Aequor, Inc. – Antibiofilm/Antifouling Platform

Bacteria adhere to surfaces and form a slimy layer known as biofilm. Biofilms cause microfouling (corrosion, contamination) and macrofouling (adhesion of eukaryotic organisms) that result in operational inefficiencies and higher labor, materials, and fuel costs. On human tissues, according to the CDC, biofilms are associated with over 80% of all chronic wound infections. Aequor, Inc. has developed platform technologies to generate novel chemical entities to prevent biofilm formation and fouling and remove existing biofilm by 99.99%. The Company's portfolio of new chemical agents represents a new technology in the four trillion dollar chemical megasector.

The Problem: Biofilm

Biofilm is an impenetrable matrix formed by microorganisms and bacteria as they colonize. Biofilm is associated with chronic bacterial infections on human, plant and animal tissues, and the adhesion of "foulers" on most industrial surfaces, particularly those in contact with fresh or salt water. Industrial "microfoulers" (bacteria, slimes, etc.) cause corrosion, contamination, and operational inefficiencies, including increased fuel consumption and CO_2 emissions. Biofilms also facilitate the adhesion of eukaryotic organisms, or "macrofoulers" (algae, barnacles, mussels, etc.). In aggregate, the recurring costs to combat biofilm and fouling in industry, agriculture, healthcare, and consumer sectors are estimated to exceed hundreds of billions annually.

Once established on a surface, bacteria relentlessly form biofilm. Manual or mechanical scraping is still considered the most effective ways to penetrate biofilm in order to remove it. Treatments with heat and chemicals (biocides, germicides, disinfectants, antiseptics) kill the underlying bacteria but cannot stop the reformation of biofilms over time. Recent studies show that chemicals in biofilm's upper layers can neutralize even the harshest disinfectants and treatments used to kill bacteria.

As known from dental plaque (a biofilm), brushing and even the strongest mouth rinses are ineffective. Regular professional scraping is effective -- but only temporarily. In the healthcare setting, biofilm is not affected by most antibiotics, heat, or the human immune system. Biofilm-forming bacteria (e.g., *Staphylococcus)* eventually form resistance to antibiotics (MRSA). Where biofilm removal is not possible or medically desirable, chronic infections can develop into diseases considered incurable (cystic fibrosis, endocarditis).

Aequor's Discoveries

Aequor's Founder, Cynthia Burzell, hypothesized that there was a natural mechanism in the marine environment that impeded the adhesion of "macrofoulers" (barnacles, mussels and algae) to surfaces. Otherwise, living surfaces would be covered with foulers.

She isolated marine bacteria from fouler-free living surfaces in the Caribbean Sea and discovered 17 novel bacteria including a new genus and several new species. These novel marine bacteria impeded the *in situ* attachment of foulers (barnacle larvae, microalgae, marine bacteria). Biofilm is the basis upon which "microfoulers" (contaminants, corroders, slimes) and macrofoulers attach to surfaces. She further extracted from these microbes new chemical compounds that effectively impeded the formation of medically relevant biofilm (S*taphylococcus aureus*, *S. epidermidis*, *Pseudomoans aeruginoa*) and oral flora biofilm (*Streptococcus mutans* and *S. gordonii*).

Aequor, Inc. is an early-stage for-profit company whose mission is to develop non-toxic solutions to biofilm infections and marine fouling. Eight years of in-house testing indicate that the bacteria produce novel non-toxic agents that prevent biofilm formation and fouling. The project builds on the theory that the surfaces of marine organisms would foul were they not protected by naturally occurring substances that are not toxic to the marine environment.

Aequor, Inc. has developed platform technologies to generate novel chemical entities to penetrate and impede biofilm formation. Aequor, Inc. has intellectual property protection on a set of novel, effective, natural chemical agents that appear to be easily incorporated in various delivery systems (e.g., washes, aerosols, creams, pastes, treatments, therapeutics, paints, coatings) formulated to combat biofilm. The Company's portfolio of new chemical agents represents a new technology in the four trillion dollar chemical megasector.

The following report describes Aequor's data and discoveries. Please note that polyphasic taxonomy was performed on the marine isolates to determine their novelty however, phylogenetic data is not disclosed at this stage.

Medical antibiofilm activity

Antibiofilm assays were performed to determine the inhibition/prevention of biofilm formation of human pathogens *Staphylococcus aureus* (ATCC 25923 and ATCC 12600), *S. epidermidis* (ATCC 12228), and/or *Pseudomonas aeruginosa* (ATCC 27853) using a standard 96-well microplate assay [Merritt et al., 2005]. Purified (80%) and semi-purified lead New Chemical Entity (NCE), as well as, crude lead NCE and supernatants from marine isolates E, F, and G and were tested. Biofilm assays were performed in triplicate unless otherwise indicated. As expected, antibiofilm activity increased as lead NCE was purified.

Semi-purified lead NCE inhibited 24-hour *P. aeruginosa* biofilm formation >99% ($p<0.001$). Purified (80%) lead NCE reduced *S. aureus* 48-hour biofilm formation an average of 95.86% ($p<0.00001$).

Confocal laser scanning microscopic images of 48-hour *S. aureus* biofilm in the presence of crude lead NCE confirm these results (Figure 1). Crude lead NCE antibiofilm activity is similar to the positive control, 1000 μg/ml penicillin G, a high dose, which is required to inhibit biofilm and is toxic to humans.

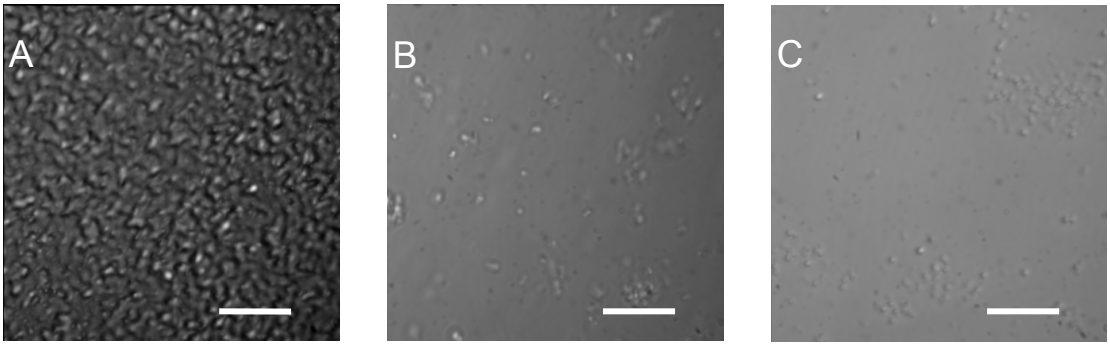

Figure 1. Confocal laser scanning microscopic images of crude lead NCE inhibiting 24-hour *S. aureus* biofilm. Negative control biofilm developed thick and tightly adhered (A). Crude lead NCE (B) and positive control (penicillin 1000 μg/ml) (C) developed small patches of thin loosely associated biofilms. Bar, 20μm.

In addition, *S. aureus* 48-hour biofilm formation was reduced 76.57% ($p<0.0001$) by crude lead NCE (Figure 2), 67.93% ($p<0.0001$) by crude isolate E supernatant, and 57.06% ($p<0.0001$) by crude isolate G supernatant. Testing was performed on four or more different occasions and results were reproducible.

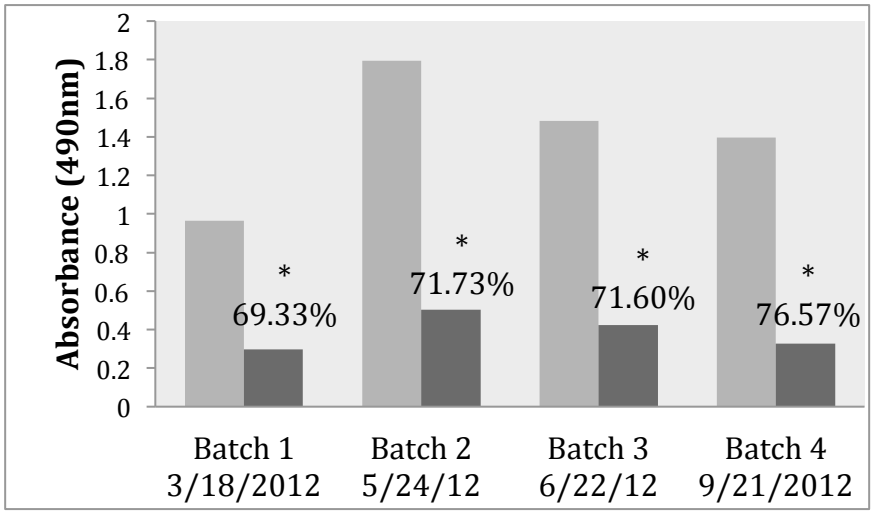

Figure 2. Reproducibility of crude lead NCE samples. Inhibition of *S. aureus* 48-hour biofilm formation by crude lead NCE on four different occasions in 2012. *, $p<0.001$.

Note: Crude and partially purified supernatants of the marine isolates were tested multiple times for the antibiofilm screen with fewer repeats for screening purposes and those tests produced similar results (data not shown).

Third Party Validation – Lonza Microbial

In addition to the **inhibition** of biofilm formation testing that Aequor has performed, Lonza Microbial performed antibiofilm activity testing of crude lead NCE against *S. aureus* to determine if crude lead NCE can **remove** pre-formed 24-hour biofilm. Lonza Microbial evaluated crude lead NCE and freeze-dried crude lead NCE at different concentrations for antibiofilm activity against *S. aureus* (ATCC 6358). The assay was performed in quadruplicate and followed the ASTM E2799 - 12 Method - Standard Test Method for Testing Disinfectant Efficacy against Biofilm using the MBEC Assay. The Biofilm was cultivated for 24hs in a rocking table at 35°C. After this, the samples were tested for 48-hour contact time and neutralized in Letheen broth. The evaluation was performed quantitatively by colony count of viable cells in TSA and qualitatively by Absorbance reading (650 nm).

Both crude lead NCE and freeze-dried crude samples removed 99.99% (4.4 log reduction) of 24-hour *S. aureus* biofilm formation after 48 hours of exposure according to the quantitative test (Figure 3). All counts were below detection limit of 1E+02 CFU/mL. In addition, both crude lead NCE and freeze-dried crude samples removed up to 62.5% of 24 hour *S. aureus* biofilm formation after 48 hours of contact time according to the quantitative test (Table 1).

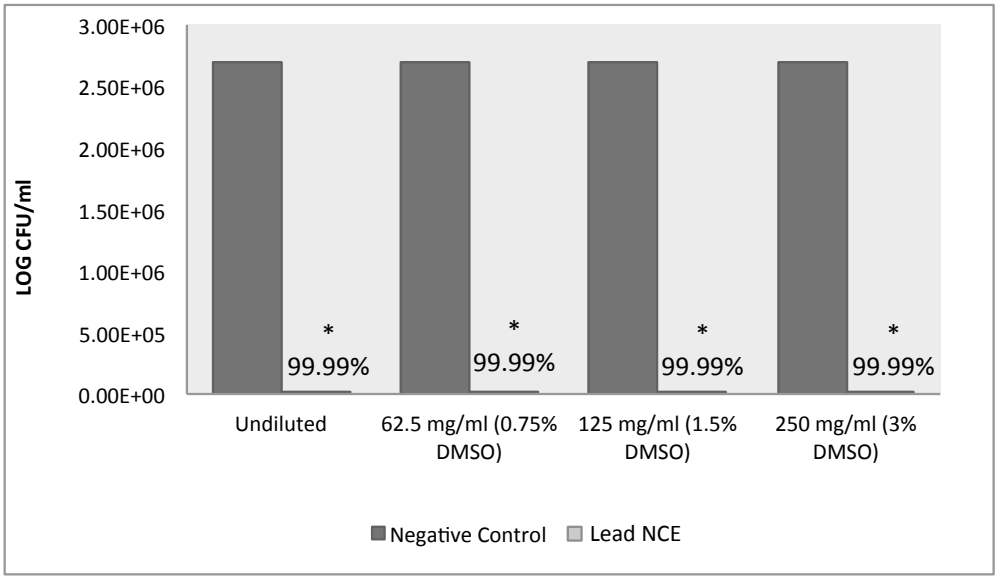

Figure 3. Removal (99.99%) of 24-hour pre-formed *S. aureus* biofilm by crude lead NCE undiluted and at different concentrations. *, $p<0.01$.

The antibiofilm results differ between quantitative and qualitative methods because the quantitative test method is an underestimation of the activity. Note: In the quantitative

test, the freeze-dried negative control media at 250mg/mL (3% DMSO) showed 2 logs reduction compared to negative control test. We believe that the 3% DMSO had an effect on the removal of pre-formed biofilm, however, once diluted to 1.5% the DMSO did not interfere with the assay. DMSO was used because of solubility. This effect was not observed in the qualitative test.

Table 1. Percentage of biofilm removal of 24-hour pre-formed *S. aureus* biofilm by crude lead NCE undiluted and at different concentrations. Results are based on average of quadruplicates at 650nm.

	% of Biofilm Removal
Freeze-dried Crude Lead NCE 250 mg/ml (3% DMSO)	62.5
Freeze-dried Crude Lead NCE 125 mg/ml (1.5 % DMSO)	58.8
Freeze-dried Crude Lead NCE 62.5 mg/ml (0.75% DMSO)	58.4
Undiluted Crude Lead NCE	61.1

Marine antibiofilm/antifouling activity

The *in situ* antibiofilm/antifouling activity was determined in the Caribbean Sea by enumerating the various natural organisms that attached to the test surface in the presence of the bacterial substances versus a negative control. An initial attachment assay incorporating samples in gels on glass slides, modified from Henrikson and Pawlik (1995), was used to screen bacterial isolates for the inhibition of 24-hour marine biofilm formation and fouling *in situ*. The slides were stained with Acridine Orange and observed under a UV microscope to determine if the attached organisms contained chlorophyll (i.e., photosynthetic). This allowed for the enumeration of photosynthetic eukaryotes (e.g., microalgae), non-photosynthetic eukaryotes (e.g., barnacle larvae), and marine bacteria.

Crude lead NCE reduced non-photosynthetic eukaryotic fouling (e.g., barnacle larvae) by 45.4% ($p<0.01$), isolate E reduced photosynthetic eukaryotic fouling (e.g., microalgae) 78.2% ($p<0.01$), and isolate F reduced marine bacterial biofilm formation 84.5% ($p<0.05$), compared to negative controls (Figure 4).

Purified (80%) lead NCE and crude supernatants from bacterial isolates E, F, and G were screened for antibiofilm activity against marine bacteria *Cobetia marina* (ATCC 25374) using a microplate assay [Merritt et al., 2005]. Biofilm assays were performed in duplicate. Purified (80%) lead NCE reduced *C. marina* 24-hour biofilm formation by 90.91% ($p<0.0001$). In addition, *C. marina* 24-hour biofilm formation was reduced 74.40% ($p<0.001$) by isolate E, 66.01% ($p<0.01$) by isolate F, and 45.48% ($p<0.01$) by isolate G.

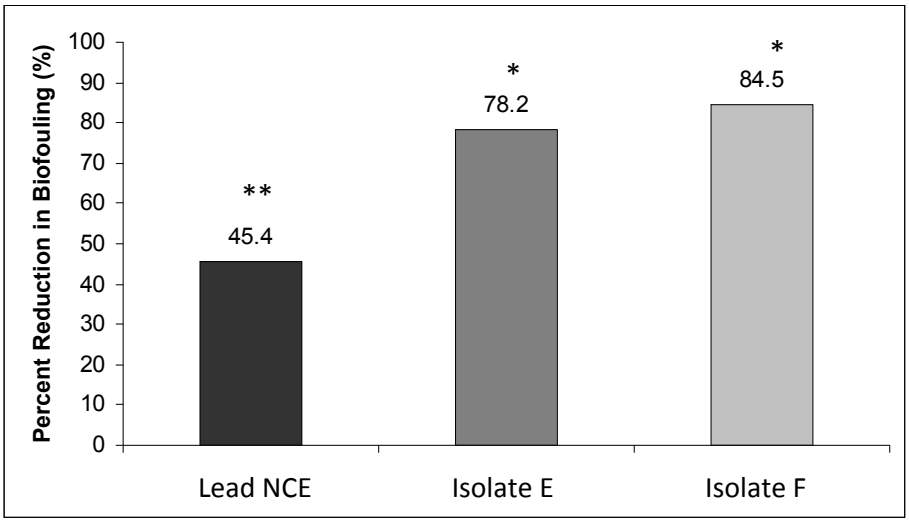

Figure 4. Inhibition of *in situ* marine fouling by Aequor's marine isolates after 24-hours deployed in gels in Caribbean Sea. Reductive effect of crude lead NCE on marine larvae, isolate E on microalgae, and isolate F on marine biofilm formation. *, $p < 0.05$; **, $p<0.01$.

Oral flora antibiofilm activity

Crude lead NCE was screened for antibiofilm activity against a mixed culture of oral bacteria *Streptococcus mutans* and *S. gordonii* using 24-hour microplate assay in duplicate [Merritt et al., 2005]. Crude lead NCE reduced *S. mutans* and *S. gordonii* biofilm an average of 51.43% ($p<0.0001$). This value is meaningful because it is likely to increase when chemical is purified.

Testing should be repeated to include additional oral flora including, *S. salivarius*, S. sanguinis, *Treponema denticola, Fusospirochetes, Veillonella, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans,* and some *Lactobacillus* species. Additional testing should be performed using oral care positive controls for comparison.

These tests indicate that the lead NCE and active agents from marine isolates E, F and G have the potential to significantly inhibit medically relevant biofilm, marine biofilm formation and fouling, as well as, oral biofilms.

Preliminary data in support of non-toxicity

The following very preliminary and simple toxicity tests were performed as the first step in toxicity testing. We recognize that further toxicity testing using animal models and eukaryotic cell lines will be required by the EPA and FDA, however, these initial results indicate a good start.

Genotoxicity was determined for crude lead NCE according to the Ames test [Ames *et al*., 1973] with three auxotrophic *Salmonella enterica* strains (previously *S. typhimurium*) (ATCC 29629, ATCC 29630, ATCC 29631). The results were considered positive if the number of revertants were at least twice as high as the negative control. Crude lead NCE reverted the *S. enterica* mutant strains the same as or less than the negative control (Table 2), therefore, the Ames test results were negative. Crude lead NCE was determined to be free from genotoxins according to the Ames test.

Table 2. Ames test results for crude lead NCE. SD, standard deviation.

Experimental condition	**Average number of revertants ± SD**		
	***S. enterica* ATCC 29629**	***S. enterica* ATCC 29630**	***S. enterica* ATCC 29631**
Positive Control	38 ± 4	101 ± 21	246 ± 77
Negative Control	5 ± 1	4 ± 1	10 ± 8
Crude Lead NCE	3 ± 2	4 ± 2	6 ± 4

Brine shrimp (*Artemia salina*) toxicity assay was performed in duplicate on crude lead NCE. Dilutions of crude lead NCE were incubated, in duplicate, with brine shrimp larvae in a total volume of 10 ml. Approximately ten brine shrimp larvae were placed in each solution and a mixture of artificial seawater was used as a negative control. The nauplii were examined after 24 and 48 hours of growth and the average number of survived larvae was recorded. The mean percentage mortality was plotted against the logarithm of concentrations. The concentration killing 50% of the larvae (LC_{50}) was determined from the graph. Crude lead NCE is non-toxic to brine shrimp with LC_{50} values >10,000 μg/ml.

These tests indicate that crude lead NCE is not toxic according to genotoxicity testing and brine shrimp toxicity assay.

Lead NCE: Isolation, Chemical Elucidation, and Novelty Validated

The lead NCE was isolated using activity guided fractionation. Supernatant from marine isolate D was fractionated and purified using High Performance Liquid Chormatography (HPLC) using C-18 Reverse Phase (RP)-CombiFlash and Phenomenex Gemini columns (data not shown). Fractions were tested for antibiofilm activity using *S. aureus* 48-hour biofilm assays (data not shown) (Figure 5).

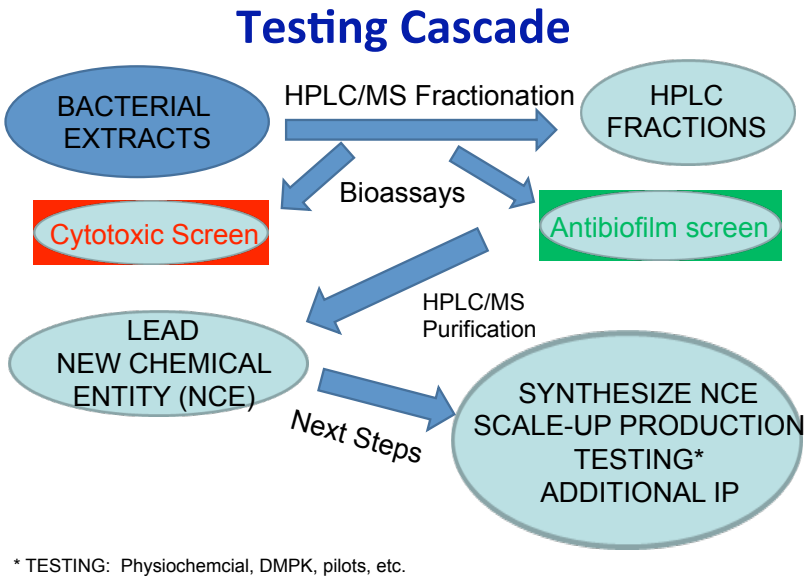

Figure 5. Testing cascade representing the steps completed: marine isolate screening, activity guided fractionation, chemical purification and elucidation of lead NCE, as well as, next steps: synthesis, scale-up, additional testing, and IP.

Structural elucidation was performed using a battery of Nuclear Magnetic Resonance (NMR) experiments [Proton NMR (1H-NMR), Carbon NMR (13C-NMR)] and 2D experiments [2D- Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Coherence (HSCQ), Heteronuclear Multiple Bond Correlation (HMBC)], combined with Mass Spectrometry (MS) (data not shown). Structural elucidation data was used to identify eight possible compounds that fit MS data molecular weight (data not shown).

Chemists at the Warner Babcock Institute for Green Chemistry (WBI) and Pacific BioLabs (PBL) performed searches to determine the novelty of the chemical. The MS data for the purified lead NCE was entered in the National Institute of Standards and Technology (NIST) standard reference database. The search resulted in no matches indicating that the active chemical was not known. In addition, our patent attorney at K&L Gates performed patent searches on the chemical that did not result in any known matches. Two Composition of Matter patents were filed on the most likely chemical structure and its biosimilars in 2012.

The initial isolation of the natural chemical generated a limited amount of purified sample that was used in these studies. Funding is needed to purify more natural chemical to confirm its structural elucidation and novelty.

These tests indicate that the lead NCE is novel according to NIST standard reference database and patent searches.

Patent Estate

Claim	**Filing Date**	**Priority Date**	**Status**
-Methods of Extraction	7/29/2013	4/30/2004	Pending
-Methods of Use	4/13/2012	10/14/2009	Pending
-Composition of Matter of 1 chemical compound and analogues	2/24/2012		Pending

K&L Gates manages Aequor's patent strategy. Our intent is not to let patents publish until additional patents have been filed on the composition of matter of other chemicals in the portfolio, methods, formulations, and on the genetically-modified source microbes, etc.