

Introduction

The Hawaii Undersea Munition and Material Assessment (HUMMA) has been an ongoing study of the environment impact of conventional and chemical munitions disposed of at site HI-5. The main focus for our testing has been the chemical munitions containing Bis(2-chloroethyl) sulfide (HD) and its degradation product Thiodiglycol(TDG). Samples were collected in close proximity to munitions found at the site by a Remote Operated Vehicle (ROV, Fig. 1). Initial analysis of the samples was conducted for HD and all its breakdown products. Samples were shipped back to Edgewood, MD for further analysis for TDG by LCMSMS. The negative results for TDG showed a need for additional research for Biological Testing for the possibility of TDG bacteria remediation. Following the chemical analysis, the ECBC BioMonitoring Team selected six representative samples, containing a range of HD levels from below the detection limit (<10 µg/kg) up to 2100(E) µg/kg, for evaluation of the microbiological communities present (Table 1). The intent of this testing was to evaluate the impact of chemical agent on the growth of bacteria near the dumped chemical weapons, and to assess the potential of the bacteria present to utilize the HD as a carbon source and thereby contribute to the ultimate breakdown of the agent in vivo.

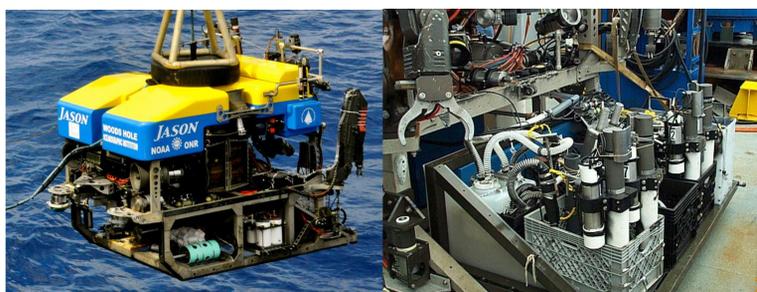


Figure 1. Left: Samples were collected using the Jason Remote Operated Vehicle (ROV) from the Woods Hole Oceanographic Institution; shown here preparing for its descent to where chemical munitions are found 500-600 meters underwater off the coast of Hawaii. Right: Jason's sample tray.

Table 1: HD Level in Samples Selected for Biological Analysis

EML Sample ID	HUMMA Sample ID	HD Level (µg/kg)
HUM140004	004S	<10
HUM140007	007S	<10
HUM140021	021S	18
HUM140038	034S	180 (E)*
HUM140059	048S	2100 (E)
HUM140192	144S	1300 (E)

*E = Result exceeds upper calibration level

Materials and Methods

Biological Analysis

Initial Inoculations. Samples were inoculated onto various selective and non-selective media and held at two different temperature and oxygenation conditions to determine the ideal growth parameters (Table 2).

Table 2: Media Used for Initial Inoculations

Aerobic media	Media
	Tryptic Soy Broth (TSB)
	Tryptic Soy agar with 5% Sheep's Blood (BAP)
	MacConkey II agar
	Chocolate agar
	Columbia CNA agar
Anaerobic media	Media
	Chopped Meat broth
	CDC Anaerobe 5% Sheep blood agar

Two sets of inoculations were prepared for each sample; one was held at 25°C and the other at 5°C to simulate growth conditions on the ocean floor. Anaerobic cultures were kept in a sealed anaerobe chamber with an anaerobe pouch to remove the oxygen and a colorimetric anaerobic indicator for verification of anaerobic conditions. Analysis of all samples containing any level of HD was done in a chemical fume hood. Plates were checked daily for growth at all conditions. When colonies became visible, they were streaked onto BAP to isolate a single type of bacteria onto each plate to obtain a pure colony. Additional isolation plates were streaked as needed until pure cultures were obtained for all species seen. If two isolates were visibly identical, only one culture was maintained. Any visible differences in colony morphology resulted in both cultures being kept.

Materials and Methods (cont'd)

Morphology and Staining

After all isolates were obtained from each sample, the isolates were assigned a consecutive number, colony descriptions were noted, and freezer stocks were prepared for preservation. The descriptions taken included colony morphology (size/shape/color/growth pattern) and Gram stains. Isolates were identified as Gram positive or Gram negative and their general shape were recorded. In addition, a photograph was taken of each isolate growing on a BAP (Fig. 2).



Figure 2. Isolate #83 growing on BAP

Ongoing Analysis: Growth on Thiodiglycol media

The unique isolates obtained are currently undergoing testing for their ability to use TDG (the key hydrolysis product of HD) as their sole carbon source for growth. This testing is using the recipe developed by Medvedeva, N. et. al² with minor modifications. The contents of the media used are in Table 3. Agar plates were prepared and poured in three ways: containing only the ingredients above, with no added carbon source; with 0.35mg/L of tryptone as a carbon rich control; and with 0.35 mg/L of TDG for the test plate. The agar media was prepared separately, autoclaved, cooled, and poured into plates that had been individually spiked with the appropriate carbon source (as applicable). This method was selected to minimize breakdown of the TDG due to the heat of the liquid agar. Each isolate was inoculated from the freezer stock onto a BAP and incubated until sufficient growth was present to prepare a sterile water suspension at a MacFarland standard of approximately 1. One hundred µL of this culture was then inoculated onto each of the plates above (no carbon, tryptone, and TDG-containing) and a BAP, and incubated in chemical containment for one week. After a week of growth, visual observations were made regarding the growth of the isolate on each media. Control plates containing TDG but not inoculated with a bacterial culture were poured at the same time as test plates, and were tested both one day after creation and approximately one week after creation to correspond to the time point at which test plates were analyzed.

Table 3: Contents of Media Used for TDG Testing

Chemical Name	Abbreviation	Concentration
Ammonium Sulfate	(NH ₄) ₂ SO ₂	4 g/L
Monopotassium Phosphate	KH ₂ PO ₄	1.5 g/L
Dipotassium Phosphate	K ₂ HPO ₄	1.5 g/L
Magnesium Sulfate	MgSO ₄	0.2 g/L
Sodium Chloride	NaCl	5.0 g/L
Agar agar	N/A	20 g/L

Chemical Analysis Methods for TDG

A Method Blank (MB), Laboratory Control Sample (LCS), and a Laboratory Control Sample Duplicate (LCS D) were prepared each day plates were to be analyzed. The MB plate was triple rinsed with Methanol (MeOH) and the rinsate was collected in a volumetric flask. The rinsate was then spiked with TDG-d8 Internal Standard and MeOH was added for a final volume of 20 mL. The LCS and LCS D were spiked with TDG and then processed the same as the MB.

TDG test plates were pre-spiked with TDG and approximately 20-40 grams of agar media was poured into each plate. Plates were inoculated and evaluated for growth of the bacteria. All plates were extracted on an average of 9 days after inoculation. The agar media was removed from the plates and added to a 50 mL conical tube. The plates were triple rinsed with MeOH and the rinsate was collected in a volumetric flask. The rinsate was spiked with TDG-d8 and MeOH was added for a final volume of 20 mL. The Rinsate was then poured into the conical tube containing the agar media. Samples were placed on a vortexer for 30 minutes. Control plates were processed the same as the test plates.

All samples were aliquoted into 0.45 µm PTFE Mini-Uniprep vials and analyzed by LCMSMS to identify whether the bacterial growth (if present) appeared to have a significant impact on the residual TDG level as compared to a control plate.

Results

Based on visual observations, the 70 unique bacterial strains isolated showed a variety of morphological characteristics, gram stain results, and preferred growth conditions. To date, the majority of the isolates showed significantly reduced or no ability to grow on media containing TDG or no carbon source. Table 4 shows the growth results for a selection of isolates tested on each type of media. Most isolates were able to grow successfully on BAP and the tryptone-containing media. In almost all cases where growth was seen on the TDG-containing media, the growth was much slower and colonies were smaller and less robust. In these cases, similar weak growth was often also seen on media with no carbon source added.

Table 4. Selected Isolate Results for TDG Media Growth

Sample #	Date innoc on TDG	BAP	NC	TR	TDG
58	7/1/2015	+	weak +	weak +	weak +
59	7/1/2015	+	-	-	-
60	7/1/2015	+	-	-	-
62	7/1/2015	+	weak +	+	weak +
63	7/1/2015	+	weak +	+	weak +
65	7/9/2015	+	-	+	-
67	7/9/2015	+	+	+	weak +
68	7/9/2015	+	-	-	-
69	7/9/2015	+	-	+	-
72	7/9/2015	+	+	+	weak +

In addition, minimal impact was seen on the level of TDG remaining in plates that showed bacterial growth. Table 5 shows the results from a selection of isolates and controls tested so far and the percentage of TDG that was recovered from each.

Table 5. Selected TDG Test Results

Test Date	Sample	Plate wt(g)	Plate +Isolate(g)	Total Isolate(g)	MeOH Amt.(mL)	Results(ug/L)	% Recovery
7/7/2015	MeOH Blank	NA	NA			0	
	MB	NA	NA			0	
	LCS	NA	NA			238.15	95.26
	LCS D	NA	NA			259.92	103.968
	control	16.24	35.56	19.32	17	155.89	62.356
	Isolate 58(spiked 7/2/15)	16.34	42.21	25.87	18	109.8	43.92
	Isolate 59(spiked 7/2/15)	16.33	41.98	25.65	17	145.05	58.02
	Isolate 60(spiked 7/2/15)	16.24	39.12	22.88	17	148.11	59.244
Isolate 62(spiked 7/2/15)	16.24	41.03	24.79	15.5	145.04	58.016	
Isolate 63(spiked 7/2/15)	16.33	37.17	20.84	16	5.67	2.268	

Discussion

Not all isolates have been tested on TDG containing plates due to programmatic limitations. The remaining isolates will be investigated when testing resumes. TDG testing so far has resulted in only one isolate that appears to have a significant impact on TDG levels, indicating that they are likely using TDG as a carbon source for growth. It is unknown why significant numbers of isolates were able to grow (if poorly) on media that contained TDG or no carbon, but no effect was seen on the resulting TDG level of the media in most of these cases. Additional investigation should be done to determine if there is a more appropriate media formulation which eliminates growth that is considered extraneous (i.e., occurs despite lack of an appropriate carbon source).

In addition, any isolates that appear to have any impact on TDG levels as compared to controls will be tested on agar that substitutes HD for TDG, to determine whether any isolates are capable of breaking down HD from its original form as present in the dumped chemical weapons. These isolates will also be sequenced for identification. This information will be used to help determine whether there are any bacterial strains which may be contributing to the breakdown of chemical weapons in the sea floor environment surrounding the Pacific Ocean dump sites.