2013

Aerobic Degradation of α-, β-, γ-Hexachlorocyclohexane by Narragansett Bay Bacterioplankton

Ian M. Rambo
ian.maguire.rambo@gmail.com

HPR 401/402

Aerobic Degradation of α-, β-, γ-Hexachlorocyclohexane by Narragansett Bay Bacterioplankton

Ian M. Rambo
Senior Honors Project
URI Biological Sciences Department
URI Spanish Department

Adviser
Dr. David C. Smith
URI Graduate School of Oceanography

Submitted May 13, 2013

Hexachlorocyclohexanes (HCHs) are a family of chlorinated organic compounds that were previously used as agricultural insecticides. HCHs are recognized as persistent organic pollutants due to their toxicity, recalcitrant properties, and tendency to bioaccumulate in food webs. Although HCH was first synthesized in 1825, its use was not widespread until the discovery of the insecticidal activity of the γ-HCH isomer in 1942. γ-HCH and its toxic waste isomers α-HCH and β-HCH were banned from production and use by the United Nations in 2009, yet these chemicals still present environmental problems due to their persistence in soils and surface waters. HCHs continue to be introduced into aquatic ecosystems via rain and groundwater. Bioremediation of HCH-contaminated soil and water by bacteria is a viable option for restoring these areas.

Aerobic biodegredation of HCH by soil bacteria has been studied extensively, yet the HCH degrading potential of aerobic bacterioplankton in the ocean water column has not. Certain bacteria are able to utilize toxic compounds like HCH as carbon sources, and in environments where HCH concentrations are high, HCH-degrading species have the potential to out-compete other members of the microbial community and alter the greater ecological landscape.

This study focuses on the effects that α, β, and γ-HCH isomers have on aerobic marine bacterial communities. These isomers were chosen due to their higher prevalence in the environment compared to other HCH isomers, as well as their toxicity. Water samples from Narragansett Bay were spiked with α, β, and γ-HCH to select for bacterial species able to tolerate or utilize these compounds. Bacteria isolated from these seawater incubations were used in subsequent HCH degradation experiments. Mass spectrometry was used to quantify the concentrations of each HCH isomer over time in order to calculate degradation rates. Comparisons of experimental and control samples highlight possible bacterial population trends in HCH-contaminated environments. Isolation of marine bacteria capable of degrading HCH *in situ* has potential for bioremediation of contaminated waters.

Hexachlorocyclohexanes (HCHs) constitute a family of organochlorine molecules whose gamma isomer, also known as Lindane, was a popular pesticide and pediculicide. HCH does not exist in nature and was first synthesized by Michael Faraday in 1825 by the photochlorination of benzene (Vijgen, 2006). Studies conducted on technical HCH (t-HCH) by Bender in 1935 and Dupire in 1940 proved its insecticidal properties, but Slade's research in 1942 proved that the gamma isomer Lindane was solely responsible for the insecticidal nature of the t-HCH mixture (Stoffbericht, 1993).

The photochlorination process of benzene to HCH yields several stereo-isomers in various proportions, but due to the exclusive insecticidal properties of Lindane (γ-HCH) this isomer is purified via extraction from the t-HCH mixture. Since Lindane is generally only 10-15% of the yield, the remaining 85-90% of inactive isomers are discarded as waste (NARAP, 2006). Although these inactive isomers do not exhibit insecticidal properties, they are still toxic and are considered to be persistent organic pollutants (United Nations, 2009).

The production and use of Lindane, α-HCH, and β-HCH were banned by the United Nations under the 2008 Stockholm Convention on Persistent Organic Pollutants (United Nations, 2009), yet the persistent nature of these compounds presents long-term environmental problems.

The provisions of the Stockholm convention do not account for the thousands of tons of waste isomers that act as chronic sources of HCH in the environment.

The cleanup of HCH stereo-isomer waste sites often involves the use of base catalyzed dechlorination via the addition of an alkali or alkaline earth metal carbonate, bicarbonate, or hydroxide (CMPS&F, 1997). This process can be quite costly and is beyond the financial reach of many developing nations (Vijgen, 2006). The use of in-situ bioremediation offers a cost-effective method for HCH isomer elimination efforts.

Several studies have been conducted on the biodegradation of HCHs by aerobic soil bacteria (Bachmann et al., 1988; Nagata et al., 2007; Phillips et al., 2005), but research regarding the biodegradation of HCHs by aerobic marine bacterioplankton is lacking. This research is the first concerning the HCH biodegradation abilities of aerobic bacteria in the Narragansett Bay water column. The intention of this study is to gain a primary understanding of the effects of Lindane, α-HCH, and β-HCH on aerobic marine bacterial community dynamics and whether or not selected species are able to degrade the aforementioned HCH isomers.

Materials and Methods

Seawater sampling. 1 L seawater samples were taken from the Durbin Aquarium at the University of Rhode Island Graduate School of Oceanography (GSO) and the Jamestown 6E Wharf on Febraury 27, 2013 and April 10, 2013, respectively. The GSO sample was collected from a water supply pipe into a 1 L amber glass bottle. Seawater from the pipe is pumped continuously from Narragansett Bay and passed through a sand filter distribution. The Jamestown sample was collected at a depth of 2 m by using a 1 L polypropylene flask attached to a rope and weighted by plastic coated lead doughnuts. The sample was stored in a 1 L amber glass bottle. Seawater samples were stored in a refrigerator at 4 °C.

α-, β-, γ-HCH stock solution. A stock solution consisting of 10 mL methanol and 10 mg of each HCH isomer crystal (alpha-BCH, beta-BCH, Lindane, 99% purity, Analabs, Inc., Charleston, WV) was prepared for subsequent experiments. HCH isomers were weighed in separate 2 mL amber glass vials. The vials were washed three times with methanol and the solutions were transferred to a single 10 mL graduated cylinder using muffled Pasteur pipettes. The graduated cylinder was placed into a sonicator to break up remaining crystals (Model 8851, Cole-Parmer, Vernon Hills, IL). Stock solution was transferred to a glass vial wrapped in aluminum foil and was stored in a refrigerator at 4 °C.

HCH test. Bacteria species capable of resisting or utilizing HCH isomers were selected for by spiking 1 mL of seawater with 10 µL of the HCH/methanol stock solution using a 10 µL glass syringe (Hamilton Co., Reno, NV). The syringe was flushed three times with 100% methanol between each spike. A control sample contained 1 mL of raw seawater and was not spiked with HCH/methanol. Samples were contained in 2 mL amber glass vials and were incubated at 23 °C for three days. Three sterile marine nutrient agar plates (15:5:1 g agar, peptone, yeast extract for 1 L seawater) were prepared in a horizontal laminar airflow workstation (NU-201, NuAire, Plymouth, MN) for both the experimental and control samples. Agar was sterilized beforehand in an autoclave at 121°C for 30 minutes. Agar plates were inoculated with 100 µL of the respective sample using an Eppendorf pipette. New tips were used for each plate inoculation. The inoculum was spread using a glass spreader sterilized via 95% ethanol and a propane torch. Inoculated agar

plates were wrapped in Parafilm (Pechiney Plastic Packaging Company, Chicago, IL) and incubated at 23 °C for three days.

Pure culture isolation. Colonies derived from the HCH tests were isolated by streaking marine agar plates and inoculating marine nutrient broth (5:1 g peptone, yeast extract for 800 mL seawater and 200 mL DI water). Inoculations were performed in a horizontal laminar airflow workstation (NU-201, NuAire, Plymouth, MN). Broth was partitioned into 5mL aliquots and transferred to 14 mL polystyrene tubes (Cat. No. 352057, Falcon, Franklin Lakes, NJ) through the use of a three-way squeeze bulb and sterile 10 mL disposable polystyrene serological pipettes (Fischerbrand, Pittsburgh, PA). The broth was inoculated with pure cultures obtained from streaking using an inoculation loop sterilized with a propane torch. The caps of the broth tubes were left in the "ajar" position to allow for airflow but still prevent contaminants from entering. Tubes were placed in a shaker (Classic Series C1 Platform Shaker, New Brunswick Scientific, Enfield, CT) at speed 60, 23°C to allow for oxygenation.

Methanol test. Pure cultures were subjected to methanol concentrations identical to those in the HCH test to determine if methanol acted as an inhibitory factor. 100 µL of each pure broth culture was inoculated into 1 mL filtered seawater (FSW) using an Eppendorf pipette. An experimental and control sample was used for each culture.10 µL pure methanol was spiked into the experimental samples using a 10 µL glass syringe (Hamilton Co., Reno, NV). Seawater was filtered using a sterile 60 mL plastic syringe (BD) coupled with a sterile, disposable 25 mm filter with 0.45-µm pore size (Fischerbrand, Pittsburgh, PA). FSW was collected in an autoclaved 100 mL beaker and transferred to 2 mL amber glass vials using a sterile 3 mL plastic syringe (BD). The vials were placed into a shaker (Classic Series C1 Platform Shaker, New Brunswick Scientific, Enfield, CT) at speed 60 for two days at 23°C. 100 µL of each sample was transferred to a respective nutrient agar plate using an Eppendorf pipette and was plated using a glass spreader sterilized with 95% ethanol and a propane torch. The plates were wrapped in Parafilm and incubated at 23°C for ten days.

Preparing cultures for HCH extraction. Pure broth cultures selected for HCH resistance (GSO Cream, GSO Opaque) were transferred to 2 mL micro-centrifuge tubes in 1.5 mL aliquots using 10 mL sterile serological pipettes (Fischerbrand). A pure streak plate colony exhibiting HCH resistance (Jamestown Cream) was inoculated with a sterilized wire loop into a 2 mL micro-centrifuge tube containing 1.5 mL FSW (0.2 µm) added with a 10 mL sterile syringe (BD). The tubes were centrifuged for 10 minutes in a micro-centrifuge (SN 0412 0449, Fischer Scientific). Broth was discarded from the centrifuge tubes and the cell pellets were washed three times with FSW (0.2 µm). 1.5 mL of FSW was then added to the tubes and the cell pellets were broken up by a sterilized inoculation loop.

HCH extraction sample sources. Three extraction sample sources were prepared by spiking 200 µL of HCH/methanol stock solution into an amber glass vial containing 20 mL of FSW (0.2 µm). Spikes were performed with a 100 mL glass syringe (Hamilton Co., Reno, NV), and the syringe was flushed three times with methanol after each spike. 100 µL of each pure culture was transferred to a respective vial with an Eppendorf pipette.

HCH extraction. HCH was extracted from the inoculated FSW using hexane (MacFarlane et al., 2007) at *t* 30 minutes, 1 hour, 5 hours, 7 hours, and 32 hours. 1 mL of each sample vial was transferred to a 2 mL amber glass vial using muffled Pasteur pipettes. The internal standard (IS) used was a 13C-hexachlorobenzene organochlorine pesticide (OCP) surrogate (4ng/mL) provided by David Adelman. 10 µL of the internal standard was added to the vials using a 100 µL glass syringe (Hamilton Co., Reno, NV). The syringe was flushed with methanol after adding the surrogate to each sample. Samples were then transferred to muffled glass vials using glass Pasteur pipettes. Remaining HCH and internal standard in the 2 mL vials were collected by the individual addition and shaking of 1 mL hexane and 1 mL milli-q water. The samples in the muffled glass vials were shaken and the water and hexane were allowed to re-separate. The top layer of hexane was extracted using a glass Pasteur pipette and was transferred to a new muffled glass vial. Sodium sulfate anhydrous was added to the hexane until free-moving crystals formed, indicating that any remaining water had been absorbed. The hexane was pipetted into a 2 mL amber glass vial with a Pasteur pipette, taking care not to take up any sodium sulfate anhydrous. The hexane samples were then condensed with nitrogen at 80 kPa via a mini-vap (6-port mini-vap, Supelco Analytical). The mini-vap was cleaned with a hexane-soaked tissue (Kimwipe) to prevent possible sample contamination. Hexane samples were condensed and transferred to new 2 mL amber glass vials three times. Condensed samples were then transferred to new 2 mL amber glass vials containing 50 µL BMI glass inserts (Supelco Analytical). 10 µL of tribromobiphenyl injection standard (IJS) (3.5 ng/µL) provided by David Adelman was added to the samples using a 100 µL glass syringe (Hamilton Co, Reno, NV). The syringe was flushed three times with hexane prior to the addition of the injection standard.

Mass spectrometry. Mass spectrometry was used to determine the concentrations of the HCH isomers at each time interval (). Samples were analyzed using a Model 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA). The program Chemstation MSD Chemstation D (Agilent Technologies, Santa Clara, CA) was used to quantify peak responses. HCH isomer concentrations were calculated by first determining the concentration of internal standard:

$$[IS] = ((Peak\ Response\ IS / Peak\ Response\ IJS) / mIS) * [IJS]$$

From this the concentration of the HCH isomers was determined:

$$[HCH] = ((Peak\ Response\ HCH / Peak\ Response\ IS) / mHCH) * [IS]$$

Degradation rates of HCH were determined through the equation:

$$\Delta [HCH] = ([HCHi] - [HCHf]) / t$$

Results

HCH tests. Plated control samples showed the presence of several bacterial species acting as a community. Based on colony morphology, only one or two species of bacteria dominated the experimental samples. Isolated colonies were labeled based on colony morphology. The non-

resistant colonies were labeled GSO-yellow, GSO-peach, and GSO-black. Resistant colonies were labeled GSO-opaque, GSO-cream, and Jamestown-cream.

Methanol tests. The 1% methanol concentration of the samples did not have any noticeable effect on the survivability of the pure cultures. It can be determined that HCH had bacteriostatic or bactericidal effects on non-resistant species.

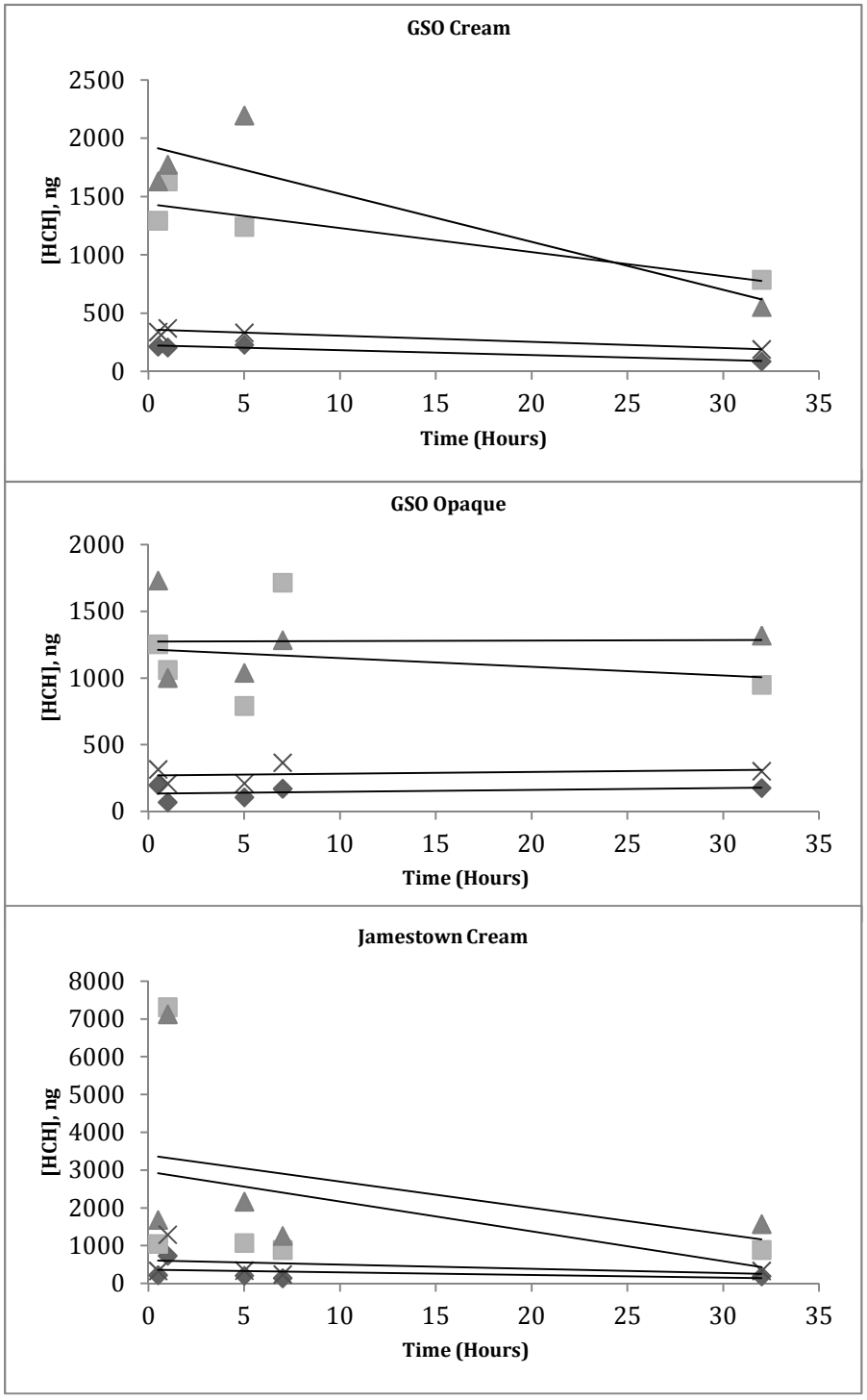

FIG. 1. Linear regression lines demonstrating the degradation of HCH isomers (♦ Alpha-HCH, ■ Beta-HCH, ▲ Gamma-HCH, x Unknown HCH) by each species over time. GSO Opaque and Jamestown Cream displayed trends of HCH resistance, and GSO Cream displayed the highest rate of HCH degradation.

Mass spectrometry. GSO Opaque and Jamestown Cream exhibited trends of HCH resistance, while GSO Cream showed trends of HCH degradation (Fig. 1). GSO Cream had the highest rate of degradation for each isomer (Table 1). An unknown HCH isomer was recorded in the mass spectrometry data.

TABLE 1. Degradation rates of HCH isomers per species, ng/hr

	Alpha	**Beta**	**Gamma**	**Unknown**
GSO Cream	3.97	15.8	33.6	4.00
GSO Opaque	.682	9.5	3.47	.428
Jamestown Cream	.997	4.94	3.47	0

Discussion

Studies involving the biodegradation potential of HCH isomers by bacteria have often dealt with identified soil-dwelling species subjected to a single HCH isomer (Nagata et al., 2007). The lack of information concerning biodegradation of HCH by aerobic marine bacteria, as well as the simultaneous use of three known HCH isomers in this study, presents several questions and challenges for future research in this area.

The initial incubation of the marine bacterial community from Narragansett Bay greatly reduced diversity of the bacterial community as determined by colony morphology. This may have been a result of the inhibition or death of the majority of the colony forming bacteria and/or the rapid growth of those bacteria that were able to utilize HCH. It is possible that HCH inhibited or killed species that did not exhibit resistance, but there is a possibility that metabolites formed during the bioconversion process of the HCH isomers had bacteriostatic or bactericidal properties (Endo et al., 2006). Identifying the metabolic pathways of HCH isomers in resistant species could help to determine if metabolites are a significant factor in the inhibition or death of non-resistant bacteria species in a community. Metabolite analysis can be aided by identifying the enzymatic pathways in HCH degradation. The enzymatic pathways of aerobic γ-HCH degradation have been revealed extensively in soil bacteria such as *Sphingobium japonicum* (Nagata et al., 2007).16S rRNA analysis could be used to determine if observed HCH-resistant aerobic marine species are related to *S. japonicum* (Ash et al., 1991). Future studies designed to identify genes that produce the same enzymes to degrade γ-HCH and form inhibitory metabolites in the degradation process are warranted. Enzymatic activity could also play an important role in the aspect of bioremediation at a community level. Identifying enzymatic pathways for different isomers in species known for HCH degradation could aid in developing a "community-based" method of bioremediation. Genetically altering resistant species to only express enzymatic pathways for a single HCH isomer could allow for a community to effectively perform remediation in areas where several isomers are present, e.g. an HCH waste isomer dump. Steps would have to be taken to ensure that each species could only utilize a specified HCH isomer as

an organic energy source. Due to the specialization of the enzymatic pathways, each species in the community would theoretically die once its food source is depleted; this could negate the risk of a single species becoming dominant in that area once the bioremediation process is completed.

The biodegradation of HCH in soils is known to require certain parameters including pH and temperature (Bachmann et al., 1988). Further research in the aerobic degradation of HCH by marine bacteria would include testing to see if factors such as pH, temperature, and salinity affect degradation rates and enzymatic activity. This knowledge is essential for future implementation of *in situ* marine bioremediation methods tailored for certain environments.

The results obtained in this study showed a low concentration of alpha-HCH and virtually no observed decrease in concentration for all three tested bacteria species. This could possibly be due to the tendency for alpha-HCH to volatize due to its vapor pressure (United Nations, 2008). This should be taken into account when preparing stock solutions containing alpha-HCH and condensing samples via hexane evaporation the future. Another issue experienced in the mass spectrometry aspect of this study involved the use of a single internal standard, which could have affected the results obtained. Future studies should utilize several internal standards to reduce error.

It is entirely possible that other species able to biodegrade HCH were present in the collected seawater samples, but due to the use of only two types of nutrient media there is a fair chance that some species were not able to be properly observed. Future studies would utilize several culture methods to account for all species with HCH-degrading potential. Observations of the marine bacterial community without the use of culturing are also a viable option for determining HCH degradation as a result of community metabolism.

Acknowledgements

I would like to thank my sponsor and friend Dr. David C. Smith for his invaluable help in making this project possible. Even in the midst of countless meetings, business trips to China, proposals, defenses, and other onuses that come with being Vice Dean, he was unwaveringly enthusiastic, helpful, and supportive during the course of this project. With his help I have gained a renewed love of science and a direction for my life.

I also wish to thank Dr. Rainer Lohmann, David Adelman, and Carrie McDonough of the Chemical Oceanography Department at GSO. The chemistry aspects of this project (and ultimately the entire project) would not have been possible without their help.

Literature Cited

Ash, C., Farrow, J.E., Dorsch, M. , Stackebrandt, E., Collins, M. (1991). Related Species on the Basis of Reverse Transcriptase Sequencing of 16s rRNA, 343–346.

Bachmann, a, Walet, P., Wijnen, P., De Bruin, W., Huntjens, J. L., Roelofsen, W., & Zehnder, a J. (1988). Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. *Applied and environmental microbiology*, *54*(1), 143–9.

Cooperation, E. (2006). The North American Regional Action Plan (NARAP) on Lindane and Other Hexachlorocyclohexane (HCH) Isomers, (November).

CMPS&F (1997). Appropriate Technologies for the Treatment of Scheduled Wastes. Review Report Number 4, (5), (November).

Macfarlane, J. K. (2007). Polyethylene Devices : Passive Samplers for Measuring Dissolved Hydrophobic Organic Compounds in, *41*(4), 1317–1323.

Nagata, Y., Endo, R., Ito, M., Ohtsubo, Y., & Tsuda, M. (2007). Aerobic degradation of lindane (gamma-hexachlorocyclohexane) in bacteria and its biochemical and molecular basis. *Applied microbiology and biotechnology*, *76*(4), 741–52.

Nations, U. (2008). *Alpha Hexachlorocyclohexane*.

Nations, U. (2009). Stockholm Convention on Persistent Organic Pollutants (POPs).

Phillips, T. M., Seech, A. G., Lee, H., & Trevors, J. T. (2005). Biodegradation of hexachlorocyclohexane (HCH) by microorganisms. *Biodegradation*, *16*(4), 363–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15865341

Services, U. S. D. of H. and H. (2005). Toxicological Profile for Alpha-, Beta-, Gamma-, and Delta-Hexachlorocyclohexane, (August).

Stoffbericht, (1993) in The Legacy of Lindane HCH Isomer Production, International HCH

& Pesticides Association, January 2006.

Vijgen, J. (2006). The Legacy of Lindane HCH Isomer Production, (January).