panel C). Numerous bacteria were observed in this glue matrix; some individual and some in small clusters but again, no observed colony formation was noted. Figure 3 panels are images of various subsamples of wallboard sample 09-840-9962-07. Figure 3 panel A is gypsum filler material and one small cluster of bacteria can be seen in the upper right quadrant of that image. Few bacteria were seen in the remaining gypsum filler material or on any of the paper liner surfaces of this sample (Figure 3, panels B through D).

Figure 4 panels A through D are images of rock sample 10-302-1140. The numerous small fluorescing spots in these panels are bacteria on the surfaces of fine rock fragments. Figure 5 panels A through D are images of fine fragments from rock sample 09-302-1498. Only a few bacteria were seen on the surfaces of fragments in this sample. Figure 6 panels A through D are images of fragments from rock sample 09-302-2624. Bacteria can be seen on the surfaces of fragments in panels A and B. Panel C depicts auto-fluorescing inorganic particulates (non-microbiologic in origin) and panel D depicts a combination of auto-fluorescing inorganic particulates and a few fluorescing bacterial cells. Figure 7 panels A through D are images of rock sample 09-302-2625. Each of the panels in this figure depicts large and fine auto-fluorescing inorganic particulates (non-microbiologic). Few bacteria were observed on the surfaces of particulates in this sample.

Quantitative Polymerase Chain Reaction

Table 1 lists the qPCR results for the first set of sample that were received and analyzed (wallboard samples 09-840-9962-07, 09-810-7339-09, 09-810-8357-03, 09-840-9673-07, 09-

840-9858-02 and rock samples 10-302-1140, 09-302-1498, 09-302-2624, and 09-302-2625). Bacteria (universal 16S qPCR) were detected in three of the five original un-enriched wallboard samples at concentrations ranging from 1.4 to 8.9 x 10^3 genomic equivalents per mm^2 (paper or tape) or per gram (gypsum filler). These detections occurred in the dark paper subsample of wallboard 09-840-9962-07, the dark paper and tape subsamples of wallboard 09-810-7339-09, and the gypsum filler subsample of wallboard 09-810-8357-03. Three of the four un-enriched rock samples were positive for bacteria at concentrations ranging from 1.2 to 6.4 x 10^4 genomic equivalents per gram of rock. In the enriched (post-sulfate reducing broth) samples, bacteria (universal 16S qPCR) were detected in all of the wallboard subsamples with the exception of the 09-840-9962-07 gypsum filler sample at concentrations ranging from 1.5 x 10^4 to 1.0 x 10^7 genomic equivalents per ml of broth, and all of the rock samples at concentrations ranging from 3.1 x 10^4 to 1.0 x 10^6 genomic equivalents per ml of broth. Amplification of suspected target organisms was detected by SRB-qPCR following enrichment in wallboard samples 09-810-7339-09 (edging tape subsample) and 09-810-8357-03 (gypsum filler subsample) and rock sample 09-302-2625 at concentrations of 1.2 x 10^2, 2.2 x 10^2 and 3.3 x 10^2 per ml of broth, respectively.

Melt-curve analyses however indicated that the melting temperatures (*Tm*) in the samples were lower by 1 to 5 C^o than what was observed in the standards or positive control. To account for the possibility of novel SRB in these samples, aliquots of amplicon (the positive control and three positive subsamples) were sent to Northwoods DNA, Inc. to attempt direct DNA sequencing. Of these samples and the positive control template, only wallboard sample 09-810-8357-03 and the positive control template produced readable sequence segments that could be compared to a database of genetic knowns (GenBank Blast). The positive control amplicon

matched to its respective ATTC organism (*Desulfovibrio desulfuricans*) and the amplicon in wallboard sample 09-810-8357-03 matched at 97% (51/53 bases) to *Desulfotomaculum aeronauticum* (GenBank identification AF273033).

Table 2 lists the qPCR results for the second set of samples received and analyzed (wallboard samples 09-302-1394-01b, 09-302-1395-11b, 09-302-2542-03b, 09-302-2544-02b, 09-302-2544-03b, 09-203-2544-04b, and 10-810-5462-01b). Bacteria (universal 16S qPCR) were detected in four of the seven un-enriched samples at concentrations ranging from 6.2×10^3 to 1.9×10^4 genomic equivalents per mm^2 of paper or per gram of gypsum filler. These detections occurred in the gypsum filler subsample of 09-302-1395-11b, the light paper and gypsum filler subsamples of 09-302-2542-03b, the dark paper subsample of 09-302-2544-02b and the light paper subsample of 09-302-2544-03b. In the enriched samples bacteria (universal 16S qPCR) were detected in six of the seven-wallboard subsamples (no bacteria were detected in the subsamples of 09-302-2544-03b) at concentrations ranging from 5.4×10^4 to 4.2×10^7 genomic equivalents per ml of broth. Sulfate reducing bacteria were not detected in any of the second set of subsamples by SRB-qPCR.

Table 3 lists the analyses of a second 09-810-8357-03-wallboard sample (post-enrichment) received, and re-analyses of two of the original enriched wallboard samples 09-810-8357-03 and 09-840-9858-02 (samples listed in Table 1 are more than two months post enrichment for this experiment. 09-810-8357-03 was originally SRB-qPCR positive and 09-840-9858-02 was SRB-qPCR negative). Sulfate reducing bacteria were not detected in any of these subsamples by SRB-qPCR.

Discussion/Conclusion

Analyses of wallboard subsamples by epifluorescent microscopy demonstrated that bacteria were present in the various samples of Chinese and North American origin. Visible cells were noted on the exterior and interior of drywall paper liner, within the gypsum filler material and in the case of the one edging tape sample (sample 09-810-7339-09), within the glue matrix. The only sample with numerous visible bacteria was sample 09-810-7339-09 where the bacteria were located within the glue matrix of the edging tape sample. In none of the samples was there evidence of profuse colonization that would be expected if wallboard material were being scavenged as a nutrient source. Small numbers of bacteria would be expected in all wallboard material since these samples have been openly exposed to various environments during and following manufacture. This is illustrated by the universal 16S un-enriched qPCR data where bacteria DNA were readily detectable in numerous subsamples (Tables 1 and 2). This universal un-enriched qPCR data report genomic equivalents per unit area or weight and do not address whether the DNA came from metabolically active or viable cells. The following experiments where subsamples were inoculated into sulfate reducing broth and incubated for ~30 days (enrichment assays) prior to universal 16S qPCR analyses do in part address whether or not metabolically active or viable cells are present. These data listed in Tables 1 and 2 demonstrate that most of the subsamples contained cultivable (viable) bacteria and includes sample where bacteria were originally detected or not detected using the direct (un-enriched) DNA extraction. The issue of being able to detect DNA from non-cultivable cells (dead or viable but non-cultivable) is illustrated in Table 2 with the 09-302-2544-03b light paper sample. In this case bacteria were not detected following enrichment due in part to dilution (only 100µl of the 25ml of sulfate reducing broth was utilized for DNA extraction) of the originally detectable DNA. It is

well known in the field of microbiology that typically less than 1% of bacteria present in environmental samples is cultivable, and while most are viable, will not grow using conventional culture assays. The enrichment data demonstrate that there were species of bacteria in most of the samples that were capable of utilizing components of sulfate reducing broth in an anaerobic setting for growth, and in light of the SRB qPCR data were not sulfate reducing bacteria. Growth of non-target microorganisms in or on 'target-specific' media is a common phenomenon in environmental sample analyses. Bacteria have evolved to utilize alternate sources of nutrients within various physical environments when primary sources are not available to ensure survival.

SRB qPCR results for the first set of samples indicated the possibility of target amplification in three of the samples as discussed. Melt-curve analyses indicated that the *Tm* for these amplicons were slightly below the positive control *Tm* and two of these three samples did not produce usable sequences for bacterial species identification. The third sample (09-810-8357-03 filler) sequence only produced a short usable sequencing segment whose data was inconclusive. To determine if this Chinese wallboard sample might harbor viable SRB, another sample (an additional wallboard segment shipped and received for analyses) was screened for SRB presence using enrichment SRB qPCR, along with aliquots of broth from the originally enriched 09-810-8357-03 subsamples and an additional originally enriched negative subsample set (Table 3). No SRB were detected in any of these samples.

Based on the overall data set and the various environments that these samples (Chinese and North American wallboard) were exposed to following manufacture, transport and use, and installation in homes, it would not be unusual to occasionally detect SRB when utilizing

sensitive molecular assays such as enriched SRB qPCR. This observation of sporadically detectable SRB in Chinese and North American wallboard samples has previously been reported (Environmental Health and Engineering, Inc. Needham, MA, Report #16512 dated March 26, 2010, titled – Draft report on preliminary microbiological assessment of Chinese drywall. Available at - http://www.cpsc.gov/info/drywall/microbio.pdf). As stated, the presence of viable bacteria in these samples was expected and they were readily detectable. Individual bacterial cells and small clusters of cells were visible via epifluorescent microscopy, but there were no visible signs of clusters of replicating cells that would be expected from a metabolically active group. These and other data indicate that the reported 'sulfur emissions' from these suspected samples are not in all likelihood due to microbial activity.

Disclaimer

Any use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government

Figure 1. Panel A illustrates SYBR Gold Nucleic Acid Gel Stain fluorescence of single and grouped bacterial cells and virus-like particles in an Afghanistan soil sample. Panel B illustrates the matrix of school notebook paper stained with SYBR Gold (note, no bacteria are visible). Magnification for these figure panels was 1000x for panel A and 400X for panel B.

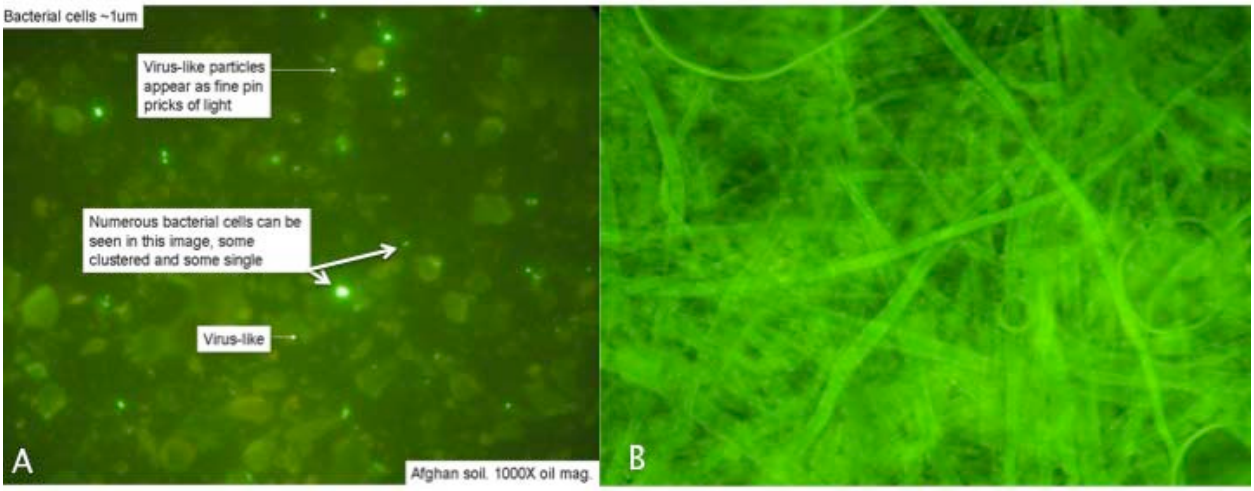

Figure 2. Drywall sample 09-810-7339-09 stained with SYBR Gold Nucleic Acid Gel Stain. Panel A is the board's gypsum filler; few bacteria were noted in this material. Panel B is the outside facing paper's gypsum filler side and no bacteria were noted. Panel C is the glue side of the edging tape located on the outside facing paper and numerous bacteria are seen embedded in the glue. While many bacteria are pictured, these are individual cells or small clusters of cells and do not represent a growing colony. Panel D is the same edging tape, but its outer face (non-glue side) and no bacteria were noted. Magnification for these figure panels was 1000x.

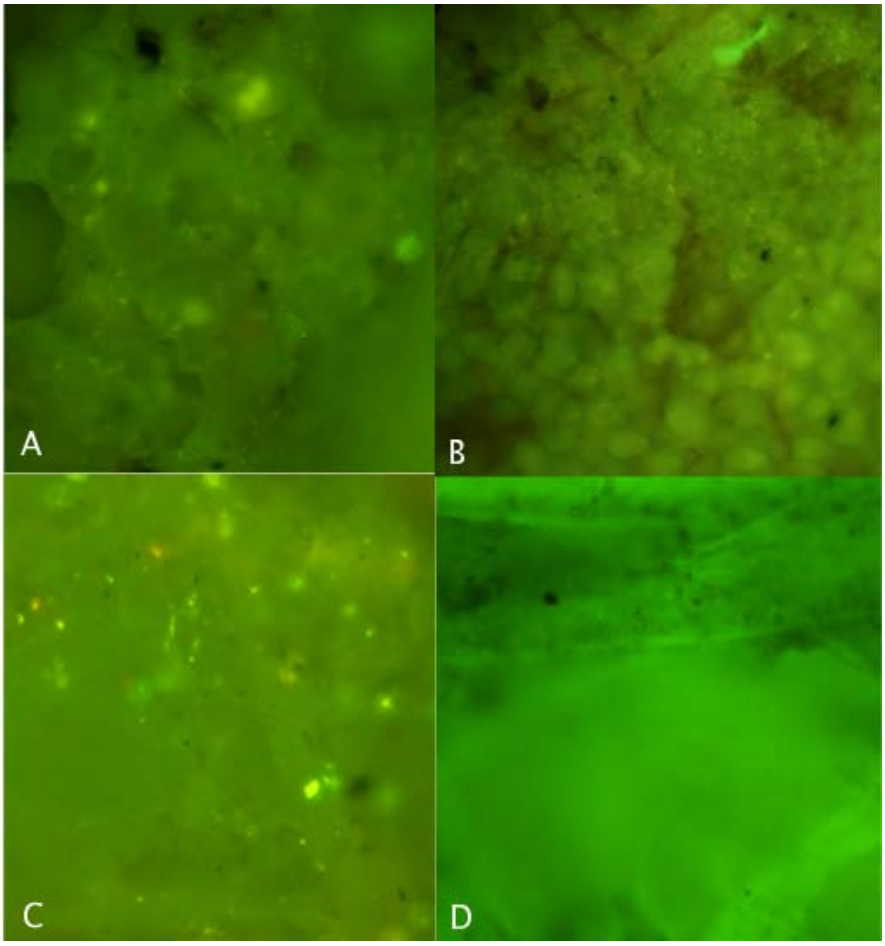

Figure 3. Drywall sample 09-840-9962-07 stained with SYBR Gold Nucleic Acid Gel Stain. Panel A is gypsum filler with one small cluster of bacteria in the upper right quadrant. Panel B is the facing paper on the gypsum filler side (no bacteria noted). Panels C and D are the external faces of the paper liner (no bacteria noted). Magnification for these figure panels was 400x for panels A and B and 1000x for panels C and D.

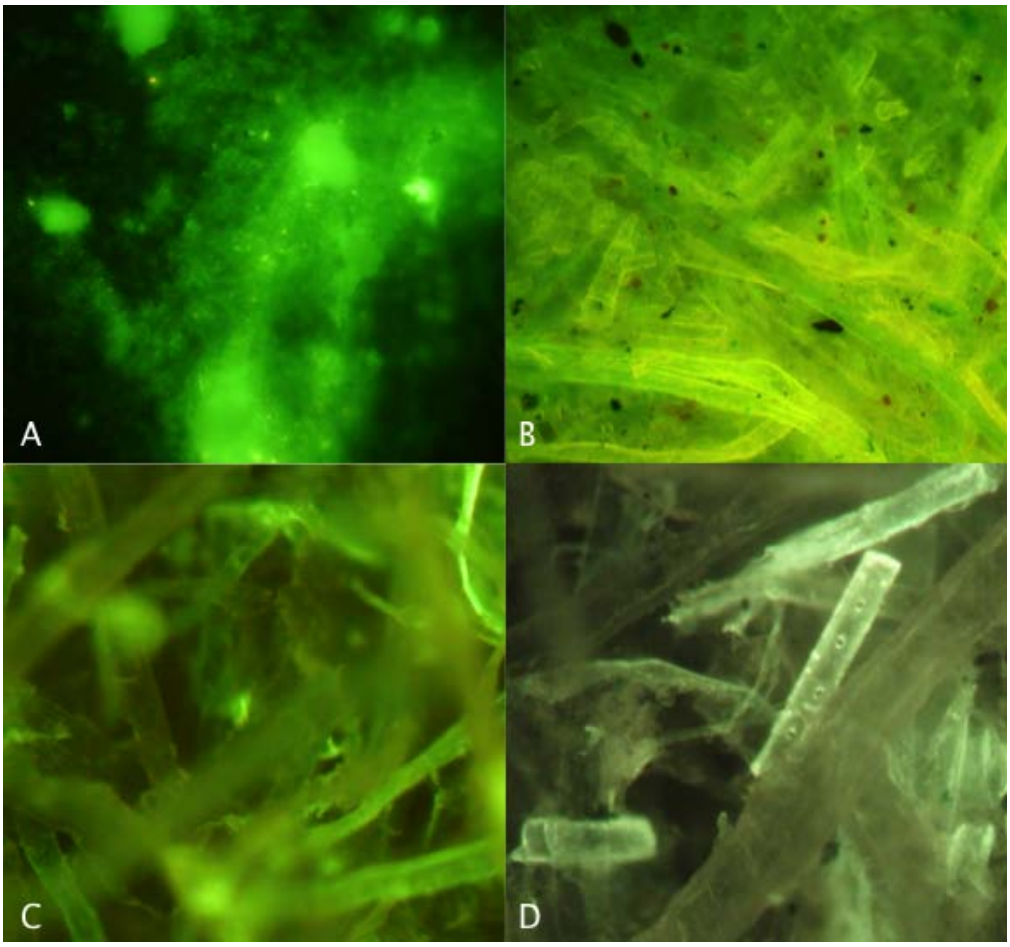

Figure 4. Rock sample 10-302-1140 stained with SYBR Gold Nucleic Acid Gel Stain. Panels A through D: numerous small bright fluorescing spots are bacteria on the surfaces of sample particulates. Magnification for these panels was 1000x.

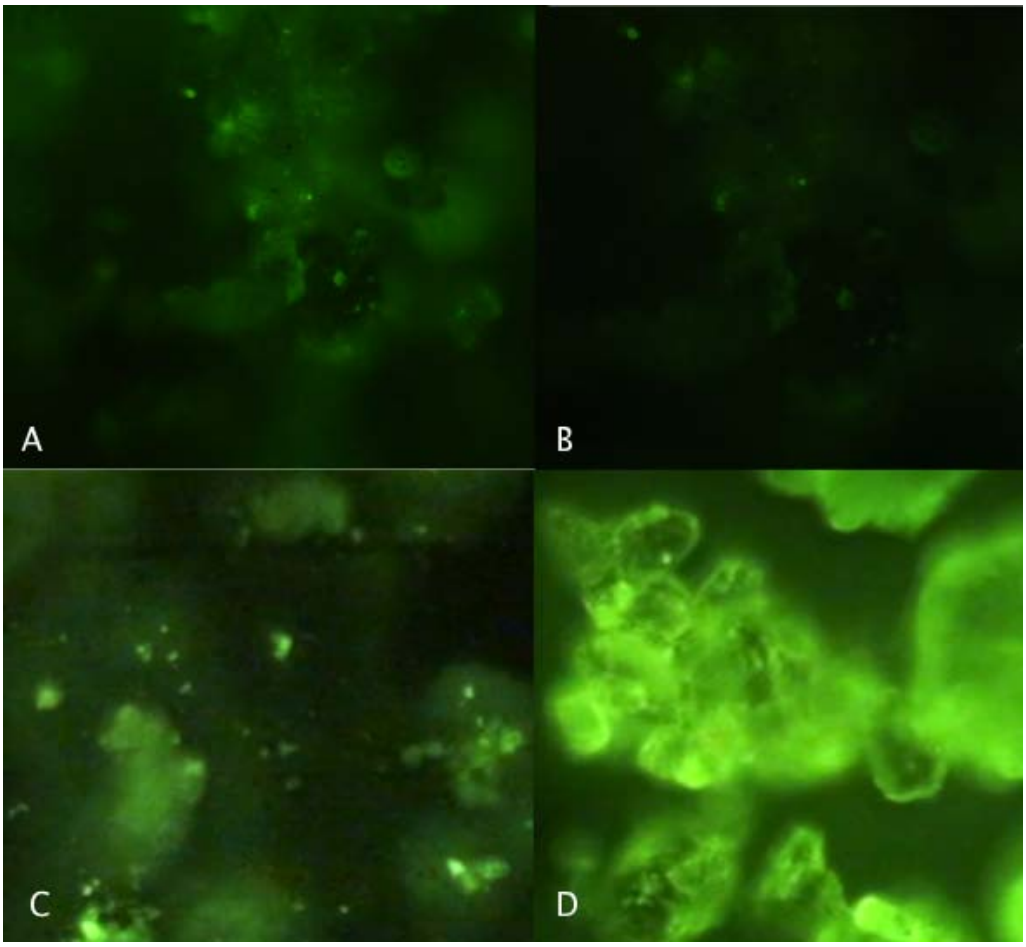

Figure 5. Rock sample 09-302-1498 stained with SYBR Gold Nucleic Acid Gel Stain. Panels A through D illustrate that bacteria were generally not noted on the surfaces of sample particulates. Magnification for these figure panels was 1000X.

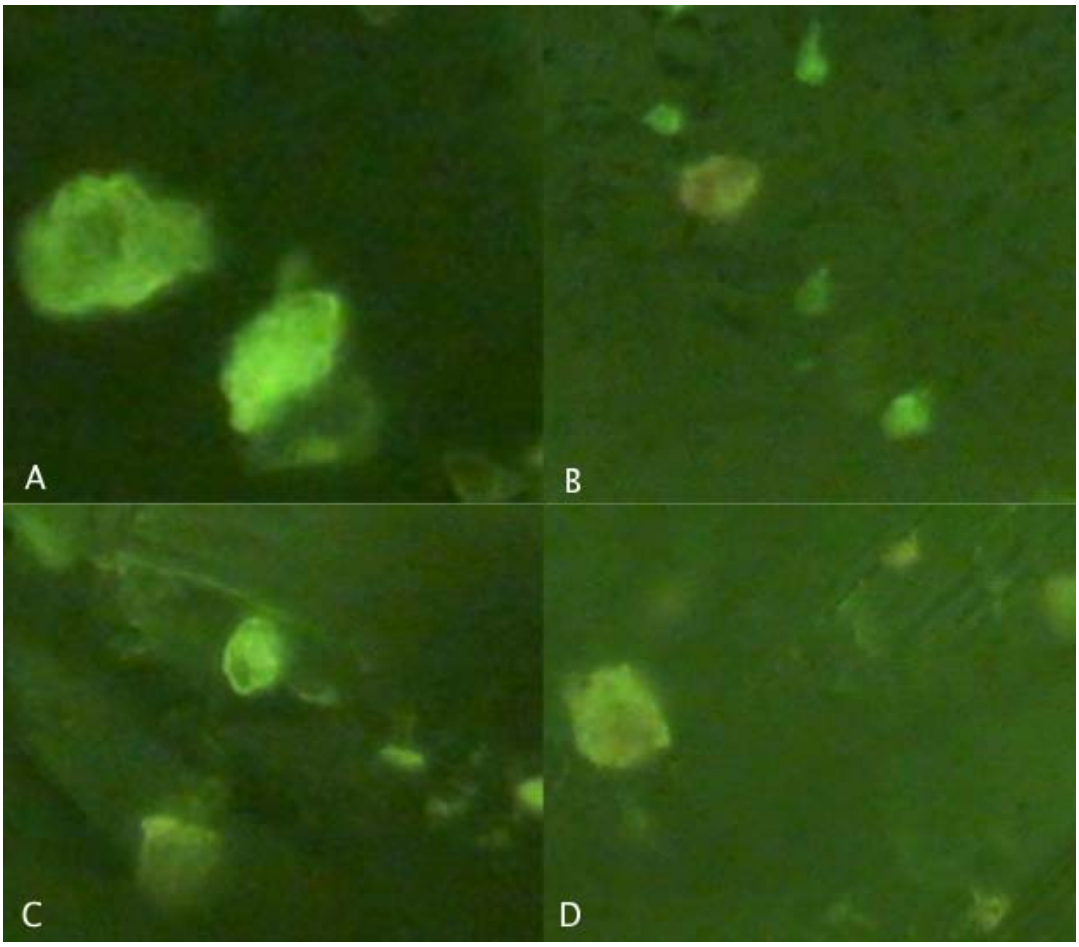

Figure 6. Rock sample 09-302-2624 stained with SYBR Gold Nucleic Acid Gel Stain. Fluorescing bacteria can be seen on the surfaces of particulates in Panels A and B. Auto-fluorescing inorganic matter (non-microbiologic) in Panel C and a mixture of auto-fluorescing inorganic matter and fluorescing bacteria in Panel D. Magnification for these figure panels was 1000x.

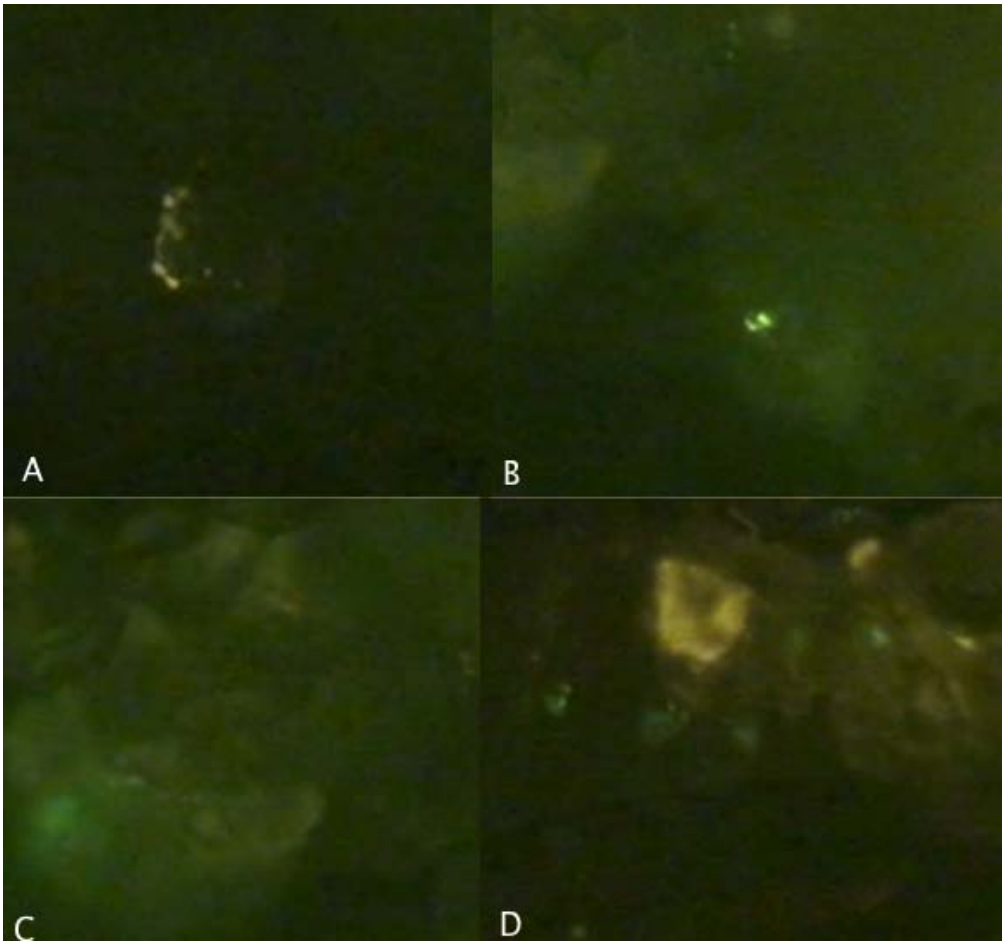

Figure 7. Rock sample 09-302-2625 stained with SYBR Gold Nucleic Acid Gel Stain. A large auto-fluorescing inorganic particle along with a few auto-fluorescing fine inorganic particles can be seen in Panel A. Panels B, C, and D. illustrate numerous auto-fluorescing fine inorganic particles on the surfaces of the gypsum rock particulates. Magnification for these figure panels was 1000x.

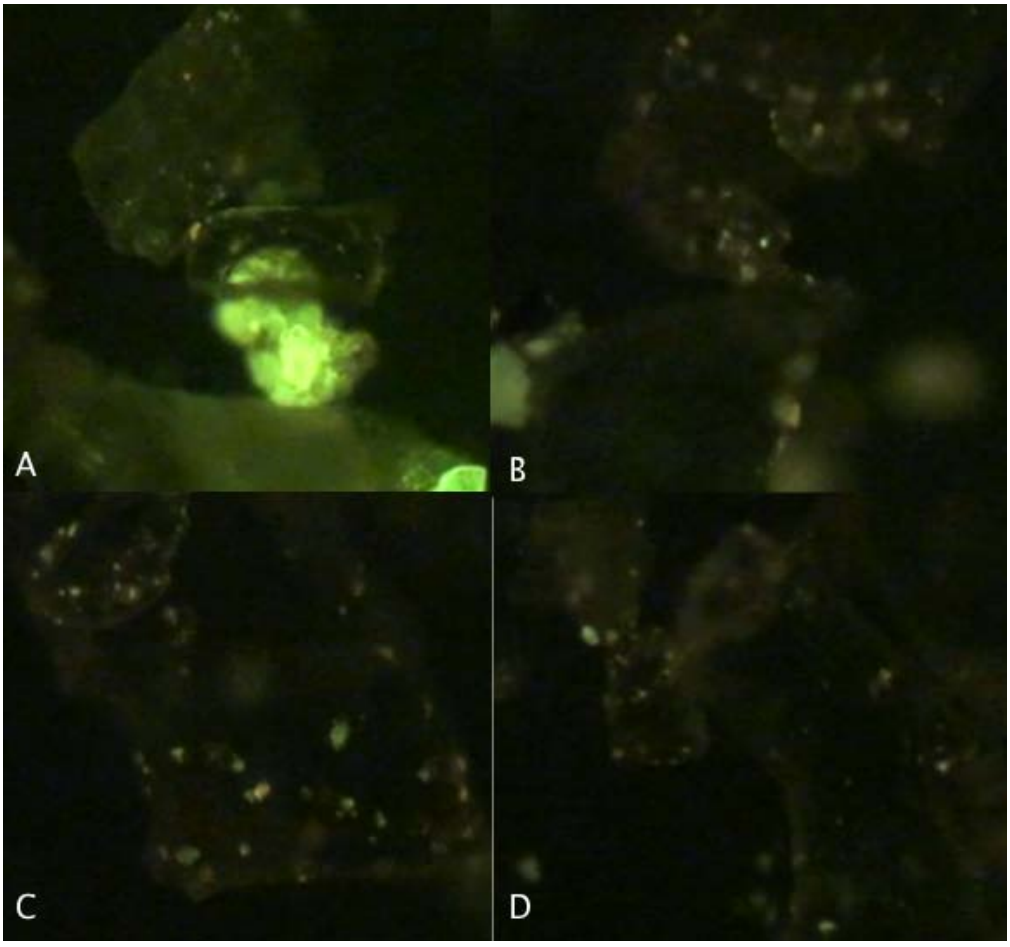

Table 1. First sample set (received August 25, 2010). Quantitative Polymerase Chain Reaction (qPCR) data for the number of bacteria (universal16S) and sulfate reducing bacteria (SRB) per sample weight, area, or milliliter of post-culture broth.

Sample identification and sub-sample type	Direct Universal 16S qPCR. Genomic equivalents per mm^2 of paper or gram of gypsum filler or rock	Post culture Universal 16S qPCR. Genomic equivalents per ml of culture broth	Post culture SRB qPCR. Genomic equivalents per ml of culture broth
09-840-9962-07 light paper	-	1.4×10^6	-
09-840-9962-07 dark paper	2.2×10^3	1.0×10^7	-
09-840-9962-07 filler	-	-	-
09-810-7339-09 light paper	-	7.8×10^5	-
09-810-7339-09 dark paper	1.4×10^3	1.6×10^6	-
09-810-7339-09 tape	3.9×10^3	2.0×10^6	1.2×10^2
09-810-7339-09 filler	-	1.5×10^4	-
Rock 10-302-1140	4.2×10^4	3.1×10^4	-
Rock 09-302-1498	6.4×10^4	1.0×10^6	-
Rock 09-302-2624	1.2×10^4	3.8×10^4	-
Rock 09-302-2625	-	5.8×10^5	3.3×10^2
09-810-8357-03 light paper	-	1.4×10^6	-
09-810-8357-03 dark paper	-	1.4×10^6	-
09-810-8357-03 filler	8.9×10^3	8.9×10^5	2.2×10^2
09-840-9673-07 light paper	-	3.0×10^5	-
09-840-9673-07 dark paper	-	1.4×10^6	-
09-840-9673-07 filler	-	9.4×10^4	-
09-840-9858-02 light paper	-	1.8×10^6	-
09-840-9858-02 dark paper	-	1.3×10^6	-
09-840-9858-02 filler	-	3.6×10^5	-
Positive/negative controls	Std's +/-	1.1×10^5/-	1.3×10^5/-

- = none detected. Std's + = standards utilized as positive controls with positive amplification, *Bacillus atrophaeus* DNA. Positive control for post–culture qPCR = DNA extract from ATCC 13541 *Desulfovibrio desulfuricans* culture tube.

Table 2. Second sample set (received November 18, 2010). Quantitative Polymerase Chain Reaction (qPCR) data for the number of bacteria (universal 16S) and sulfate reducing bacteria (SRB) per sample weight, area, or milliliter of post culture broth.

Sample identification and sub-sample type	Direct Universal 16S qPCR. Genomic equivalents per mm^2 of paper or gram of gypsum filler or rock	Post-culture Universal 16S qPCR. Genomic equivalents per ml of culture broth	Post-culture SRB qPCR. Genomic equivalents per ml of culture broth
09-302-1394-01b light paper	-	1.6×10^5	-
09-302-1394-01b dark paper	-	-	-
09-302-1394-01b filler	-	-	-
09-302-1395-11b light paper	-	-	-
09-302-1395-11b dark paper	-	1.2×10^5	-
09-302-1395-11b filler	6.2×10^3	9.0×10^4	-
09-302-2542-03b light paper	1.9×10^4	4.5×10^5	-
09-302-2542-03b dark paper	-	5.4×10^4	-
09-302-2542-03b filler	9.3×10^3	7.4×10^4	-
09-302-2544-02b light paper	-	3.9×10^7	-
09-302-2544-02b dark paper	8.8×10^3	6.6×10^4	-
09-302-2544-02b filler	-	-	-
09-302-2544-03b light paper	7.8×10^3	-	-
09-302-2544-03b dark paper	-	-	-
09-302-2544-03b filler	-	-	-
09-302-2544-04b light paper	-	4.3×10^5	-
09-302-2544-04b dark paper	-	7.9×10^4	-
09-302-2544-04b filler	-	6.7×10^4	-
10-810-5462-01b light paper	-	4.2×10^7	-
10-810-5462-01b dark paper	-	3.2×10^5	-
10-810-5462-01b filler	-	-	-
Positive/negative controls	Std's +/-	1.2×10^5/-	5.3×10^6/-

- = none detected. Std's + = standards utilized as positive controls with positive amplification, *Bacillus atrophaeus* DNA. Positive control for post–culture qPCR = DNA extract from ATCC 13541 *Desulfovibrio desulfuricans* culture tube.

Table 3. Analyses of another fragment of sample 09-810-8357-05 (received February 22, 2011) and repeat of qPCR for the original cultures of samples 09-810-8357-03, 09-840-9858-02 and 09-302-2625. Quantitative Polymerase Chain Reaction (qPCR) data for sulfate reducing bacteria (SRB) per milliliter of post culture broth.

Sample identification and sub-sample type	Post-culture SRB qPCR. Genomic equivalents per ml of culture broth
09-810-8357-05 new sample/culture - light paper	-
09-810-8357-05 new sample/culture - dark paper	-
09-810-8357-05 new sample/culture - filler	-
09-810-8357-03 original culture - light paper	-
09-810-8357-03 original culture - dark paper	-
09-810-8357-03 original culture - filler	-
09-840-9858-02 original culture - light paper	-
09-840-9858-02 original culture - dark paper	-
09-840-9858-02 original culture - filler	-
Rock 09-302-2625 original culture	-
Positive/negative controls	Std's +/-

- = none detected. Std's + = standards utilized as positive controls with positive amplification, *Bacillus atrophaeus* DNA.

References:

Burdack-Freitag, A., F. Mayer, et al. (2009). "Identification of odor-active organic sulfur compounds in gypsum products." CLEAN - Soil, Air, Water **37**(6): 459-465.

Curtis, M. E., P. R. Jones, et al. (2009). "Determination of the presence or absence of sulfur materials in drywall using direct analysis in real time in conjuction with an accurate-mass time-of-flight mass spectrometer." Journal of the American Society for Mass Spectrometry **20**(11): 2082-2086.

Hooper, D. G., J. Shane, et al. (2010). "Isolation of sulfur reducing and oxidizing bacteria found in contaminated drywall." International Journal of Molecular Sciences **11**(2): 647-655.

Nadkarni, M. A., F. E. Martin, et al. (2002). "Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set." Microbiology-Sgm **148**: 257-266.

Noble, R. T. and J. A. Fuhrman. (1997). "Virus decay and its causes in coastal waters." Applied and Environmental Microbiology. 63(1):77-83

Wagner, M., A. J. Roger, et al. (1998). "Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration." Journal of Bacteriology **180**(11): 2975-2982.