

# **CHALLENGING TRADITIONAL BIODEGRADATION TESTS: THE BIODEGRADATION OF GLUTARALDEHYDE**

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## **ABSTRACT**

Biocides play an important role in oilfield operations with regards to corrosion and souring control. However, their potential to impact the environment in a negative manner can lead to concerns about where and when to use them, and can limit how much is used. To address these concerns, it is imperative for biocide manufacturers to determine the biodegradation profile and environmental impact of their products. Traditional biodegradation tests, however, are not well equipped to determine the biodegradation potentials of molecules whose sole purpose is to kill microorganisms. Choosing which biodegradation test to run is often determined by the regulatory requirements of a particular geographic region and the end use applications of the biocide. Success is often difficult as many biodegradation protocols clearly indicate that the test is designed to provide a limited opportunity for biodegradation to occur. It is indeed possible for biocides to pass the requirements of a biodegradation protocol, but to do so requires a thorough understanding of the test protocol, coupled with complete knowledge of the mechanism of action of the biocide.

Glutaraldehyde has been used as a biocide in oilfield operations for many years and its environmental fate profile has been extensively studied. While it is an effective biocide, it is also been shown to be biodegradable as defined by a number of different and widely accepted biodegradation protocols. For example, glutaraldehyde satisfies the criteria of ready biodegradation as defined by the OECD 301A test, and it also exceeds the passing criteria of the OECD 306 test. Prior to initiation of both of these protocols, extensive lab studies were performed to determine the concentration of glutaraldehyde that would be used in each of these tests. Details of the test protocols and the lab studies as they pertained to each of the biodegradation studies will be discussed.

# Introduction

Biocides are typically used in industrial applications to control the growth of microorganisms that can cause a variety of problems in process water systems. These problems can range from corrosion of metal surfaces and souring of oil and gas in oilfield applications, to biofilm formation and public health problems such as Legionnaires Disease in industrial water treatment settings. Ironically, microorganisms may also be called upon to degrade or detoxify solutions containing these chemicals in either the environment or a wastewater treatment plant. It is for this reason that determining the biodegradation potential of biocides is an inherently difficult process. The challenge in evaluating the biodegradation potential of a biocide comes in balancing the amount of biocide used in the test versus the potential to kill or inhibit the microorganisms. Further, the biodegradation tests themselves are designed to provide limited opportunities for the test substance to biodegrade. Even though many challenges exist when evaluating the biodegradation potential of a biocide, exceeding the test criteria for a successful outcome requires a thorough understanding of the parameters of the test protocol, as well as knowledge of how the biocide will function in the presence of the test medium and microbes.

Glutaraldehyde is a well-known biocide that has found uses in many different industrial applications. It is used in water treatment and paper making applications, as well as hospital settings as a sterilant for medical instruments that cannot tolerate heat sterilization. It is perhaps best known as an industrial crosslinking agent for proteins, leather, and photographic films. In the oilfield, it is used as a biocide on both the injection and the production side of both on-shore and offshore oilfields. As a biocide, there is always the potential for negative impact should the environment be unintentionally exposed to solutions of glutaraldehyde. To address those concerns, we have undertaken the task of determining the environmental fate and biodegradation potential of glutaraldehyde under various conditions. Given the breadth of end-use applications for glutaraldehyde, the choice of which biodegradation test to run was governed primarily by the regulatory requirements of the particular geographic region in which a glutaraldehyde-containing product is being used. Once the appropriate regulatory requirements were identified, the process of reviewing the various biodegradation protocols could begin. This paper will describe the challenges that were presented by evaluating glutaraldehyde biodegradation in both the OECD 301A and 306 biodegradation protocols.

## Glutaraldehyde Chemistry

Chemically, glutaraldehyde is 1,5-pentanedial. As an aldehyde, it is a reactive molecule that undergoes reactions that are typical of any aldehyde (1). Aldehydes react with amines to form imines. Imines are reactive species that are usually unstable and can undergo addition chemical reactions. Glutaraldehyde will react readily, and in an irreversible manner, with ammonia and primary amines, but the reaction with secondary amines is not nearly as facile as seen with primary amines (2, 3 - 8). Glutaraldehyde is stable in the presence of tertiary and quaternary amines. The reactivity with primary amines is how glutaraldehyde functions as a biocide; by reacting with and crosslinking

those primary amine containing amino acids that compose either the cell wall or the essential proteins associated with the cell (9). The other major pathways of reaction for glutaraldehyde include oxidation or reduction. Glutaraldehyde can be oxidized to glutaric acid, or reduced to 1,5-pentanediol as shown in Figures 1 and 2. Indeed, these reaction pathways were the means by which glutaraldehyde was found to be metabolized under aerobic and anaerobic conditions, respectively, in an aquatic sediment-river water metabolism study (10, 11).

The reaction of glutaraldehyde with primary amines can, in some instances, be problematic. For instance, a minimum inhibitory concentration of glutaraldehyde against a particular microorganism cannot be determined if the test involves growing the organism in the presence of protein based nutrient broth. The glutaraldehyde will react with the amines provided by the nutrient broth and will not be available to react with the microbes. Another problem that was found arising from the reaction of glutaraldehyde with ammonia was that the product of this reaction was found to be more difficult to biodegrade than glutaraldehyde itself (12). These observations are noteworthy since many growth mediums for biodegradation tests require the use of a nitrogen source for proper microbial growth. It is usually the case that the form of nitrogen that is used is an ammonium ion source such as ammonium chloride.

## OECD 301A READY BIODEGRADATION TEST

The Organization for Economic Cooperation and Development (OECD) has categorized biodegradation testing protocols into a tiered system that begins with relatively fast and easy screening methods and progress' to more complex methods (13). The 301 series of biodegradation tests are screening tests also known as ready biodegradation tests. These are rigorous tests that usually use the test substance as the sole carbon source for the microbes and are designed to provide limited opportunities for the substance to be biodegraded. Passing these tests requires that two criteria be met. The test substance must (i) either exhibit 70% removal of dissolved organic carbon (DOC) or 60% O<sub>2</sub> uptake or CO<sub>2</sub> evolution with the 28-day testing time, and (ii) it must reach this degree of biodegradation within 10 days of reaching 10% biodegradation. The OECD guidelines specify that a chemical that exceeds the test criteria may be assumed to rapidly biodegrade in the environment. Failure to pass the 301 series test does not mean that the substance is not biodegradable, but that additional testing is required or the chemical is not well suited for this type of test. In that case, the 302 series tests, which measure inherent biodegradation, may be a better test for the substance in question.

We chose to evaluate the biodegradation potential of glutaraldehyde in the OECD 301A protocol. We felt that this test was the best suited for evaluating glutaraldehyde despite several concerns that we had. First, we were worried about the reaction of the glutaraldehyde with the ammonium ions in the test medium. Our concerns were tempered by the knowledge that the test called for a fairly high concentration of test substance (from 10 – 40 mg/L as dissolved organic carbon or 16 – 66 mg/L of active

glutaraldehyde) to be used in the protocol while the ammonium ions were present in the medium at low concentrations (approximately 5 ppm). After studying the protocol and evaluating the residual stability of glutaraldehyde in the presence of the test medium, we felt confident that only a minor amount of glutaraldehyde would be lost to the reaction with ammonium ion. Second, we were concerned that a substantial portion of the glutaraldehyde would react with proteinaceous material present in the test medium and would be considered non-biodegraded. Since the OECD 301A protocol allows for the use of high populations of test organisms, typically greater than  $10^5$  CFU/ml, this concern for glutaraldehyde was relevant. However, this situation is addressed by the 301A protocol in that its pass criteria are more stringent than the other tests in the 301 series. The 301A protocol requires 70% removal of DOC, whereas the 301D test requires 60% oxygen consumption, and the 301B test requires 60% CO<sub>2</sub> evolution. Lastly, we were somewhat apprehensive about the effect of the high concentration of test substance (glutaraldehyde) on the viability of the microbes used in the test. Experience had told us that glutaraldehyde concentrations of 40 mg/L would surely kill the majority of the test organisms and lead to a failure in the test. We then set out to determine the effect of moderate concentrations of glutaraldehyde on high concentrations of both viable and non-viable microorganisms.

## **Effect of Glutaraldehyde on the Population of Test Organisms**

To understand the effect of glutaraldehyde on the population of microbes used in the test, we picked 30 mg/L as the concentration of glutaraldehyde that we would evaluate. We then prepared a mixed culture of *E. coli*, *Pseudomonas aeruginosa*, and *Enterobacter aerogenes*. Samples containing various populations of microorganisms were prepared by suspending varying amounts of the microbes in saline solution. The concentration of microorganisms in the samples was estimated by the turbidity of the sample (as reported in Klett units) and was directly determined by plating a portion of the sample onto agar plates. (The samples of microbes with the higher Klett numbers correspond to samples with a higher population of microorganisms.) Each microorganism sample was split in half, with half killed by autoclave and the other half treated with 30 mg/L glutaraldehyde. When the autoclaved sample had cooled to room temperature, 30 mg/L of glutaraldehyde was added to it. This gave 2 sets of samples, one with live and one with dead microorganisms, each containing approximately 30 mg/L glutaraldehyde. In addition, 5 mg/L of NH<sub>4</sub>Cl was added into one of the samples to study the additional affect of ammonia on the glutaraldehyde concentration. We monitored the stability of the glutaraldehyde over the next 24 hours and sampled (for plate counts) the live microbes 24 hours after the addition of glutaraldehyde to determine the affect of the glutaraldehyde on the microorganism population. The results are shown in Tables 1 and 2. The residual concentration of glutaraldehyde, as shown in Table 1, does indeed indicate that high populations of viable microbes affect glutaraldehyde concentration over time. From the results shown in Table 2, it appears that populations of viable microbes in the low  $10^7$  CFU/ml range are most affected by glutaraldehyde (i.e. glutaraldehyde showed some efficacy). However, the samples that contained mid to high  $10^7$  CFU/ml were not significantly affected by the glutaraldehyde. These results correlate well to the fact that the glutaraldehyde was consumed by the viable cultures (as shown in Table 1). Also, non-viable cultures at these populations did not consume the glutaraldehyde. Lastly, it appears that the presence of the 5 mg/L of ammonia did not significantly affect either the stability or the consumption of the glutaraldehyde.

## **OECD 301A Testing**

With the confidence of knowing that high concentrations of viable microorganisms would probably survive a dosage of approximately 30 mg/L of glutaraldehyde, we recommended that this concentration be used in the testing. The 301A guidelines specify a concentration of the test substance of between 10 – 40 mg/L of dissolved organic carbon (DOC). The suggested concentration of glutaraldehyde of 30 mg/L of active material corresponds to a DOC concentration of approximately 18 mg/L. The 301A testing was performed at Wildlife International of Easton, Maryland and was run under GLP conditions and in accordance with the protocol. Figure 3 shows the extent of biodegradation of both the glutaraldehyde and the sodium benzoate control as determined by the disappearance of DOC. As was expected, there was some initial inhibition of the microbes due to their exposure to the glutaraldehyde. However, the microbes recovered and biodegradation of the glutaraldehyde proceeded quickly from that point. The test was terminated after only 9 days since the DOC removal had reached a plateau and the pass criteria had been exceeded. Therefore, glutaraldehyde exceeded the criteria of the OECD 301A test and may be considered readily biodegradable.

## **OECD 306 BIODEGRADATION IN SEAWATER**

Since the OECD 301 series of tests use sewage treatment microorganisms as the source of the inoculum, it is tempting to say that these tests reflect the ultimate fate of a chemical should it be introduced into a municipal waste water treatment plant. The biocide applications that may result in the disposal of biocide containing waste streams into such a facility would include industrial water treatment (cooling towers, closed loop cooling systems, etc), paper making applications, and medical applications (i.e. hospital sterilant uses). For those applications that may dispose of waste streams into a marine environment, the 301 series of tests are not representative tests for determining the biodegradation potential of chemicals in that environment. To address this discrepancy, the OECD has developed an alternative test that is called the OECD 306 Biodegradation in Seawater test. Unlike the 301 or 302 series tests that add microbial inoculum in the form of sewage effluent or activated sludge, the OECD 306 test uses seawater as both the test medium and the sole source of microorganisms. Since there is no supplementing the test medium with additional microorganisms, this is not a ready biodegradation test. Those chemicals that pass this test cannot be called readily biodegradable solely based upon the result of this test. Rather, the chemical is termed to have passed the test and as such has the potential to biodegrade in the marine environment.

### **Glutaraldehyde and The OECD 306 Test**

As we did in the OECD 301A test, we had several concerns about the use of glutaraldehyde in the 306 test. First and foremost was our concern that the relatively low populations of microorganisms that are present in seawater would be killed by the glutaraldehyde. Given that there is no flexibility with regards to microorganism population, our choice of the concentration of glutaraldehyde to use was all the more important. Secondly, the effect of the ammonium ions in the test medium on the concentration of glutaraldehyde was also viewed as problematic especially in light of the

fact that low concentrations of test substance are called for in the test. In an earlier evaluation of glutaraldehyde in the OECD 306 test, 1.5 mg/L of active glutaraldehyde was used. While glutaraldehyde exhibited 52% biodegradation in the test, this was not sufficient to satisfy the passing criteria. We attributed the failure to the fact that (i) a portion of the glutaraldehyde probably reacted with the ammonium ions to form a less biodegradable product, (ii) background hydrolysis of glutaraldehyde had occurred, and (iii) the population of microorganisms in the seawater was low. To help give us a better chance of passing the test, we ran studies in the laboratory to determine how the test medium would affect the residual stability of glutaraldehyde.

The test medium used for the 306 test is supplemented with a minimal nutrient media that consists primarily of minerals. The inclusion of approximately 5 mg/L of ammonium chloride was cause for concern. In order to evaluate the residual stability of glutaraldehyde in this growth medium, we prepared samples of the medium with and without the ammonium ions and added either 3 or 5 mg/L of glutaraldehyde to each. We then determined the residual concentration of glutaraldehyde over 20 days. This was done by taking 1 ml aliquots of each sample, derivatization with dinitrophenylhydrazine (DNPH) and determining glutaraldehyde concentration by HPLC analysis. The results are shown in Figure 4. As expected, the ammonium ions reacted with the glutaraldehyde to the point where very little glutaraldehyde was left in either the 3 mg/L or 5 mg/L sample by the end of the test. In contrast, the samples that contained no ammonium ions showed very little change from the initial glutaraldehyde concentration over the 20 days of the test. Although we were worried about the decrease in glutaraldehyde concentration in the presence of ammonium ions, we felt that the residual stability of the glutaraldehyde was good enough that 3 mg/L of active glutaraldehyde would work well in the actual test.

## **OECD 306 Testing**

To pass the OECD 306 test, the test substance must show 60% biodegradation (when determined by dissolved oxygen removal) within the 28 days of the test. The protocol also requires the test substance be present at a concentration of 2 – 10 mg/L. Knowing that the glutaraldehyde would be stable in the test medium for at least 7 days, we felt comfortable recommending that 3 mg/L of glutaraldehyde be used in the test. The testing was performed by Aqua Survey of Flemington, NJ and was run under GLP conditions and in accordance with the protocol. Figure 5 shows the extent of biodegradation over the 28 days of the test. As we had expected, there was an initial lag in biodegradation for the first several days, presumably due to the shock of the microbes being exposed to the glutaraldehyde. However, the microbes recovered and by day 10, the 60% threshold had been exceeded. Ultimately, glutaraldehyde had attained 73% biodegradation by the end of the test.

## **SUMMARY**

Determining the biodegradation potential of a biocide is a difficult process. The widely accepted biodegradation protocols are not well suited to evaluate molecules whose primary use is to kill those microorganisms that are considered problematic. However, as the results in this paper demonstrate, it is possible to evaluate the biodegradation potential of a biocide and exceed the passing criteria of these tests. However, to do so requires extensive efforts to understand the requirements of the testing

protocols, and a complete and thorough knowledge of how the biocide will impact the testing media and how the media will impact the biocide. Based upon the tests that are described herein, glutaraldehyde exceeds the passing requirements of both the OECD 301A and 306 test's, and as such is a readily biodegradable molecule that has the potential to biodegrade in the marine environment.

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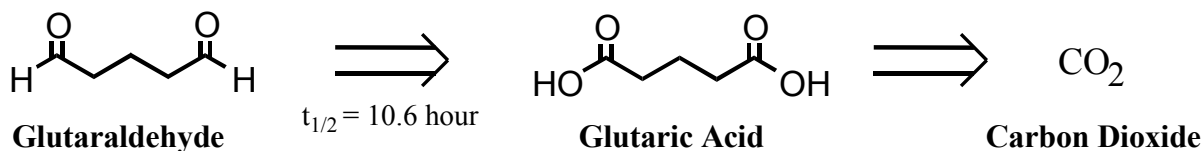
**Table 1.** Residual Glutaraldehyde Concentration (30 mg/L active initial level) in the Presence of Viable and Non-Viable Microorganisms

Time (Hr)	Viable Microbes				Non-Viable			
	Sample #	5K	13K	13K+NH4	24K	Sample #	13K	24K
0		31.4	32.5	31.6	34.6		28.1	30.4
1		27.3	22.8	22.1	15.5		--	--
3		23.9	16.7	14.2	11.3		32.1	30.8
5		22.6	11.6	11.4	5.9		30.3	29.9
24		14.1	0.6	0.6	0.2		31.3	29.8

**Table 2.** Microorganism Counts Before and After Glutaraldehyde Treatment

Sample	Initial Counts	Counts after 24 hours
5K	$3.2 \times 10^7$	$1.0 \times 10^4$
13K	$6.7 \times 10^7$	$9.7 \times 10^6$
13K w/NH4	$6.7 \times 10^7$	$1.0 \times 10^7$
24K	$8.7 \times 10^7$	$1.2 \times 10^7$

**Figure 1.** Aerobic Aquatic Metabolism of Glutaraldehyde



**Figure 2.** Anaerobic Aquatic Metabolism of Glutaraldehyde

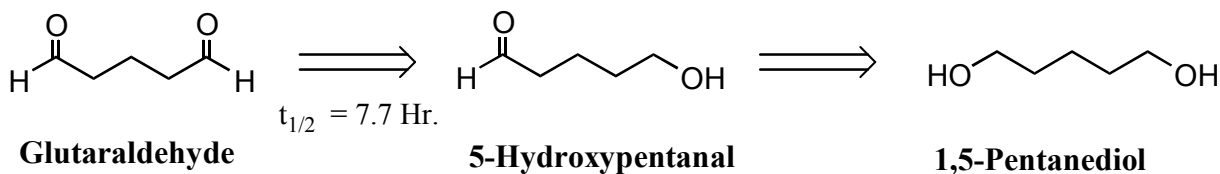


Figure 3. Biodegradation of Glutaraldehyde in OECD 301A Test

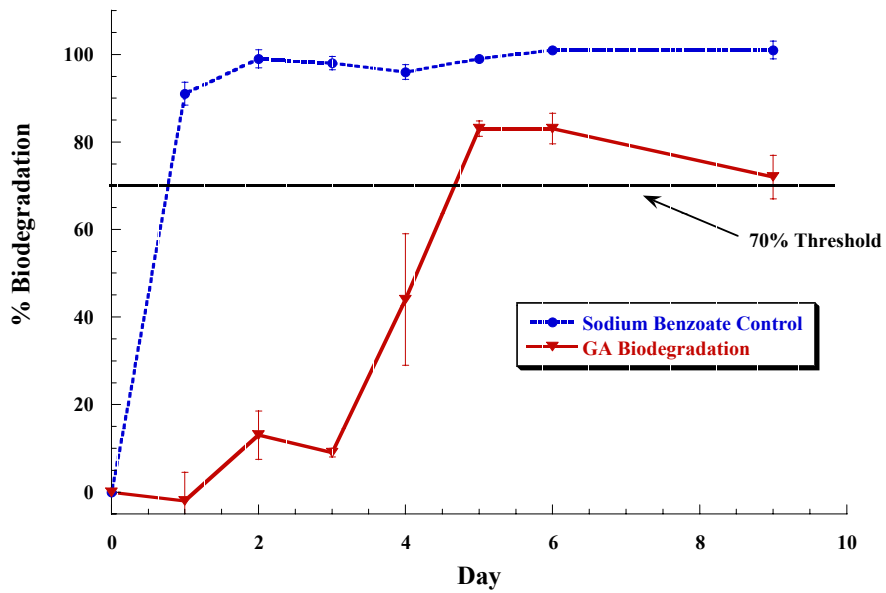


Figure 4. Stability of 3 and 5 mg/L GA in the OECD 306 Test Medium with and without 5 mg/L Ammonium Chloride

