Quantification and Degradation of 2,2-Dibromo-3-Nitrilopropionamide (DBNPA) in Bioethanol Fermentation Coproducts

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Research Article

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Abstract

2,2-Dibromo-3-Nitrilopropionamide (DBNPA) has been used as a biocide in industrial water applications due to its instantaneous antimicrobial activity and rapid chemical breakdown. In this study DBNPA is considered as a potential alternative for antibiotics used for bacterial control during corn to ethanol fermentation. A method using LC/MS/MS was developed to accurately quantify DBNPA in water. When this method was applied to quantify DBNPA concentration in a fermentation matrix, DBNPA was found to be unstable and to decay rapidly, preventing validation of the method or quantitation. This method was then used to evaluate the degradation rate of DBNPA in whole stillage, which is the nonvolatile residue produced by removal of ethanol from corn-based fermentation beer by distillation through the relative decrease in measured signal. In addition, a method was developed and validated to quantify bromide, one of the degradation products of DBNPA, in whole stillage using LC/MS/MS. The degradation rate of DBNPA in whole stillage was found to display first order kinetics with a calculated half-life of 85 min. Laboratory analytical chemistry results on DBNPA degradation were confirmed in field trials.

Introduction

Fuel-ethanol production in the U.S. has grown from less than 2 billion gallons per year in 2000 to 15.8 billion gallons in 2019.¹⁰ A major byproduct of ethanol production from corn is distiller's dried grains with solubles (DDGS). DDGS is used primarily as animal feed and consists of the nonfermentable components of the corn kernel (*e.g.*, protein, oil, and fiber), unconverted starch, and nonvolatile fermentation products (*e.g.*, yeast biomass, glycerol).²⁴ During corn fermentation, bacterial contamination and spoilage of product are common problems since chemical and biological processing are not performed under aseptic conditions.²⁶ To prevent and treat bacterial infections, antibiotics, such as virginiamycin and penicillin, are often added to the fermentations.^{2,16,27} Recently, the FDA has become more concerned about antibiotic residues in DDGS,¹² and as a result, interest in alternative strategies for controlling bacterial infections has increased in the ethanol industry. One such strategy is to apply a biocide such as DBNPA.^{29,30,31}

2,2-Dibromo-3-nitrilopropionamide (DBNPA) is a brominated acetamide¹⁵ originally applied as a seed fungicide.²² More recently, DBNPA has been used effectively to limit bacterial growth in different water applications, such as cooling water and paper processing.^{5,17,25} In these applications, favorable characteristics of DBNPA included instantaneous antimicrobial activity and rapid chemical breakdown into relatively non-toxic byproducts.^{11,29} These characteristics would also be beneficial in the ethanol industry,^{29,30,31,32} but the behavior of DBNPA has not been studied in this application. In particular, DBNPA decomposition in water treatment applications is dominated by two reaction pathways: pH dependent hydrolysis, and light-catalyzed reaction with reducing nucleophiles.¹¹ However, the relevance of these pathways in ethanol processes and operations is not known.

Whole stillage is comprised of nonvolatile residues produced by removal of ethanol from corn-based fermentation beer by distillation. It typically contains 95% water and 5% residual material from corn fermentation. This includes fermentation byproducts, residual fermentable sugars, and nonfermentable components of corn, such as protein, triglycerides and free fatty acids, and corn fiber. Because whole stillage is a critical intermediate product of the operations that eventually result in production of DDGS, it was selected as the sample matrix for this study. Previous methods have utilized HPLC with UV detection to quantitate DBNPA in wastewater¹¹, but interference from other components of whole stillage precluded the use of this method in this matrix. Similar interference issues were encountered when conductivity detection was used to attempt to quantify bromide, a major decomposition product of DBNPA, in whole stillage. To achieve the analytical goal of this study to measure DBNPA degradation in fermentation medium, we developed and validated the quantification methods for DBNPA as well as bromide using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Subsequently, we introduced DBNPA to the fermentation process in field trials and measured the presence or absence of this biocide downstream in the corn-to-ethanol process by bioluminescence.

Materials And Methods

Chemical Standards and Reagents DBNPA was purchased from Sigma Aldrich (St. Louis, MO, USA) at 96% purity. Field trials used 20% active ingredient dissolved in propylene glycol as manufactured by DOW Chemical Co. (Midland, MI.) LC/MS grade methanol, formic acid, ammonium acetate, sodium bromide, and N,N-1,4-diethyl-*p*-phenylenediamine (DPD) were also purchased from Sigma Aldrich. Purified (DI) water, with a measured resistance of 18.2 MΩ, was produced using a Millipore Synergy 185 (Millipore, Billerica, MA, USA). High-purity nitrogen, which was used as the nebulizing, sheath, and collision gas, was provided by a nitrogen generator (Peak Scientific, Billerica, MA, USA).

Microorganisms Frozen cultures of the bioluminescent bacterium, *Allivibrio fischeri*, were thawed in a water bath at 15.0±0.5°C., hydrated, adjusted osmotically to 2% NaCl with 22% NaCl in ultrapure water, mixed aseptically, and utilized immediately for tests on the Microtox 500 Analyzer, according to the instructions of the manufacturer (Azur Environmental, Carlsbad, CA). The photometric reading of the light production by *A. fischeri* was measured in the Microtox 500 ($\lambda = 490$ nm) and immediately recorded. The light output radiated by this bacterium is known as an indicator that is directly proportional to bacterial cell metabolic activity.²¹

Whole stillage supernatant For laboratory studies a whole stillage sample was chosen from archived samples that were generated during a pilot plant trial that used typical conditions for production of fuel ethanol and DDGS from corn. The pilot plant trial was performed at NCERC, and the sample was stored frozen at -20° C until it was used in this study. Whole stillage supernatant was prepared by centrifuging approximately 50 ml of whole stillage at 22° C at 3000 rpm for 5 minutes. The supernatant was filtered through a 0.45-μm syringe filter to remove colloidal suspended solids. The pH of the whole stillage sample was 4.6. (Field samples of whole stillage are covered below.)

DBNPA Identification and Quantitation DBNPA was analyzed in a methanol/water matrix with a Shimadzu SPD 20 HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of an autosampler, a degasser, two dual head pumps, and a thermostated column oven set at 40° C connected to a triple quadrupole mass spectrometer (3200 QTrap, AB Sciex) equipped with an ESI turbo ion source. A binary solvent system was used: solvent A was 0.1% (w/v) formic acid in water and solvent B was 0.1% (w/v) formic acid in methanol. An Inertsil ODS-4 C18 column (6 mm x 250 mm, 5 µm; GL sciences, Torrance, CA, USA) and a guard column (7.1 mm x 2.1 mm) were used for the chromatographic separation. The chromatographic separation was performed at a flow rate of 0.9 mL min⁻¹ using a gradient elution program. (T=0 min, A:95% B:5%; T=10 min, A: 75% B: 25%; T=10-17 min A:75% B:25%; T=17-21 min A:60% B:40%) The mass spectrometer was operated at negative mode under software control (*Analyst*, version 1.5, AB Sciex). DBNPA was detected using the 238.8, 240.8, 242.8 m/z ions, corresponding to [M-H]⁻ ion of DBNPA with various bromide isotopes.

The method was validated by evaluating linearity, accuracy, and precision. Five external calibration standards were prepared through dilutions of a 200 ppm DBNPA stock solution prepared by dissolving an appropriate amount of DBNPA in 50/50 methanol/water mixture using a volumetric flask producing the following concentrations: 25 ppm, 20 ppm, 10 ppm, 5 ppm, 2 ppm. The correlation coefficient, r^2 , (or coefficient of determination) between the detector response and the concentrations of the external standards was 0.998. Due to the rapid degradation of DBNPA in whole stillage, the method could not be validated in this medium. Instead, the method was validated using a water/methanol matrix, in which DBNPA is relatively stable. The accuracy of the method was determined from the recovery of DBNPA in a series of spiked samples. Aliquots were made from the 200 ppm DBNPA stock solution in the following amounts: 500 µl, 100 µl, 50 µl, and 25 µl. The aliquots were then diluted with 50/50 methanol/water creating spiked samples in the following concentrations: 100 ppm, 20 ppm, 10 ppm, and 5 ppm. In the case of spiked samples which were spiked with a concentration above the calibration range, the sample was diluted by a factor of 10 before submission for analysis. (See Table 1.)

Table 1
LC/MS/MS results from aliquots made from 200 ppm stock DBNPA solution and then diluted in 50/50 methanol/water. (See Materials and Methods.) Validation results of DBNPA method for both accuracy and precision. Accuracy results are reported as a percent of the spike value detected in the spiked sample. Precision results are reported as the relative standard deviation of 5 injections from the same sample.

Accuracy		Precision		
Sample ID	Recovery (%)		n	RSD (%)
100 ppm	93.2	5 ppm	5	2.56
20 ppm	103.4	60 ppm	5	0.89
5 ppm	104.2			
2 ppm	98.3			

Bromide Identification and Quantitation Bromide concentration in whole stillage was determined with the same chromatograph and detection instruments as were used for DBNPA. Before instrumental analysis, the whole stillage sample was cleaned using a solid phase extraction procedure. The SPE cartridge (C18, Agilent, Santa Clara, CA, USA) was conditioned with methanol, then 50/50 methanol/water, and the whole stillage sample was filtered through the cartridge and the eluent was collected for direct injection on LC/MS/MS. A Dionex RFIC Ionpac AS22 (4 mm x 250 mm) ion chromatography column was used with 0.2% (w/v) ammonium acetate in water solution as the mobile phase. Identification and detection of analytes was performed by a triple quadrupole mass spectrometer (3200 QTrap LC/MS/MS, AB Sciex) equipped with an APCI ion source operated at negative mode with the mass transition of 79/79.

The method was validated by evaluating its linearity, accuracy, and precision. Five external calibration standards were prepared at concentrations of 12.4, 5.0, 2.5, 1.2, and 0.5 ppm of bromide ion. The correlation coefficient (or coefficient of determination) of the external calibration curve was 0.999. Spiked samples were prepared to test the accuracy of the method by spiking an aliquot of concentrated sodium bromide into whole stillage supernatant. Spikes were prepared in the following concentrations: 6.8, 4.3, and 1.8 ppm bromide ion. Recovery for each spiked whole stillage sample was determined. Instrument repeatability was evaluated based on five injections of the 1.2 ppm standard and 4.2 ppm spiked sample. Relative standard deviations are shown in Table 2 for both sample types.

Table 2

Validation results of bromide method for both accuracy and precision. Accuracy results are reported as a percent of the spike value detected in the spiked sample. Precision results are reported as the relative standard deviation of 5 injections from the same sample. See ==

Accuracy		Precision		
Sample ID	Recovery (%)		n	RSD (%)
6.8 ppm	104.4	1.2 ppm	5	2.144
4.3 ppm	108.3	4.3 ppm	5	3.905
1.8 ppm	99.1			

DBNPA Degradation Time Trials A whole stillage supernatant sample was spiked with DBNPA to make a 40 ppm DBNPA sample for the degradation study. The sample was kept at 15°C in the dark within the autosampler between injections due to the photosensitivity of DBNPA. The sample was analyzed using the LC/MS/MS method developed and validated for the DBNPA in methanol/water.

A duplicate sample of whole stillage supernatant was spiked with DBNPA to an initial concentration of 40 ppm DBNPA for the degradation study. Instead of determining DBNPA concentration, bromide concentration was monitored. The method was modified to remove the SPE pretreatment phase due to the time requirements of the experiment.

Field trials Field sites in four states were surveyed in order to run proprietary trials. Ethanol plants were sought specifically where personnel had experience in working with biocide techniques (as opposed to non-biocide, clean-in place technologies) but also where personnel were not bound by contract or were not given exclusivity rights to test uncharacterized chemicals or biochemicals from another company or agency. Two corn-to-ethanol plants agreed to carry out a confidential trial. Before proceeding, they cleaned their equipment with permeate water from their reverse osmosis system. Absence of chlorine in the water was assured by using standard DPD testing in a HACH DR5000^{TM} spectrophotometer. DBNPA was introduced into the fermenters at 200 ppmv small quantity dosages as product (20% active).²⁹ Temperature of the first fermenter was 52-53 °C., 4.6±0.1, for 42 h; the second plant maintained its fermentation at 55 °C., 4.5±0.1, for 46 h. In both fermentation trials, field samples were drawn by experienced plant personnel and dispensed into opaque, covered containers in order to avoid photodegradation of DBNPA. Samples were pre-filtered through Whatman No. 4 filters, and then centrifuged and filtered as described above. The presence of DBNPA was determined by its effect on the bioluminescence radiated by *A. fischeri* culture, as measured in the Microtox 500 Analyzer,²¹ and results from the trial samples were compared with the control samples.

Results

Degradation of DBNPA was determined using LC/MS/MS. Table 1 lists the recovery for each spiked sample. Instrument repeatability was determined from five injections out of a standard (5 ppm) and spiked sample (60 ppm). For each, the relative standard deviation based on peak area was calculated for the set of runs and the results are shown in Table 1. The limit of quantitation was not determined for this method.

Table 2 lists the recovery for each spiked whole stillage sample. Instrument repeatability was determined from five injections of the 1.2 ppm standard and 4.2 ppm spiked sample. Relative standard deviations are shown in Table 2 for both sample types.

Both developed analytical methods displayed a high degree of accuracy and reproducibility during validation. For both methods, recovery of the spikes at all levels was within 90-110% recovery of the spike. The RSD for both methods, at both high and low analyte concentration, was below 5%. The results of the validation are shown in Tables 1 and 2 for DBNPA and bromide respectively.

Figure 1 shows the change in relative DBNPA signal area as a function of time in whole stillage. The relative signal area of DBNPA decreased to approximately 20% of its initial value when incubated for 3 hours at 15°C in the dark, and the rate appeared to be first order in DBNPA concentration. The best-fit degradation rate coefficient (0.0081 min^{-1}) corresponds to a half-life of about 85 minutes. Results in Figure 2 indicate that, even though DBNPA degradation started immediately, bromide was only produced after a delay of about 150 minutes. After this relatively long lag period, bromide was rapidly released as a free ion.

Field trial results In each field trial DBNPA was introduced as 200 ppmv in each fermenter. After completion of fermentation, samples were drawn from whole stillage and were prepared for analysis using the Microtox 500 instrument (See Methods.). Bioluminescence measurements were used to determine the presence of DBNPA in the stillage and compared with laboratory controls. Exposure to a microbicide such as DBNPA causes a change (decrease) in luminescence which is a byproduct of cellular respiration of the photoluminescent bacteria. Change in respiration directly relates to the toxicity of the biocide and to the inhibition of *A. fischeri* bioluminescence.²¹ The results of two field trials at corn-to-ethanol plants are provided in Figure 3. Laboratory samples with and without DBNPA under ambient conditions were tested at 60 min, and 0 min and 60 min, respectively. Samples from whole stillage that were treated with DBNPA were tested at the end of the fermentation run. The results of the field samples were compared with those of the laboratory samples. The results in Figure 3 clearly indicate that the bioluminescence radiated by *A. fischeri* is strong for the negative controls before (1a) and after (2b) the field tests. In contrast, the bioluminescence is weak in the presence of 200 ppm DBNPA treated laboratory samples (1b), indicating that the DBNPA was present and had a strong effect in killing the *A. fischeri*.
Whole

stillage sample results consistently indicate strong bioluminescence indicating that biocidal activity of the DBNPA waned. Lack of biocidal activity in samples of whole stillage are almost identical to the

negative controls, meaning the DBNPA has degraded (95% degradation in trial #1; 98% in trial #2). The effect of DBNPA on fresh samples was tested in duplicate microbiologically to determine its efficacy at 0 to 60 min, but the results verifying biocidal efficacy and lack thereof are not shown.

Discussion

Chemically, two well-characterized degradation pathways have been described for DBNPA: pH dependent hydrolysis and light-catalyzed reaction with nucleophiles.⁵ However, in the case of this study, neither of these mechanisms seem likely. Because the sample was kept in the dark during the degradation study, the light-catalyzed reaction cannot occur. The pH-dependent degradation mechanism is rapid at pH >7; however, pH of the whole stillage used in this study was 4.6 where literature indicates DBNPA is relatively stable. Also, the observed degradation rate at this pH was much faster than would be predicted based on the previously reported rate coefficient.¹¹ Instead, DBNPA may have reacted with nucleophilic compounds in whole stillage such as sulfhydryl groups derived from corn protein or yeast metabolic byproducts. Physiologically it is in fact known that DBNPA attacks sulfhydryl groups of bacterial cell proteins, rendering them inactive.⁴ The lag between DBNPA disappearance and bromide appearance suggests that one or more intermediate products accumulate during the degradation mechanism followed by a degradation step which releases bromide ion, but more work needs to be done to define the chemical pathways.

Based on the analytical chemistry results in Figures 1 and 2, we conclude that DBNPA decayed rapidly in whole stillage that is typical of the material made in the production of fuel ethanol from corn. The DBNPA was not persistent. Using LC/MS/MS, the degradation rate of DBNPA in whole stillage was found to display first order kinetics with a calculated half-life of 85 min. During decomposition, an intermediate product forms and, ultimately, decays to free bromide and other (unidentified) compounds by an unknown reaction mechanism. The measured decay rate of DBNPA ignored the effects of sunlight, a major route of rapid decomposition of DBNPA. In addition, whereas in whole stillage at the pH (4.6) where DBNPA is expected to be relatively stable and does not undergo rapid degradation through a mechanism of hydrolysis, photolytic and hydrolytic effects may increase the rate of DBNPA decomposition in a manufacturing environment. Based on the compelling results of the analytical chemistry studies of DBNPA degradation, then, we pursued further testing in corn-to-ethanol processing plants.

To carry out these field studies, we applied experiences that we gained in testing by bioluminescence the degradation of brominated biocides such as 2,2-dibromo-3-nitrilo-propionamide and 2-bromo-2-nitro-propane-1,3-diol in cooling water and paper processing industries. These tests were done by investigating the cellular toxicity of their residuals on *Aliivibrio fischeri* by measuring the change in bioluminescence spectrophotometrically. Likewise, in this study we conducted field trials on DBNPA persistence in corn-to-ethanol processing using bioluminescence of *A. fischeri* as our photometric indicator determined by a Microtox 500 Analyzer, which determines toxicity as a sensitive test.^{9,20,23}

Based on the results in Figure 3, the effect of DBNPA in whole stillage samples was not detected. In light of experience of the effect of biocides on *A. fischeri* bioluminescence we can state that DBNPA was indeed degraded. These field study results measuring DBNPA loss of activity by bioluminescence also correspond well with those of other process water studies. For example, in previous work, DBNPA added to paper machines was found 99-100% degraded in whitewaters prior to their effluent being discharged into freshwater (Wiatr and Burns, unpublished data). Agreement of DBNPA degradation in laboratory and field studies confirm that the application of DBNPA in fermentation can be done without fears that the biocide would carry over into and contaminate DDGS.

Discoveries of the efficacy of DBNPA^{29,30,31,32} against bacteria coupled with the DBNPA degradation found early in the corn-to-ethanol process, as provided by the evidence in this paper, can allow this biocide to replace antibiotics in the in the corn-to-ethanol biofuel industry. That is of significant value. Not only do these results indicate that DBNPA can be used to protect against bacterial infection of the corn-to-ethanol process, saving on costs of raw materials, finished products, and post bacterial infection clean-outs, but they also suggest that use of this biocide can help prevent antibiotic resistance.

Overuse, underdosage, and other misapplications of antibiotics are known to increase antibiotic resistance by bacteria in the field, regardless of the concentration of antibiotics.^{8,14,28} Moreover, antibiotic resistance is known to be more likely when bacterial biofilms develop, such as those formed on equipment surfaces.^{1,7,18} Repeated use of antibiotics in animal feed translates to microorganisms being exposed to sub-lethal doses of and developing resistance to these drugs. This is a long-time concern, that antibacterial resistance originates in farm animals¹⁹ and continues, particularly when the antimicrobials are present at subtherapeutic levels¹³ in the food chain.

Moreover, residual antibiotics in meat in the food chain were previously found to disrupt its fermentation and also increase risk of infection, making pathogens less susceptible medically to treatment with antibiotics.¹⁸ It appears that antibiotics found at low concentrations at the end of the ethanol process³ can likewise cause high levels of antimicrobial resistance.^{6,33} Application of an effective biocide instead of an antibiotic to control bacteria in the ethanol process, however, represents a significant advance to the field because, as demonstrated in this paper, DBNPA breaks down prior to the end of the process and thus cannot enter DDGS. This means application of DBNPA can circumvent the bacterial antibiotic resistance problem of FDA concern,¹² making it a successful alternative to antibiotics. Then the agricultural use of DDGS in feed represents a safer practice because DBNPA would be degraded and thereby would not be present to induce antibiotic resistance in bacteria in bovine, swine, and poultry food applications in the food chain.

Declarations

Competing interests.

This work on 2,2-dibromo-3-nitrilopropionamide degradation is primarily academic.

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References

- 1 Abebe, G. M. (2020) The Role of Bacterial Biofilm in Antibiotic Resistance and Food Contamination. International J. Microbiol. <https://doi.org/10.1155/2020/1705814>
- 2 Aquarone, E. (1960) Penicillin and tetracycline as contamination control agents in alcoholic fermentation of sugarcane molasses. Appl. Microbiol 8, 263-8.
- 3 Bischoff, K.M., Zhang, Y., Rich, R. O. (2016) Fate of virginiamycin through fuel ethanol product process. World J. Microbial Biotechnol. 2:76. doi.10.1007/s11274-016-2026-3.
- 4 Chapman, J. S. (2003) Biocide Resistance Mechanisms. International Biodeterioration & Biodegradation. 51:2 133-138.
- 5 Chervenak, M. C.; Konst, G. B.; Schwingel, W. R. 2004) Non-traditional use of the biocide 2,2-dibromo-3-nitrilo-propionamide (DBNPA) in coatings manufacture. Proc. Annu. Meet. Program FSCT/82nd, 58/1-58/12
- 6 Ching, C. and Zaman, M.H. (2020) Development and selection of low level multi-drug resistance over an extended range of sub-inhibitory ciprofloxacin concentrations in *Escherichia coli*. Nature Research. <https://doi.org/10.1038/s41598-020-65602-z>
- 7 Donlan, R. M. (November-December, 2000) Role of Biofilms in Antimicrobial Resistance, ASAIO Journal: 46(6) S47-S52
- 8 Donlan, R. M. Biofilms: microbial life on surfaces. (2002) Emerg Infect Dis. 8(9):881-90. doi: 10.3201/eid0809.020063
- 9 Dutka, B.J. and Kwan, K.K. (1981) Comparison of three microbial toxicity screening tests with the Microtox test. Bull. Environ. Contam. Toxicol. 27 (6): 753-757.
- 10 Ethanol Fuel Organization.(accessed February 16, 2021) Historic U.S. fuel Ethanol Production. <http://www.ethanolrfa.org/pages/statistics/#A>
- 11 Exner, J. H; Burk, G. A.; Kyriacou, D. (1973) Rates and products of decomposition of 2,2-dibromo-3-nitrilopropionamide. J. Agric. Food Chem. 21, 838-42.
- 12 Food and Drug Administration. (December 21, 2010) The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals. Draft Guidance #209

- 13 Franco, D.A., Webb, J, amd Taylor, C.E.(1990) Antibiotic and sulfonamide residues in meat: implications for human health. J. of Food Protection. 53: 178-185.
- 14 Hawkey, P. M. (1998) The origins and molecular basis of antibiotic resistance. *BMJ*. 317(7159):657-660. doi:10.1136/bmj.317.7159.657
- 15 Hesse, B. C. (1896) On malonnitrile and some its derivatives. [machine translation]. Amer. Chem. J. **1896**, 18, 723-751.
- 16 Hynes, S. H.; Kjarsgaard, D. M.; Thomas, K. C.; Ingledew, (1997) W. M. Use of virginiamycin to control the growth of lactic acid bacteria during alcohol fermentation. J. Ind. Microbiol. Biotechnol. 18, 284-291.
- 17 Kiuru, J.; Tsitko, I.; Sievanen, J.; Wathen, R. (2010) Optimization of biocide strategies on fine paper machines. BioResources. 5, 514-524.
- 18 Kjeldaard, J., Cohn, M.T., Casey, P.G., Hill, C, and Ingmer, H. (2012). Residual antibiotics disrupt meat fermentation. 50: 616-620ion and increase risk of infection. mBio 3(5). doi:10.1128/mBio.00190-1
- 19 Levy, S. B. (1987.)Antibiotic use for growth promotion in animals:ecologic and public health consequences. J. Food Protection 626-620.
- 20 McFeters, G.A.; Bond, P.J.; Olson, S.B.; Chan, Y.T. (1983) A comparison of microbial assays for the detection of aquatic toxicants. Water Research. 17(2): 1757-1762.
- 21 Microtox 500: Industry-leading toxicity testing. N.D. (Retrieved May 28, 2014).
- 22 Nolan, H. K.; Hechenbleikner, I.(1947) Seed and Plant Disinfectants. U.S. Patent 2,419,888,
- 23 Riva, M.C.; Ribo, J.; Gilbert, G.; Alanon, P. (2007) Acute toxicity of leather processing effluents on *Vibrio cholera* and *Brachydanio rerio*. Afinidad. 528: 182-188.
- 24 Rosentrater, K.A. (2006) Some physical properties of distillers dried grains with solubles (DDGS). Appl. Eng. Agric. 22: 589-595.
- 25 Skinner, K. A.; Leathers, T. D. (2004) Bacterial contaminants of fuel ethanol production. J. Ind. Microbiol. Biotechnol. 31, 401-408.
- 26 Stroppa, C.T., Andrietta, M.G., Andrietta, S.R., Steckelberg, C., and Serra, G.E. (2000) Use of penicillin and monensin to control bacterial contamination of Brazilian alcohol fermentations. Int. Sugar J. 102: 78-79, 81-82.
- 28 Wiatr, C.L.(2016) Bacterial resistance to antimicrobials in a cooling water system, Part 1. The Analyst. 23:11-16.

- 29 Wiatr, C. L., Corcoran, M.L., McNeel, T.E., Clark, R. A., Bortoto Porto, R. DeCassia, and Oppong, D. (22September 2011) Processes Using Antibiotic Alternatives in Bioethanol Production. PCT Int. Appl. WO 2011116042. 1-64.
- 30 Wiatr, C. L., Corcoran, M. L., McNeel, T.E., Clark, R. A., Bortoto Porto, R. DeCassia, Oppong, D., and Hoekstra, P. (April 30, 2015.) Processes Using Antibiotic Alternatives in Bioethanol Production. Patent 20150118726.
- 31 Wiatr, C.L., Corcoran, M. L , McNeel, T. E., B. Porto, R. DeCassia., Oppong, D., Hoekstra, P. (June 6, 2018) Processes using antibiotic alternatives in bioethanol production. Patent EP2 547 777 B1.
- 32 Wiatr, C.L., Corcoran, M. L., McNeel, T.E., B. Porto, R DeCassia., Oppong, D., Hoekstra, P (September 18, 2018). Processes using *in situ* generated antibiotic alternatives in bioethanol fermentation. CA Patent 2,793,614.
- 33 Wistrand-Yuen, E., M. Knopp, K. Hjort, Koskiniemi, M., Berg, O., Anderson, D. (2018) Evolution of high-level resistance during low-level antibiotic exposure. Nat. Commun. 9: 1599. doi:10.1038/s41467-018-04.
- 34 Wolf, P. A. and Sterner, P. W. (1972) 2,2-Dibromo-3-Nitrilopropionamide, a Compound with Slimicidal Activity. Appl. Environ. Microbiol. 24: 581-584.

Figures

Figure 1

Decay of DBNPA in whole stillage. Each data point is a replicate injection from the same spike whole stillage sample.

Figure 2

Change in the concentration of bromide in whole stillage detected as a function of time. Each data point is a replicate injection from the same whole stillage sample spiked with DBNPA.

Figure 3

2,2-dibromo-3-nitrilo propionamide (DBNPA) was dosed as 200 ppmv of Bronam 20 in laboratory samples and plant fermentation media. Bioluminescent bacteria, *Aliivibrio fischeri*, were tested at 0 min to obtain a negative control reading (Control 1a) under ambient conditions. Samples that were exposed to DBNPA for 60 min in the in the laboratory yielded a decrease in bioluminescence (Control 1b) whereas readings of control samples remained the same. Similarly, after the fermentation process was completed in the plant, whole stillage was harvested, and tested. The results of DBNPA degradation in whole stillage were compared with the controls. Bar 2b represents a check of viability of fresh laboratory cultures prior to testing the whole stillage samples.

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