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Screening of Crude Microalgal Extracts for Antimicrobial Activity

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SCREENING OF CRUDE MICROALGAL EXTRACTS FOR ANTIMICROBIAL ACTIVITY

by

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SCREENING OF CRUDE MICROALGAL EXTRACTS FOR ANTIMICROBIAL ACTIVITY

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University of the Incarnate Word, 2019

Microbial biofilm associated infections are a significant threat to patients with medical devices and are partially responsible for the increased resistance seen in nosocomial infections. The biofilm chemical and physical properties restrict access of chemotherapeutic agents. Therefore, there is a need to enhance the antimicrobial effects of current chemotherapeutic agents. Microalgae live in competitive environments that include film-forming, opportunistic pathogens *S. maltophilia* and *C. albicans*. Extracts from 5 diverse species of microalgae were screened for antimicrobial enhancing effects against established biofilms with two different extraction solvents, 3:1 hexanes and isopropyl (HIPA) and 2-methyltetrahydrofuran (2-MTHF).

Three species, *B. braunii*, *C. danica*, and *N. oculata* showed statistically significant reduction in cell viability of *S. maltophilia* in an established biofilm ($p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). An extract of 2-MTHF, *N. oculata* [M], showed enhancing effects of ceftriaxone against *S. maltophilia* in an established biofilm. Furthermore, *B. braunii*, *C. danica*, and *N. oculata* extracts showed a statistically significant reduction in cell viability of *C. albicans* in combination with fluconazole ($p = 0.003$, $p = 0.002$, and $p = 0.021$, respectively). Based on our findings, additional research should focus on the organic components of *N. oculata*, *B. braunii*, *C. danica* that contribute to reduced microbial cell viability in established biofilms. Furthermore, data suggests that 2-MTHF is a viable solvent for future extraction processes.

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Background

Bacteria

Bacteria are an expansive group of microscopic prokaryotic organisms. They are found everywhere, including environments previously thought to uninhabitable.[1–3] Despite their immense genetic diversity, bacteria share common structural features (see Figure 1.0). One of the most studied and medically important structures is the bacterial cell wall. All bacteria species have a cell wall made of a peptidoglycan, a sugar and protein polymer. The cell wall is rigid to provide structural support, and has components that are vital to cell-to-cell communication.[4]

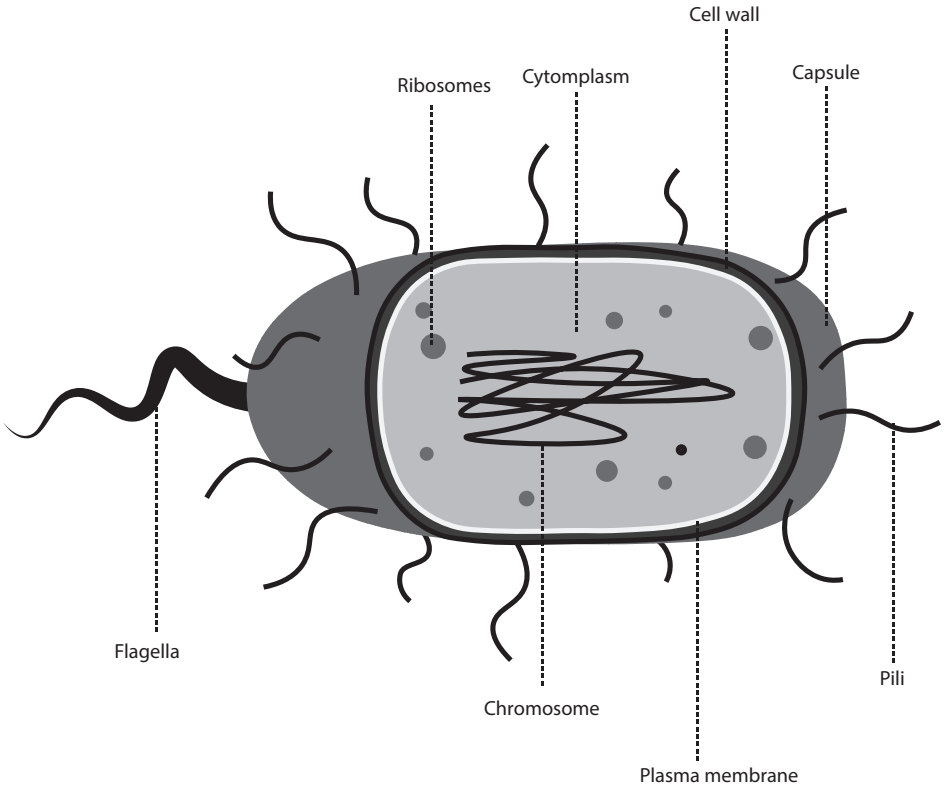

Figure 1. General bacterial cell and significant structures. Original image created with Adobe Illustrator.

Gram Negative. The broadest way to categorize bacterial species is based on their cell wall structure. The thickness of this cell wall helps categorize bacteria as gram-positive or gram-negative. A gram-positive bacterium has a thick peptidoglycan cell wall that easily retains crystal violet (see Figure 2a). Conversely, a gram-negative bacterium has a significantly thinner cell wall. In addition, gram-negative organisms have a lipopolysaccharide (LPS) outer membrane which covers the cell wall (see Figure 2b). The differences in cell wall structure are crucial in drug development, as[5–7] antibiotics target the cell wall[OBJ] must be transported through the cell wall.[5–7]

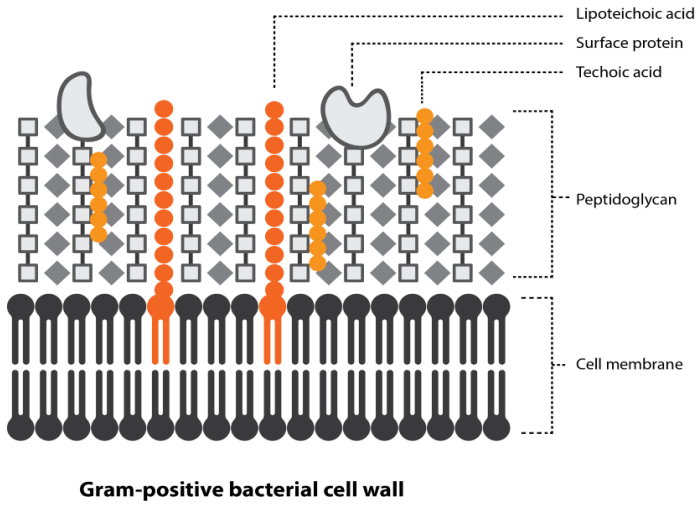

Figure 2. Gram-positive cell wall structure derived from Irving's Medical Microbiology.[4] Original image created with Adobe Illustrator.

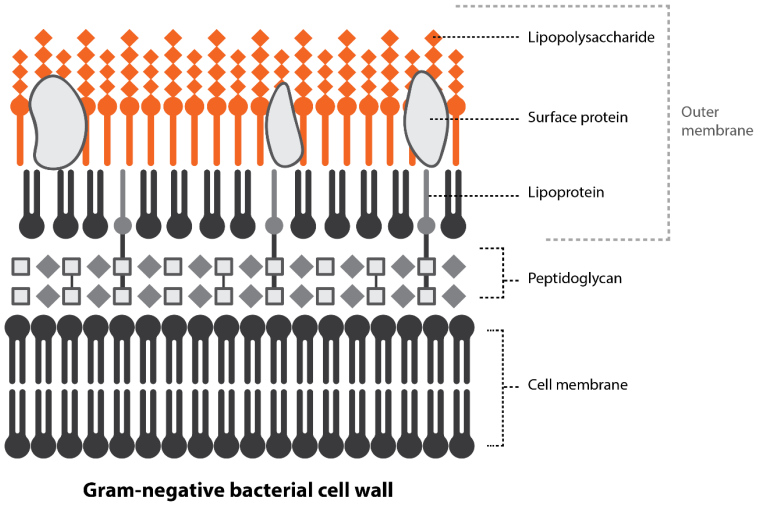

Figure 3. Gram-negative cell wall structure derived from Irving's *Medical Microbiology*.[4] Original image created with Adobe Illustrator.

Bacterial Resistance. As early as the 1940s, when penicillin was introduced to the market, there was already limited evidence of a reduced sensitivity to the beta-lactam over time.[8,9] These first signs of insensitivity to antibiotics foreshadowed the imminent rise of the modern resistance era. Yet, the long-term effects of resistance were largely dominated by our ability to find new antibiotics. However, in the 1960's drug discovery slowed greatly while resistance continued to accumulate (see Figure 3).[10,11] Now, the world is facing a surge in antimicrobial resistance which is, again, shifting our paradigm of treatment options.

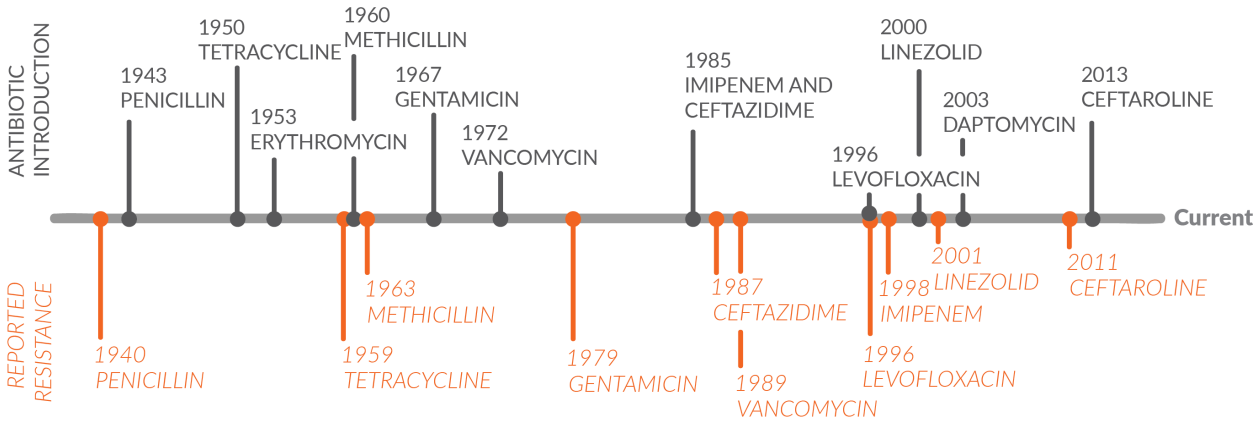

Figure 4. Timeline of drug discovery vs. the emergence of resistance to select antibiotics. Timeline was created with Adobe Illustrator. Data for the timeline was obtained through the CDC's 2013 report "Antibiotic Resistance Threats in the United States" [12]

Physical Resistance. Many antibiotics target the rigid, peptidoglycan cell wall of bacteria.[13] Through cell wall disruption, the bacteria's structure becomes unstable and vulnerable to lysis from external pressures. However, gram-negative bacteria have an outer-membrane that restricts access to the cell wall through pores. The LPS is a significant physical barrier for novel antibiotics, and has limited the number of effective compounds against gram-negative bacteria.[5,6,14] The physical membrane surrounding the cell provides a rudimentary level of resistance while bacterial genes provide more sophisticated mechanisms to evade antibiotic destruction.

Genetic Resistance. In recent decades, genetic resistance has exploded into a global crisis due to the exponential increase in incidence and accumulation of resistance genes within microbes.[12] Resistance genes can be integrated into the single chromosome of a bacteria or, more often, are located on an extrachromosomal plasmid (R plasmid).[15,16] As reviewed by Van Hoek, resistance genes have varying degrees of effectiveness, specificity, and often number in the hundreds.[17] R plasmids can accumulate resistance genes; in turn, making an organism multidrug resistant (MDR). Through the selective pressures of antibiotics, R plasmids are now seen at much higher rates in nature.[15]

Horizontal gene transfer. Horizontal gene transfer (HGT) is a ubiquitous mechanism of almost all bacteria and involves the exchange of genetic material across species or strains.[18–20] HGT is vital to the evolutionary progression of bacteria. Nearly all resistance genes in bacteria are found on transposable elements or linked to transposable elements within the genome. The transposable nature of resistance genes means that they are capable of being easily integrated into a genome (see Figure 4).[15,16,18,20–22] Thus, through HGT a resistant pathogen can transfer the associated resistance genes to another species. HGT is not limited to resistance genes. It is also attributed in conferring virulence factors that make non-pathogenic bacteria pathogenic.[15,16,20]

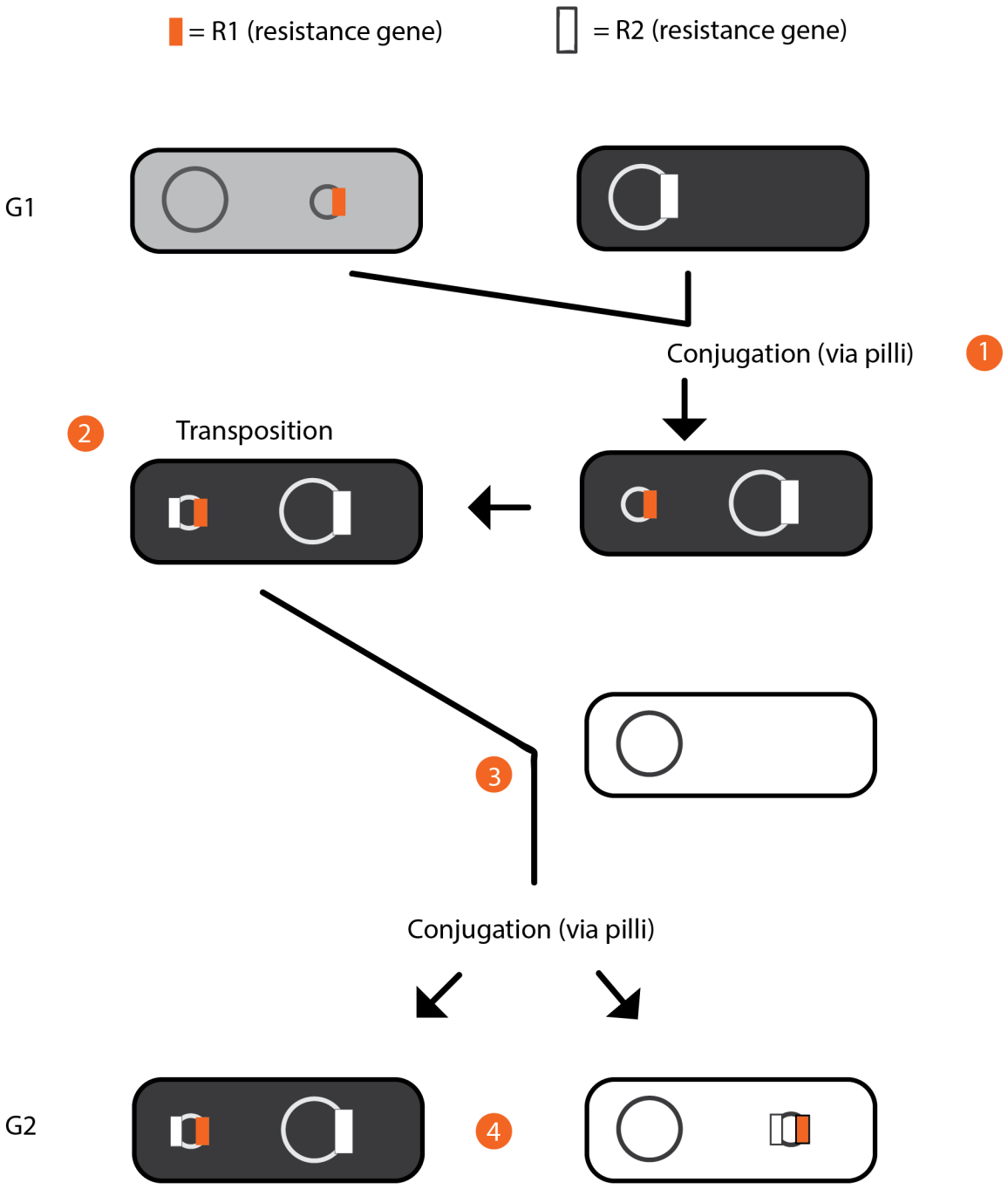

Figure 5. Accumulation of genetic resistance via conjugation. 1) When bacterial cells come within proximity, they can exchange genetic information through a pili. This method of genetic exchange is called, conjugation. Bacterial cells can transfer copies of their plasmid to a cell that lacks a plasmid. 2) Resistance genes are located on transposable elements which can easily be translocated back and forth between the chromosome and plasmid. A plasmid can accommodate multiple genes to confer multi-drug resistance. 3)A plasmid with resistance genes can be transferred to a non-plasmid cell, again, through conjugation. 4) Resistance genes have now been transferred to a previously non-resistant cell.[15] Original image created with Adobe Illustrator.

Fungi

Fungi represent a completely different biological set of microbes and are classified under the Eukarya domain accordingly. Their life cycle, molecular structures, and metabolisms are radically different than bacteria. Unlike bacteria, fungi have defined membrane bound organelles, which are also found in animal cells (see Figure 5).[4] Due to the cellular similarities between fungi and humans, treatment options for serious fungal infections are limited and carry a high risk of toxicity.[23–25]

Often, fungal infections are associated with superficial skin infections, but they can cause much more serious illnesses through invasive fungal infections (IFI). Diagnosis of fungal infections is often delayed because they are relatively less common in contrast to viral and bacterial infections but trigger similar initial symptoms. All these factors lead to dangerous mortality rates among mycotic associated infections, as high as 95% in some instances.[26,27]

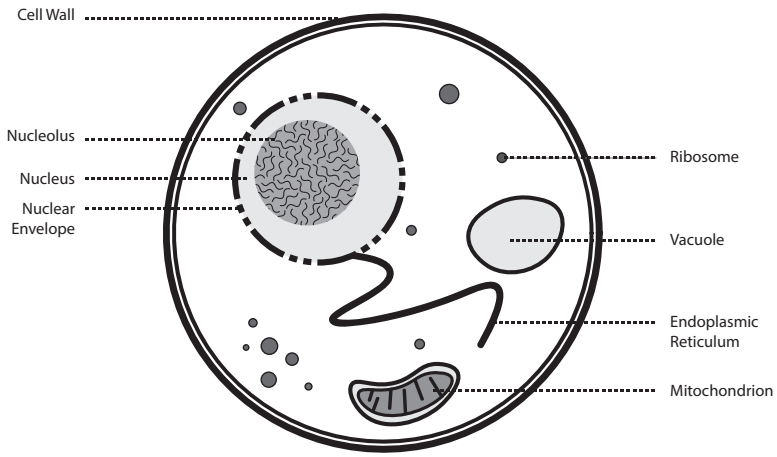

Figure 6. General structure and components of a fungal cell derived from Irving's *Medical Microbiology*.[4] Original image created with Adobe Illustrator.

Most fungal species live commensally in the environment and on other organisms. When disturbances occur in the normal flora of an organism or its immune system becomes compromised, these commensal fungi can cause dysfunction or disease. In most instances, invasive fungal infections are seen in immunocompromised or immunosuppressed patient populations: HIV/AIDS patients, cancer patients, and transplant recipients.[26–28] Misdiagnosis of invasive fungal-related deaths has made it difficult to accurately estimate its impact, but, as reviewed by Brown, IFIs account for more deaths than malaria or tuberculosis annually.

Fungal Resistance. The number of effective antifungal agents is significantly less than antibiotics, with a total of five different classes used for IFIs: azoles, echinocandins, polyenes and pyrimidines.[24] Nevertheless, antifungal agent discovery and resistance share motifs with antibiotics and bacterial resistance. Many antifungal drugs were discovered in the 60s, but many IFIs are now showing decreased sensitivity to them.[25,28,29] Fungal resistance is of great concern because of the limited number of safe, effective drugs available.

As opposed to bacterial binary fission, fungi undergo sexual reproduction. Thus, the resistance genes do not spread through HGT, but through recombination of genes. The genes can then be carried through populations without dominant expression. There are several genetic based mechanisms that help fungi avoid destruction by chemotherapeutic agents, mainly drug efflux pumps.[28,30,31] However, another mechanism of clinical significance is its robust ability to form biofilms, which are discussed shortly.

Biofilms

Bacteria and fungi are capable of adhering tightly to biotic and abiotic surfaces through the formation of biofilms.[32–35] Biofilms are complex infrastructure made of extracellular proteins, polysaccharides, and genetic material. The biofilm creates a protective haven for the

founding organisms and other microbes, bacterial or fungal. Through the formation of a biofilm, a diverse community of organisms can exchange genetic information, store nutrients, and protect themselves from physical removal and chemotherapeutic agents (Figure 6).[28,30,33,35–37]

Formation. A major challenge to combating resistant microbes are their ability to form biofilms. Bacterial biofilm formation is moderated by cellular appendages like flagella and pilli (see Figure 1). Biofilms are established in the presence of a viable surface and hospitable

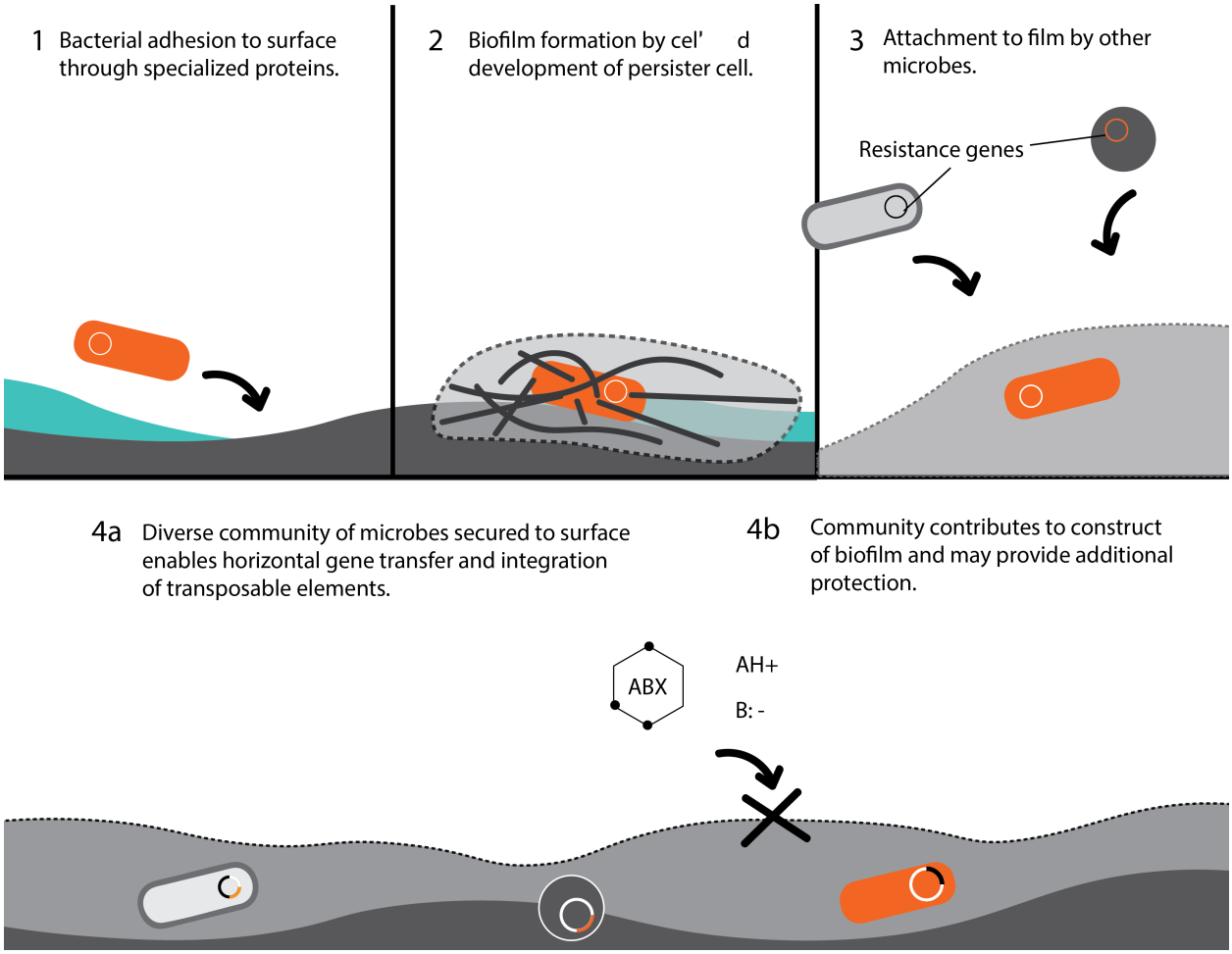

Figure 7. The process of biofilm formation and its contributions to antibiotic resistance. 1)In favorable conditions, bacteria can begin the process of adhesion to a surface, 2) once attached, the bacteria begins excreting the components to form a biofilm, 3) the newly formed surface promotes adhesion of other transient bacteria, 4a) the microbial community shares genes through horizontal gene transfer, 4b)the mature biofilm confers resistance to antibiotics (ABX), disinfectants (AH+ and B-), and other environmental stressors.[33] Original image created with Adobe Illustrator.

living conditions, such as a water source. Thus, medical devices are a primary source of biofilm related infections.[31,38–40]

Once a surface is detected, the organism begins to undergo genetic changes that promote film-specific cellular processes. The organism adheres to the surface through excretion of surface binding proteins.[30,35] In addition to the binding proteins, the organism may secrete a range of long polysaccharide chains, extracellular proteins, genetic material, and fatty acids creating an extracellular matrix (ECM).[30,33,41] The ECM encases the cells—creating a protective barrier from the environment and a favorable surface for additional cellular adherence. As more cells attach and new generations form, they contribute to the construct of the biofilm. The film can store nutrients, send cellular signals, and protect the microbes physical and chemically. Additionally, the biofilm shields microbes from therapeutic drug concentrations and the host immune cells.[33,35,37,41]

Persister Cells. Upon establishment of an ECM, some cells will undergo drastic changes in their genetic expressions.[30,33,35,42] They lower their metabolic activity and begin to upregulate proteins specific to biofilm maintenance. These metabolically inactive cells are called persister cells, and exist as a fundamental element in many fungal and bacterial biofilms.[43,44] The ECM protects persister cells from exposure, and their slowed metabolism greatly reduces the efficacy of chemotherapeutic agents. Their presence helps to continue cell proliferation within the biofilm.

Economic Implications of Resistant Infections

The economic burden of resistant infections is difficult to measure, and there continues to be debate on how to properly calculate its effects. However, according to a report released by the CDC, the direct cost of resistant infections is estimated at $20 billion with an additional $25

billion of indirect cost.[12,29] In response to the inflating cost associated with resistance, the US has increased regulations on antimicrobial prescriptions. Regulatory solutions like this may help curb the rapid growth of resistance across microbes, but ultimately has dampened interest in antimicrobial research.[45–47] If novel compounds are discovered, they likely will be used as a last resort—limiting their profitability.

Health Disparities. Many first-line antimicrobials can be produced on a commercial scale at relatively low cost; providing an efficient means of delivering healthcare to low-income and middle-income citizens, especially to those in developing nations. Due in part to their widespread, unregulated use, antibiotic resistance is becoming more common among such populations.[12,18] Since treatment for resistant organisms is often more expensive, the progression of resistance is creating a more rigid health disparity worldwide.

Antimicrobial Agents

Antibiotics. As far back as 300 CE, there is evidence that humans had discovered mysterious, natural healing powers among soil dwelling fungi.[22] However, our understanding of the microbial world and the ability to isolate such compounds limited their potential until the early 20th century. With the commercialized success of penicillin in 1942, the "Golden Age" of antibiotics had begun.[8,14,46]

The widespread success of penicillin in preventing infection related deaths was quickly followed by the discovery of streptomycin in 1943, which became the first treatment effective against tuberculosis.[7,48] While penicillin was synthesized from a fungus, *Penicillin notatum*, streptomycin was synthesized from a soil bacterium, actinomycetes. These first discoveries provided a framework for future antibiotic research and led scientists to scour soil microenvironments for additional antibiotic producing microbes.[14] Through the 1960's hundreds

of antibiotics were discovered this way, representing up to twenty different classes. The scope of this research includes agents from two of these classes, monobactams and cephalosporins which are structurally related.

β-lactams. β-lactams are a copious class of antibiotics that are all feature derivatives of a beta-lactam ring. Due to the numerous members in this broad class, they are further divided into four sub-classes based on chemical substituents around the beta-lactam ring: penicillins, cephalosporins, carbapenems, and monobactams (see Figure 7a-d).[7] They all share a similar mode of action—inhibiting cell wall synthesis through interactions with penicillin binding proteins (PBPs).[11] PBPs are membrane bound proteins that are essential to the growth of a bacterium. As the cell divides through binary fission, it must rapidly synthesize the growing cell wall. The cell wall, made mainly of a complex sugar peptidoglycan, is synthesized in part by PBPs which help cross-link rigid, foundational units. β-lactams are, structurally, similar to these foundational units; thus, PBPs have an affinity to bind with them.[7,10,49,50] In turn, the enzyme becomes inactivated, which ultimately prevents further cross-linking within the cell wall.[15] The result is a weakened cell wall that is susceptible to external pressures and cell lysis.

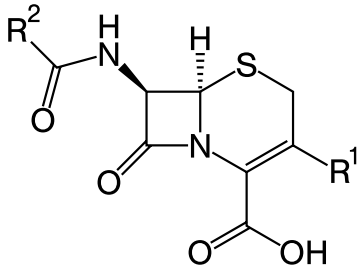

Figure 8. General structure for penicillins. Public domain image.

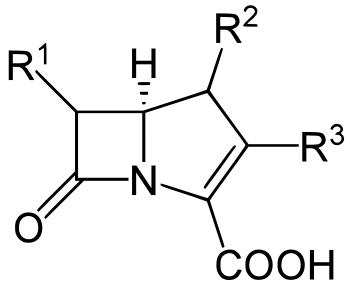

Figure 9. General structure for carbapenems. Public domain image.

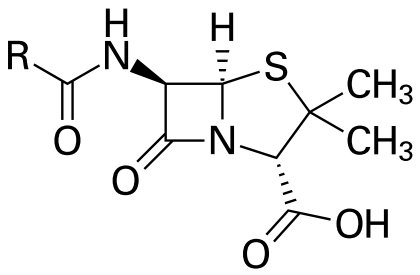

Figure 10. General structure for cephalosporins. Public domain image.

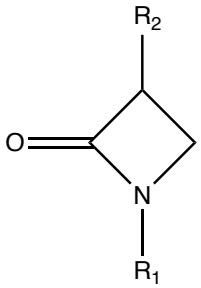

Figure 11. General structure for monobactams. Created with ChemDraw Professional 16

Aztreonam. Monobactams, are a relatively small subclass of beta-lactams. They are easily identified by the singular four-membered beta-lactam ring. They are of significant clinical significance for a couple of reasons: 1) their relatively simple structure enables total synthesis, and 2) they are highly specific to penicillin-binding protein 3 (PBP-3).[7,11,13,49] Aztreonam (see Figure 8) was the first of this subclass to be introduced in 1986. Its fully synthetic nature lowers instances of allergic reactions found in traditional beta-lactams that are produced by fungi—penicillins and cephalosporins.[7,9,11] It is freely soluble in water, and has a narrow specificity for mostly aerobic, gram-negative bacteria due to its interaction with PBP-3.[51] This feature helps to reduce disruptions in normal flora of the host, which occurs commonly with other antibiotics.

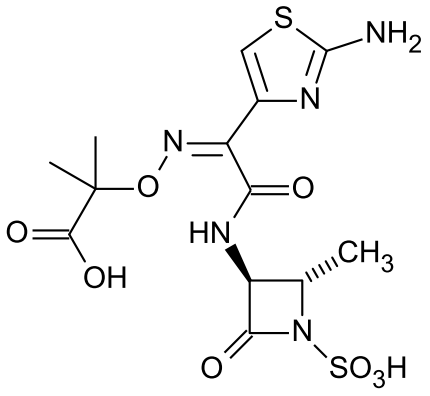

Figure 12. Chemical structure of aztreonam. Note the beta-lactam ring with substituents, and no additional fused ring. Public domain image.

Ceftriaxone. Analogous to the sub-class of penicillins, cephalosporins were discovered as a natural product of a fungus, *Cephalsporium.*[52] As with other β-lactams, they comprise of a beta-lactam ring with extensive modifications to the side-chains that interfere with cell wall synthesis. Cephalosporins have progressed since their first discovery in the 1940s through synthetic modifications, and are now classified as generations: first, second, third, and fourth.[52,53] Ceftriaxone (see Figure 9), a water-soluble, third-generation cephalosporin, is known for its effectiveness against gram-negative bacteria through interaction with PBPs.[53–55] Its introduction in the 1980's helped treat bacterial infections that had become insensitive to penicillins.

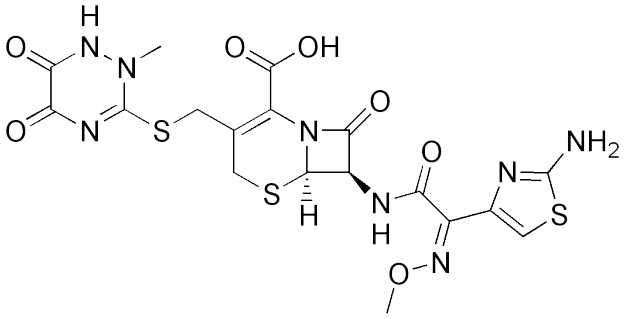

Figure 13. Chemical structure for ceftriaxone. Note the central beta-lactam ring with an additional fused ring and side chains. Public domain image.

Antifungals. As mentioned, antifungals present a greater challenge in therapeutic use because of the cellular similarities between fungal cells and animal cells. Thus, many effective antifungals pose a serious risk of toxicity to patients. Rising resistance to first-line treatment and limited classes of safe antifungal agents has begun to strain the capacity for positive outcomes from IFIs.[28,31,56–58]

Fluconazole (see Figure 10) is part of the azole class of antifungal agents that target the ergosterol biosynthesis pathway by inhibiting a cytochrome P450 (CYP51) enzyme lanosterol 14-α-demethylase (EC 1.14.13.70).[25,58] Ergosterol is an essential membrane component unique to fungi. However, CYPs exhibit widespread expression among eukaryotes, and are particularly

important in human liver cells where they participate in xenobiotic metabolism and detoxification. Thus, interactions of azole antifungals with P450 enzymes present drug interaction risks stemming from their interference with the metabolism of other drugs.[59,60] It remains a common first line treatment because of its low cost and broad specificity. Fluconazole's widespread use has led to increasing resistance among common fungal genera, especially *Candida*— with 7% of *Candida* clinical isolates currently demonstrating resistance to fluconazole.[61]

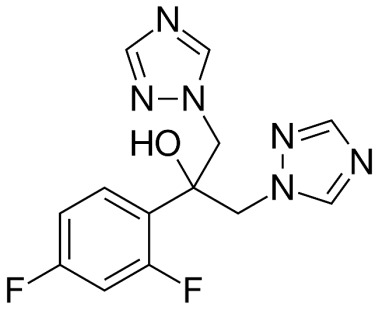

Figure 14. Fluconazole chemical structure. Public domain image.

Microalgae

Microalgae represent an incredibly diverse group of photoautotrophic and heterotrophic unicellular organisms that live in marine, freshwater, or damp soil environments.[62,63] Compared to other microbial species, microalgae grow slowly and are labor intensive to maintain. However, interest in natural products has returned in recent years as a means of offsetting biofuels production costs with secondary products and applications. Microalgae species are now used in an array of applications including: biofuel production, wastewater treatment, livestock feed, cosmetics, nutraceuticals, and antimicrobial compounds.[23,64–69]

Algal Extracts. Macroalgae and microalgae species have been shown to possess quorum-quenching metabolites, and to interact with bacterial populations.[70,71] Quorum-quenching molecules are capable of disrupting microbial cell-signaling. Moreover, studies have identified

that algal-associated bacteria are often capable of producing quorum-quenching and bactericidal compounds.[72] Extracts of algal cells are rich in known compounds (antioxidants, flavonoids, and polyphenols) that exhibit diverse effects such as: anti-inflammatory, anti-cancer, or antimicrobial activity.[67,68,73–78] Methods for extracting such active components are as diverse as the algae themselves.

Antioxidants. Antioxidants represent a broad class of molecules capable of neutralizing reactive oxygen species (ROS) and are found naturally in many plants and algae. Through routine cellular processes, ROS can readily form and cause damage to cellular components. For this reason, a great deal of research in has focused on using antioxidants to prevent cellular damage and reduce cancer risks.[73,79] Yet, antioxidants have also been recognized for their antimicrobial activity in plants. For example, phytoalexins are a group of polyphenolic compounds that accumulate in localized fungal, viral, or bacterial infections in plants.[80–82]

Polyphenols. Polyphenols are a well-studied, diverse group of secondary metabolites produced in plants. They are produced as biproducts of metabolic cellular processes, but are considered essential for the plant's survival— in particular, as part of their arsenal of defense mechanisms against fungi, bacteria, viruses, and herbaceous predators.[83–86] Polyphenols are released in response to stressors, infection or predation as a means of preventing further damage.[87,88]

According to Stéphane Quideau, a polyphenol must be derived from a phenylpropanoid and/or the polyketide pathway, feature more than one phenolic unit, and be deprived of a nitrogen-based functionality.[84] With these guidelines, polyphenols are further divided into subgroups based on chemical structure: flavonoids, nonflavonoids, tannins, and lignins.[89,90] In

general, these compounds are soluble in polar solvents but exhibit limited water solubility. Therefore, they are typically extracted using methanol or ethanol (see Figure 11a-c).[66,85]

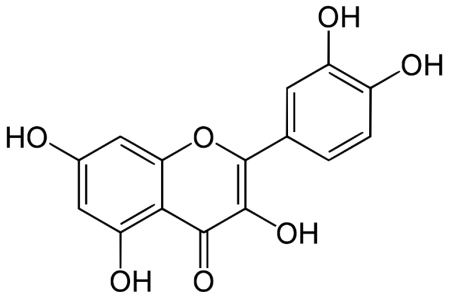

Figure 15. Quercetin. A flavonol found in many vegetables. Public domain image.

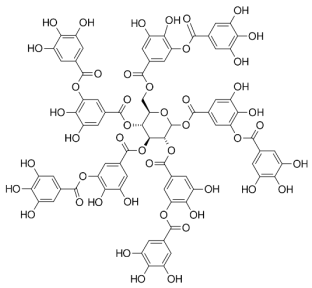

Figure 16. Quercitannin. A tannin found in oak tree bark. Public domain image.

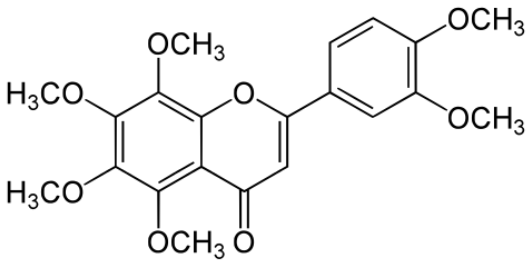

Figure 17. Nobiletin. A flavonoid found in citrus fruit. Public domain image.

Context of Study

Stenotrophomonas maltophilia

S. maltophilia is an aerobic, gram-negative, film-forming bacterium that ubiquitous in humid or wet environments and colonizes biotic and abiotic surfaces. It is abundant on many natural surfaces including human skin but is typically non-pathogenic. However, *S. maltophilia* has emerged as an increasingly common opportunistic pathogen, especially among immunocompromised populations.[38,91–93] Since it is a gram-negative bacterium, there are limited

effective chemotherapeutic agents available to combat *S. maltophilia* infections. Furthermore, genomic analysis has indicated potential resistance mechanisms for a number of antibiotic classes that are well conserved.[91] With limited treatment options and mortality rates for chronic *S. maltophilia* infections as high as 70 percent, *S. maltophilia* is evolving in both an attractive and urgent target for intervention. [91,94,95]

Biofilm Properties. *S. maltophilia* forms robust biofilms on surfaces, biotic and abiotic which greatly increase its resistance to antibiotic targeting.[94,96–98] It's adherence to surfaces is dependent on hydrophobic properties. Studies show that the flagellum, pilli, and fimbriae increase its ability to attach to abiotic, hydrophobic surfaces. Visualization of the 3-D EPS structure shows complex layering of cells and extracellular components with localized hydrophobic regions.[99,100] Cells within the biofilm have an increased hydrophobicity relative to those grown planktonically.[99] The hydrophobic nature of the cells and biofilms may account for reduced sensitivity to antibiotics since most drugs must be hydrophilic to be distributed throughout the body.[101]

At-Risk Populations. *S. maltophilia* is an emerging pathogen that continues to be isolated more frequently in hospital acquired infections due to its affinity for damp environments and ability to colonize a wide variety of surfaces.[12] It's efficient development of resistance means that the regular use of prophylactic broad-spectrum antibiotics in these patients helps creates a non-competitive growth environment for *S. maltophilia* to colonize and infect.[102] Medical devices are a common means of transmission, including endoscopes, suction hoses, and central venous catheters.[38,92,98] Unsurprisingly, most clinical isolates are found among critical care patients with compromising injuries where these devices are in frequent use, along with broad spectrum antibiotics, for long periods of time. Recent findings suggest that the

prophylactic use of broad-spectrum antibiotics may contribute significantly to resistant *S. maltophilia* infections.[94]

Like other opportunistic pathogens, *S. maltophilia* is a serious concern for patients without an active immune system—a population that is rapidly growing in modern healthcare settings.[27] High-risk patients include those with organ or tissue transplantation, HIV/AIDS, cystic fibrosis, and cancer. *S. maltophilia* in these patients is most commonly isolated from upper respiratory infections; but is also linked to bacteremia, urinary tract infections, and meningitis.[91]

Candida Albicans

Candida albicans is a fungus that is ubiquitous in the environment that often lives commensally on skin and areas of the gastrointestinal tract.[103] Normally, *C. albicans* goes unnoticed and does not disturb its human hosts. However, it is also the main cause of candidiasis, which can present in the mouth (thrush), vagina (yeast infection), or systemically (invasive candidiasis).[104] Invasive candidiasis has a particularly high mortality rate of 19-24%. Furthermore, resistance to the first-line drug, fluconazole, is increasing, due in part to the ability of *C. albicans* to form biofilms.[61]

C. albicans forms robust biofilms on biotic and abiotic surfaces, and is the most common fungal species isolated from clinical biofilms.[104] Through the establishment of a biofilm, the fungus can evade both the immune system and antifungal agents. As with *S. maltophilia*, a primary source for nosocomial invasive candidiasis are medical devices such as central venous catheters, urinary catheters, heart valves, and endotracheal tubes.[103]

It has been well documented that *C. albicans* naturally has a hydrophobic cellular surface through the expression of the cellular surface hydrophobicity (*CHS1)* gene.[105–107] *C. albicans* hydrophobicity is enhanced through the process of biofilm formation, and is linked to increased

virulence.[101] Current research demonstrates that hydrophobic compounds have had impacts on biofilm formation in *C. albicans* species.[108]

S. maltophilia and *C. albicans* are two very different opportunistic pathogens. Yet, they are both capable of forming biofilms with hydrophobic properties as a prelude to developing drug resistant, both can reside competitively in similar natural environments, and both organisms afflict the same human populations.

Microalgal Extracts

Many studies have begun investigating natural sources to enhance antimicrobial activity of current treatments to pathogens.[109–113] Microalgae have already repeatedly shown antibacterial activity.[67,114–117] Algal extracts offer an ideal alternative to increasing traditional interventions because many species have been labeled as generally accepted as safe (GRAS) and are fully biodegradable.[63] Furthermore, they offer an efficient, environmentally friendly method of producing potentially complex molecules.[118,119] However, there remains large classes of species which have evaded investigation. The current study uses several species that represent diverse classes of microalgae (see Figure 13 and Table 1). Our goal is to evaluate known algae species to determine novel applications and stimulate additional research interest for their antimicrobial use. We evaluate their efficacy on planktonic growth and established biofilms as compared to current antibiotics. Additionally, we determine if algae extracts offer enhancing effects when combined with antibiotics or antifungals, against *S. maltophilia* and *C. albicans*, respectively. It's theorized that components of the algal extracts can increase the effectiveness of the select antimicrobials by negating resistance features of bacterial biofilms and their hydrophobic effects. Recent studies have shown that extracts from lipid producing species such as *B. braunii*, *C. danica*, and *N. oculata* have measurable antimicrobial effects.[115,117,120] However, most of these

studies have not evaluated the impact on organisms in biofilms, and have not included the gram-negative bacterium *S. maltophilia* or fungus *C. albicans*.

Table 1. **Algae Species of Interest to the Present Study.**

Algae Species	Aquatic Environment	Current Interest or Use
Botryococcus braunii	Freshwater	Biodiesel[121,122]
Tisochrysis lutea	Marine	Aquaculture feedstock[123]
Rhodomonas lens	Marine	Aquaculture feedstock[124,125]
Chlorochromonas danica	Freshwater	Treatment of waste products[126,127]
Nannochlropsis oculata	Freshwater	Biodiesel[128,129]

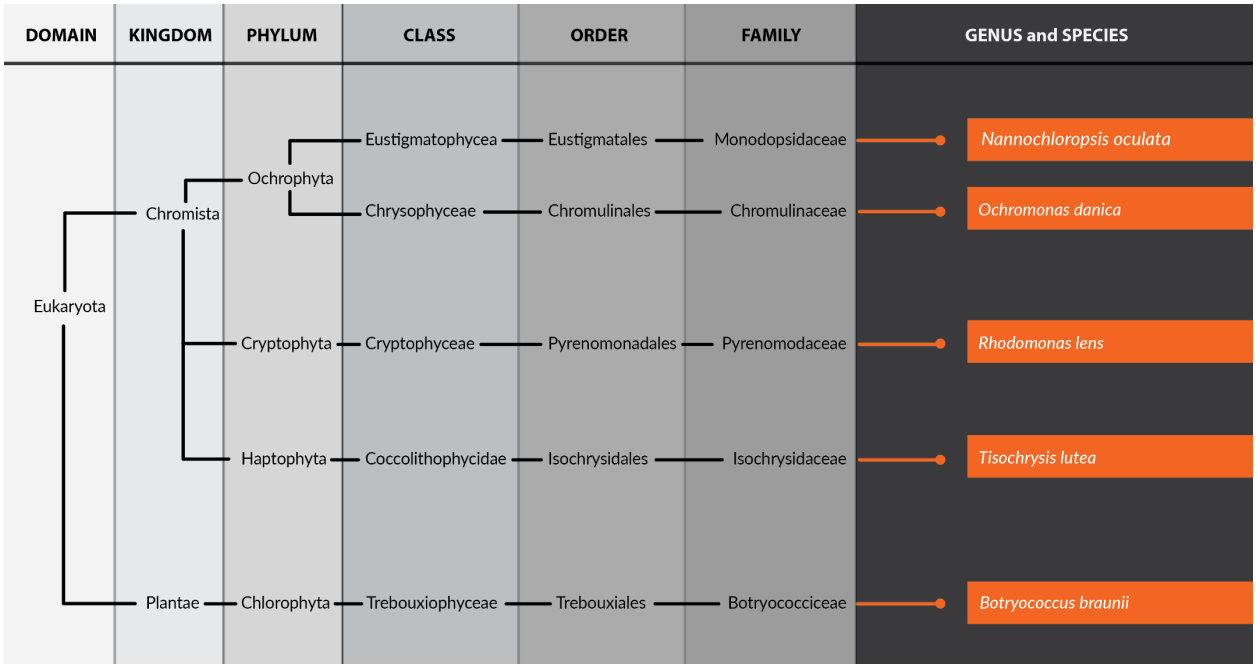

Figure 18. Taxonomical reference of algal species used in current study. Figure is a visual representation of taxonomy for species and does not depict actual genetic distance or variation between species shown. All data was collected from algaebase.org, April 1, 2017. Original image created with Adobe Illustrator.

Extraction Solvents

As with many natural extract experiments, the choice of solvent plays a crucial role in proper evaluation. A majority of active components are found in organic layers of extraction. However, it is known that the naturally produced polyphenols and flavonoids have antimicrobial

activity, and are extracted best with slightly polar solvents.[84,130] Therefore, most extraction methods use a mixed solvent system, using a small amount of polar solvent with a large amount a non-polar counterpart. One common application is a 3:1 or 3:2 ratio of hexanes-isopropyl or chloroform–methanol.[131] To simplify this process, 2-methyltetrahydrofuran (2-MTHF) is proposed as a singular solvent in comparison with a mixed solvent method employing hexanes-isopropanol (3:1). 2-MTHF is significantly more polar than hexanes, but less polar than isopropyl alcohol according to the solvent polarity scale.[132,133] Thus, it should perform similarly or slightly better than traditional HIPA solvents in extraction of active organic components. Additionally, 2-MTHF is considered a green-solvent because of its low environmental impact, its recovery capacity in reactions, and its renewable sourcing.[132,134,135]

Material and Methods

Microalgae

All species of algae were obtained from NCMA Bigelow Laboratory and UTEX as axenic cultures. Algal cultures were maintained as axenically as possible in media and lighting conditions described by the manufacturer to produce a 4-liter culture with a cell concentration of 6×10^6 per mL. Prior to pelleting, algal cultures were tested for the presence of bacteria using PCR (Appendix A). Species used in this study include: *Botryococcus braunii* 572, *Nanochloropsus oculata* 2164, *Rhodomonas lens* 739, *Tisochrysis lutea* 463 and *Chlorochromonas danica* 3279. Algal growth media, enriched seawater (L1), modified Bold's 3N medium (MB3N), Erdschreiber's medium, tris-acetate-phosphate (TAP), and Ochromonas media, were prepared in lab using recipes obtained from the National Center of Marine Algae (NCMA) Bigelow Laboratory or the University of Texas Culture Collection (UTEX) and sterilized.

Table 2. **Algae Species and Extracts**

Algae Species (UTEX/NCMA ID)	**Extraction Solvent**	**Label**
Tisochrysis lutea (463)	HIPA	*T. lutea* [H]
Tisochrysis lutea (463)	2-MTHF	*T. lutea* [M]
Rhodomonas lens (739)	HIPA	*R. lens* [H]
Rhodomonas lens (739)	2-MTHF	*R. lens* [M]
Botryococcus braunii (572)	HIPA	*B. braunii* [H]
Botryococcus braunii (572)	2-MTHF	*B. braunii* [M]
Chlorochromonas danica (3279)	HIPA	*C. danica* [H]
Chlorochromonas danica (3279)	2-MTHF	*C. danica* [M]
Nanochloropsus oculata (2164)	HIPA	*N. oculata* [H]
Nanochloropsus oculata (2164)	2-MTHF	*N. oculata* [M]

Bacterial Cultures

Bacterial cultures were grown and stored at 30^{o} C in nutrient broth (NB). *Stenotrophomonas maltophilia* 13637 was obtained from ATCC and grown in nutrient broth overnight to a McFarland standard of 0.5.

Fungal Cultures

Cultures of *Candida albicans* wild-type SC5314 were generously donated by Dr. Cristopher Pierce at the University of the Incarnate Word, Biology Department. Yeast cultures were grown at 30^{o} in YPD.

Chemicals

All chemicals used were reagent grade, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2'-azinobis-(3ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu's reagent, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium persulfate, gallic acid, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (quercetin), crystal violet (CV), 2-methyltetrahydrofuran (2-

MTHF), and aluminum chloride ($AlCl_3$). All buffers, media, antibiotics, and solvents were obtained from Sigma Aldrich or Cayman Chemicals.

Pelleting and Extraction

Extraction was completed as described in Pane with slight modification.[67] Briefly, upon reaching a threshold of 6 x 10^6 cell per mL in a 4-liter volume, algal cultures were centrifuged for 20 min at 3500 rpm and -4^o C. The resulting cell pellets were stored at -80^{o}C for no longer than 4 weeks. Each pellet was evenly divided for extraction with either 3:1 hexanes/isopropanol (HIPA) or 2-methyltetrahydrofuran (2-MTHF). Extraction with either solvent was initiated with a volume of solvent equal to the volume of the pellet to be extracted. Each pellet was then sonicated for 5 minutes on ice, and centrifuged at 5000 rpm for 10 min at -4^o C. The process was repeated with additional equivalents of solvent until the pellet showed significant color loss. The resulting organic layer was stored in a clean glass vial and its solvent was evaporated under stream of air. The final crude extraction residue was reconstituted in 2mL of acetonitrile and stored at -60^o C.

Determining Crude Extraction Component Concentration

The mass of the extracted residue from each algal culture was determined by transferring 500µL of its reconstituted extract to a pre-weighed glass vial and evaporating the solvent to yield a portion of the original extracted residue. This residue was weighed to obtain a crude extracted mass, then reconstituted in 500µL of acetonitrile.

Antioxidant Capacity Assay. The total antioxidant capacity (TAC) of extracts was evaluated using an $ABTS^{\bullet+}$ method specialized for evaluating highly pigmented algal extracts.[136,137] A standard curve was generated using a water-soluble vitamin E derivative, Trolox (4µM-0.05µM; $R^2 = 0.9581$). Absorbance was measured at 734nm, for 6 minutes. The TAC

reported for each extract was obtained by determining the difference in the absorbances at 6 min for the extract and the corresponding absorbance for the ethanol control (negative quench control) Difference in absorbances was then used in reference to Trolox standards, and the results expressed as micromolar Trolox equivalents per gram of crude extracted residue (mcM TE/g).

Estimation of Phenolic Content in Crude Algal Extracts. Total phenol concentration was determined using Folin-Ciocalteu method.[136] A 1:10 dilution of Folin-Ciocalteau reagent (F-C) was made with water. Briefly, 100µL of F-C was added to either 10µL of extract and 10µL of ethanol or 20µL of ethanol (negative phenolic control) in triplicate wells of a 96-well polystyrene plate and incubated at room temperature for 4 minutes. Then, 80µL of saturated sodium carbonate was added to all wells, and the plate incubated at room temperature for an additional 2 hours. Absorbance was measured at 765 nm. A standard curve was generated using a gallic acid (0.40-0.025 mg/mL) with an R^2=0.9452. Measurements were then expressed as milligram equivalents of gallic acid per gram crude extracted residue (mg GAE/g).

Determination of Flavonoid Content. The flavonoid content was estimated according to the method of Ordonez *et al*.[138] A stock solution of quercetin was created in ethanol (0.25g/500mL), and a 2% w/v solution of AlCl3. Briefly, 10 µL of extract or 10 µL of ethanol (as negative control) were added to 40µL of ethanol and 50 µL of AlCl3 solution (2% w/v) a 96-well polystyrene plate and incubated for 1 hr at room temperature. A standard curve was prepared using dilutions of quercetin (0.025mg/mL-0.001mg/mL; R^2=0.9175). Flavonoid content is expressed as milligram equivalents of quercetin per gram crude extracted residue (mg QE/g).

Biofilm Formation Assay

Biofilm formation assays using crystal violet staining were carried out in 96-well polystyrene plates as previously described by O'Toole.[30] Control samples contain 10µL of acetonitrile instead of 10µL of extract. Absorbance measurements were made in stained wells at 570nm. Each extract and control were carried out in triplicate for two separate trials.

Established Biofilm Assay. *S. maltophilia* biofilms were prepared in 96-well polystyrene plates according to O'Toole and verified by absorbance of rinsed wells at 600nm to verify value greater than 0.5. Dilutions of antibiotics were prepared in NB to make the following media concentrations: 128µg/mL Aztreonam, 64µg/mL Ceftriaxone, and 32µg/mL of Gentamycin. Each antibiotic media (100µL) was added to filmed wells with either 10µL of extract or 10µL of ethanol (negative control). Additional control groups were maintained with 10µL of pure solvent, acetonitrile. Plates were incubated for 24 hours, washed with PBS three times, and stained for 15 minutes with crystal violet. Stained wells were rinsed three times with water and the remaining stain solubilized in 30% acetic acid. Absorbance measurements were performed at 570 nm. Results are the average of triplicates from two trials.

The established biofilm assay procedure was followed for *C. albicans*, with the following minor modifications: a cell count of 6 x 10^8 cells/mL was established for the overnight *C. albicans* stock culture grown in yeast extract peptone dextrose (YPD); and YPD medium was replaced with RPMI for biofilm assays and overnight incubation at 37^o C.

Cell Viability Assay. Cell viability was measured in biofilm formation assays and in established biofilm assays by treatment of live cultures with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the manufacturer's protocol. The procedures

for both assays were as described above but with MTT treatment and incubation in place of CV staining. Absorbance measurements were performed at 570 nm.

Planktonic Growth Assay. Overnight cultures of *S. maltophilia* were grown in NB at 30oC, diluted (1:100) in NB, and added (100 µL) to each well of a 96-well polystyrene plate. To the diluted culture was added either 10µL of extract, 10µL of extract dilution (1:1, 1:10, 1:100), 10 µL of acetonitrile (negative control) or 10 µL of an antibiotic dilution (256µg/mL, 128µg/mL, 64µg/mL, and 32µg/mL). Plates were incubated for 16 hours at 30ºC. Absorbance measurements were made at 570nm to determine planktonic growth. Planktonic growth assay data is given in Appendix B.

Statistics. Spectroscopy measurements were made using Tecan Infinite 200 Pro plate reader. Results are displayed as means with error bars representing standard deviation. All experiments were carried out twice, independently. Biofilm assays were completed in triplicates, while planktonic growth rate was completed in duplicates. All data was compiled and analyzed using IBM SPSS 25. Analysis was conducted using one-way analysis of variance (ANOVA) with post-hoc Dunnets and Tukey's test, Kruskal-Wallis, and Mann-Whitney U tests. All statistical significance was determined at $p < 0.05$. Statistical analyses are presented in Appendix C.

RESULTS

Comparison of Solvents

The mass of extracted residue from each algal culture pellet and each of the extraction solvents, 3:1 (v/v) hexanes/isopropanol (HIPA) or 2-methyltetrahydrofuran (MTHF) was determined as described in the Materials and Methods section (Table 3). The amount of crude material extracted from the selected species may reflect the differential solubility of molecular

components expected from the selected algal species in these two solvent systems. Both B. braunii and C. danica gave significantly less crude extract with HIPA as extraction solvent than with MTHF. Extraction masses were similar between the two solvents for the other species tested.

Table 3. **Crude Extracted Mass**

Algal Extract	Extracted Mass (mg)
T. lutea [H]	21.2
T. lutea [M]	25.2
R. lens [H]	7.6
R. lens [M]	9.2
B. braunii [H]	14.8
B. braunii [M]	23.6
C. danica [H]	12.0
C. danica [M]	29.2
N. oculata [H]	10.4
N. oculata [M]	10.8

As described in Table 4, the MTHF extracts had significantly higher polyphenol concentrations relative to the corresponding HIPA extracts for all species tested. ANOVA indicates that solvents had a statistical significance when comparing total phenolic concentration ($p = 0.008$). The extraction solvent did not demonstrate significant impacts on behavior in antimicrobial assays.

Table 4. **Evaluation of Antioxidant Capacity and Phenolic Contents of Extracts.**

Algal Extract	Total Antioxidant Capacity[a] **mcMol TE/g**	Total Phenolic Concentration[b] **mg GAE/g**	Total Flavonoid Concentration[c] **mg QE/g**
T. lutea [H]	330.3 ± 1.7	39.3 ± 3.8	10.3 ± 5.7
T. lutea [M]	324.7 ± 3.1	56.7 ± 6.0	6.2 ± 2.8
R. lens [H]	102.4 ± 1.8	26.4 ± 0.6	9.7 ± 2.3
R. lens [M]	187.9 ± 3.4	111.7 ± 3.3	7.4 ± 2.0
B. braunii [H]	289.7 ± 1.6	58.6 ± 0.7	4.7 ± 0.6
B. braunii [M]	303.9 ± 2.7	116.7 ± 3.1	2.2 ± 6.6
C. danica [H]	111.0 ± 0.3	34.1 ± 0.4	3.8 ± 0.6
C. danica [M]	229.2 ± 1.4	111.2 ± 5.6	10.4 ± 3.4
N. oculata [H]	105.9 ± 2.3	52.5 ± 2.0	4.4 ± 0.6
N. oculata [M]	126.8 ± 2.8	81.4 ± 2.3	6.5 ± 0.4

[a]Standardized to Trolox regression and presented as µM Trolox equivalents (TE) per g.
[b]Standardized to gallic acid regression and presented as mg gallic acid equivalents (GAE) per g.
[c]Standardized to quercetin regression and presented as mg quercetin equivalents (QE) per gram.
± indicates standard deviation.

Impact on Planktonic Growth

Planktonic cultures of *S. maltophilia* were treated with crude algal extracts according to the method described by the Clinical and Laboratory Standards Institute as a means of assessing the sensitivity of free-living cells to growth inhibition or bactericidal activity in the extracts. The undiluted extract along with three dilutions (1:1, 1:10, and 1:100) in acetonitrile were examined. For verification, the same method was used to determine a minimum inhibitory concentration (MIC) for gentamicin, aztreonam, and ceftriaxone. None of the algal extracts tested showed significant planktonic growth inhibition, and the sensitivity of planktonic *S. maltophilia* to the

antibiotics tested was as expected, with aztreonam demonstrating the least effective growth inhibition of *S. maltophilia* (Appendix B).

Impact on Biofilm Formation in *S. maltophilia*

Because biofilm formation in S. maltophilia has been demonstrated to involve signal mediation through the diffusible signal factor (DSF) system, the disruption of the filming process by a crude algal extract may indicate interference with the DSF machinery by components within the extract.[139] Therefore, the formation of biofilm by *S. maltophilia* was evaluated with and without the presence of crude algal extracts, examining both the relative mass of formed biofilm and the cell viability within the formed film.

As indicated in Table 5, there was not a significant reduction in the formation of the biofilms created by *S. maltophilia*. The addition of extract before the formation of a biofilm had little effect on the organism's ability to generate a biofilm.

Table 5. **Impact of Extracts on Biofilm Formation by *S. maltophilia*.**

Extract	Normalized Film Biomass[ab]
T. lutea [H]	83.8% ± 6.6
T. lutea [M]	83.5% ± 3.4
R. lens [H]	89.4% ± 4.8
R. lens [M]	92.5% ± 11.9
C. danica [H]	73.3% ± 6.8
C. danica [M]	80.3% ± 11.2
N. oculata [H]	85.5% ± 12.0
N. oculata [M]	84.6% ± 17.5
B. braunii [H]	82.0% ± 10.7
B. braunii [M]	81.2% ± 8.6

[a]All percentages are normalized to untreated *S. maltophilia* and ± represents standard deviation.
[b]Biomass is estimated from CV absorbance and indicates the formation of a biofilm.

Impact on Established Biofilms

Overnight biofilms were generated and incubated with 10µL of algal extract. Two measurements were taken to compare the overall biomass using CV stain with viable cells using MTT. Using a Dunnett test, each sample's measurements were then compared to the control group to determine statistical significance at $p < 0.05$. With extract only treatments, there were no significant reductions in biomass, as evidenced by the CV percentages. *B. braunii* [H] ($p < 0.001$), *B. braunii* [M] ($p < 0.001$), *C. danica* [H] ($p < 0.001$), *C. danica* [M] ($p = 0.001$), *N. oculata* [H] ($p < 0.001$), and *N. oculata* [M] ($p = 0.001$) all showed statistically significant effects against viable cells as measured by their respective MTT percentages.

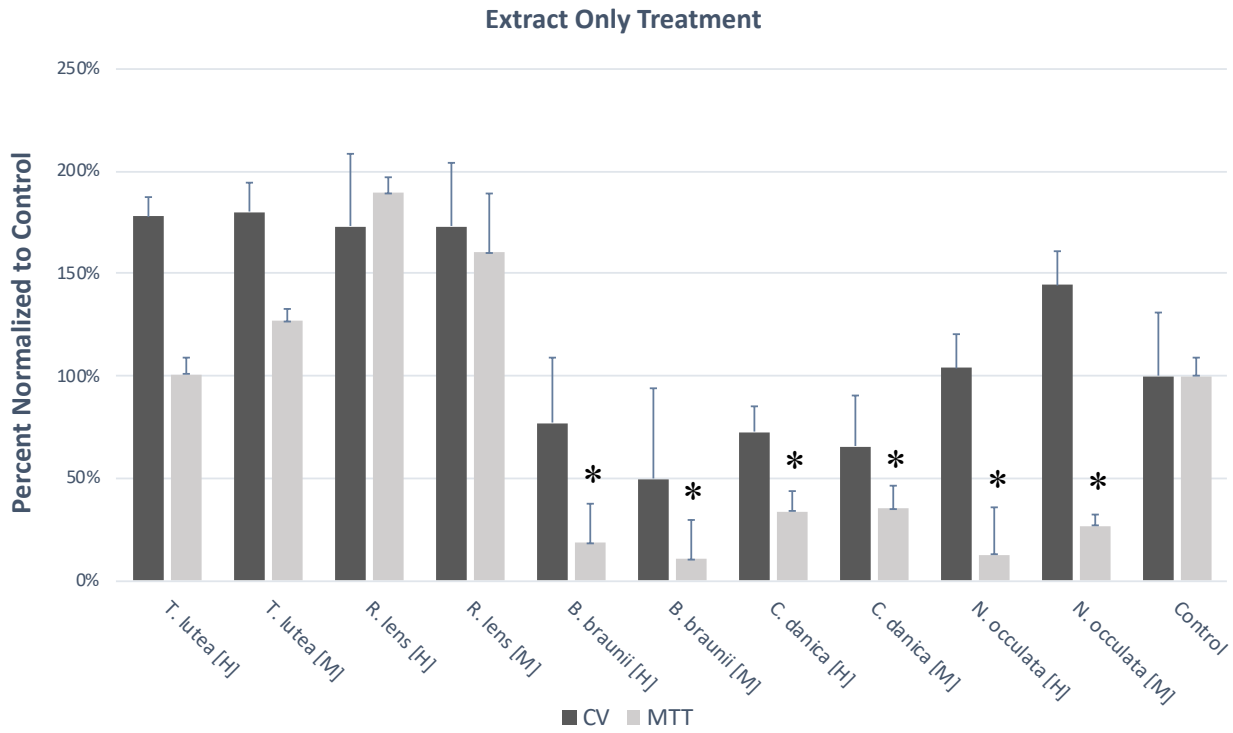

Figure 19. Overnight treatment with algal extracts on established biofilms. Resulting static biofilm formation of *S. maltophilia* and cellular metabolism after treatment with algal extracts overnight. Ratios are generated to indicate general effectiveness of treatment against cells within the biofilm. * indicates statistically significant values at p <0 .05.

Second, the algal extracts were applied in combination with the monobactam antibiotic, Aztreonam at 128µg/mL. Control groups were treated with only 128µg/mL of Aztreonam and

acetonitrile (ACN), the algal extract medium. As in the previous data, the respective CV and MTT measurements were compared to their control groups to produce a percentage. The antibiotic control groups showed almost no change compared to the non-antibiotic control in the previous experiment, which was expected. Again, *B. braunii* [H] ($p < 0.001$), *B. braunii* [M] ($p < 0.001$), *C. danica* [H] ($p < 0.001$), *C. danica* [M] ($p = 0.003$), *N. oculata* [H] ($p < 0.001$), and *N. oculata* [M] ($p = 0.001$) all showed statistically significant effects on cell viability. Interestingly, *C. danica* [H] ($p = 0.04$) showed statistically significant reductions in measurable biomass also with the addition of Aztreonam at the significance level of ($p < 0.05$) in the Dunnett's analysis.

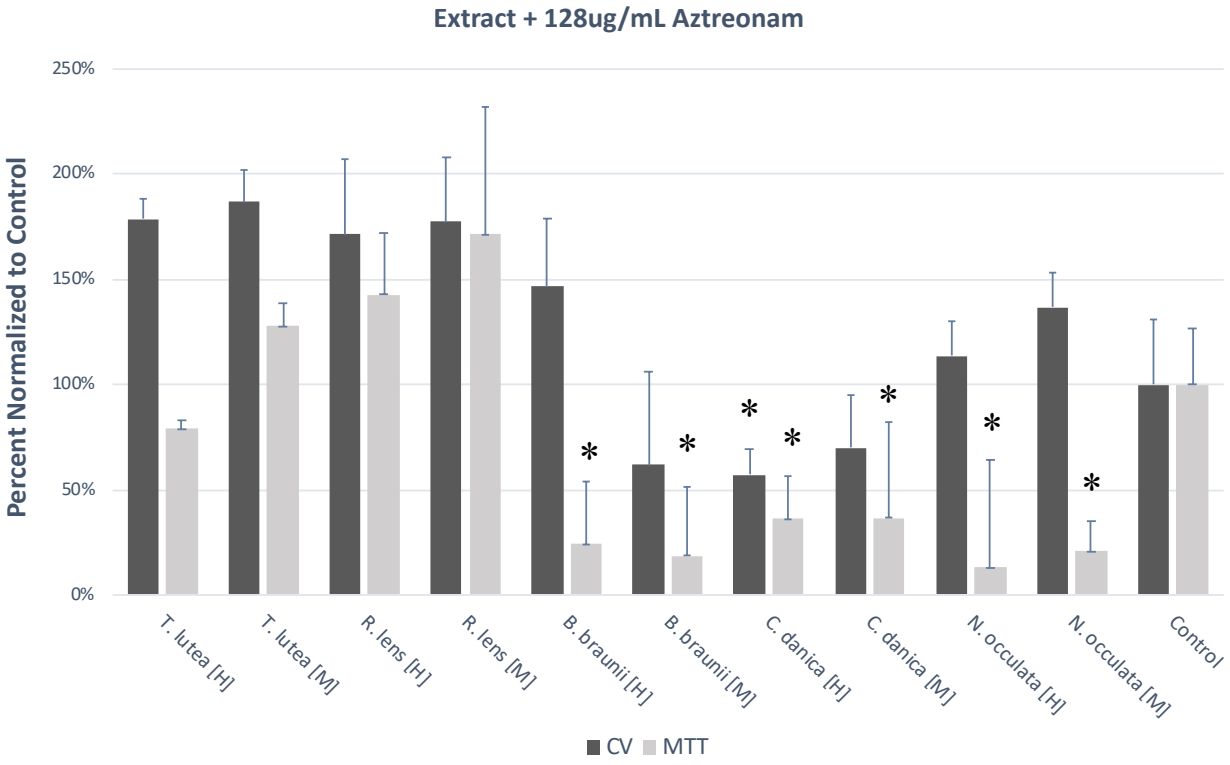

Figure 20. Overnight treatment with algal extracts and aztreonam on established biofilms. Resulting static biofilm formation of *S. maltophilia* and cellular metabolism after treatment with algal extracts and aztreonam overnight. Ratios are generated to indicate general effectiveness of treatment against cells within the biofilm. * indicates statistically significant values at $p < 0.05$ compared to control group of 128ug/mL of aztreonam only.

Lastly, the cephalosporin, ceftriaxone at 64µg/mL, was used in combination with algal extracts. Again, the control group was treated with 64µg/mL of ceftriaxone only with acetonitrile. As in previous results, the CV and MTT measurements were compared to the control group to produce a percentage. Again, *B. braunii* [H] ($p = 0.003$), *B. braunii* [M] ($p < 0.001$), *C. danica* [H] ($p = 0.001$), *C. danica* [M] ($p = 0.005$), *N. oculata* [H] ($p < 0.001$), and *N. oculata* [M] ($p = 0.001$) all showed statistically significant results against cell viability with reduced MTT percentages.

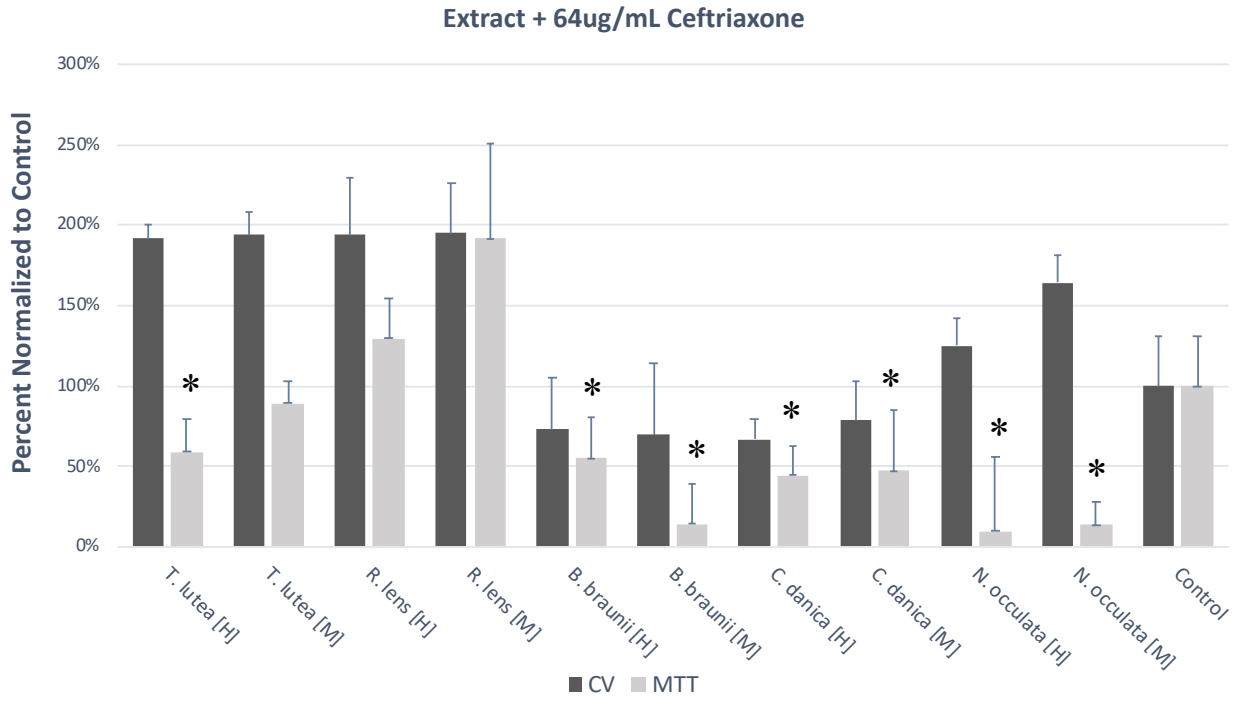

Figure 21. Overnight treatment with algal extract and ceftriaxone on established biofilm. Resulting static biofilm formation of *S. maltophilia* and cellular metabolism after treatment with algal extracts and ceftriaxone overnight. Ratios are generated to indicate general effectiveness of treatment against cells within the biofilm. *indicates statistically significant values at $p < 0$ .05 compared to control group of 64ug/mL of ceftriaxone only.

Gentamycin was also tested. However, the sensitivity to gentamycin made it difficult to effectively evaluate statistically significant impacts. To test significant interactions between algal extracts and the addition of antibiotics against cell viability an ANOVA with a multiple comparison Tukey test was performed. Resulting analysis showed that there was no statistical

difference in cell viability across control groups—meaning antibiotics alone were not able to significantly reduce cell viability in established biofilms. *N. oculata* [M] showed statistically significant increase in reduction with the addition of ceftriaxone versus extract only reduction ($p = 0.011$); indicating an enhancement of ceftriaxone activity. No other statistical significance was found in determining enhancement activity of algal extracts with antibiotics.

To understand if the observed effects in *S. maltophilia* biofilms could be applied to other film-forming organisms, *C. albicans* was tested in the same manner described for *S. maltophilia*. The extracts tested did not significantly impact the relative film mass accumulation by *C. albicans* either alone or in combination with fluconazole (see Appendix B). However, extracts from *B. braunii, N. oculata*, and *C. danica* improved sensitivity of established *C. albicans* biofilms to fluconazole. As shown in Figure 16, cell viability was significantly reduced relative to the fluconazole only control in the presence of extracts from these three algae. Treatment combinations with negative impacts on biofilm cell viability in *C. albicans* include combinations of 128µg/mL of fluconazole with extracts from *C. danica* [H] ($p = 0.003$) and *N. oculata* [H] ($p = 0.006$), [M] ($p = 0.009$); and combinations of 256µg/mL of fluconazole with extracts from *B. braunii* [M] ($p = 0.004$), *C. danica* [H] ($p = 0.002$), *C. danica* [M] ($p = 0.021$), and *N. oculata* [M] ($p = 0.042$).

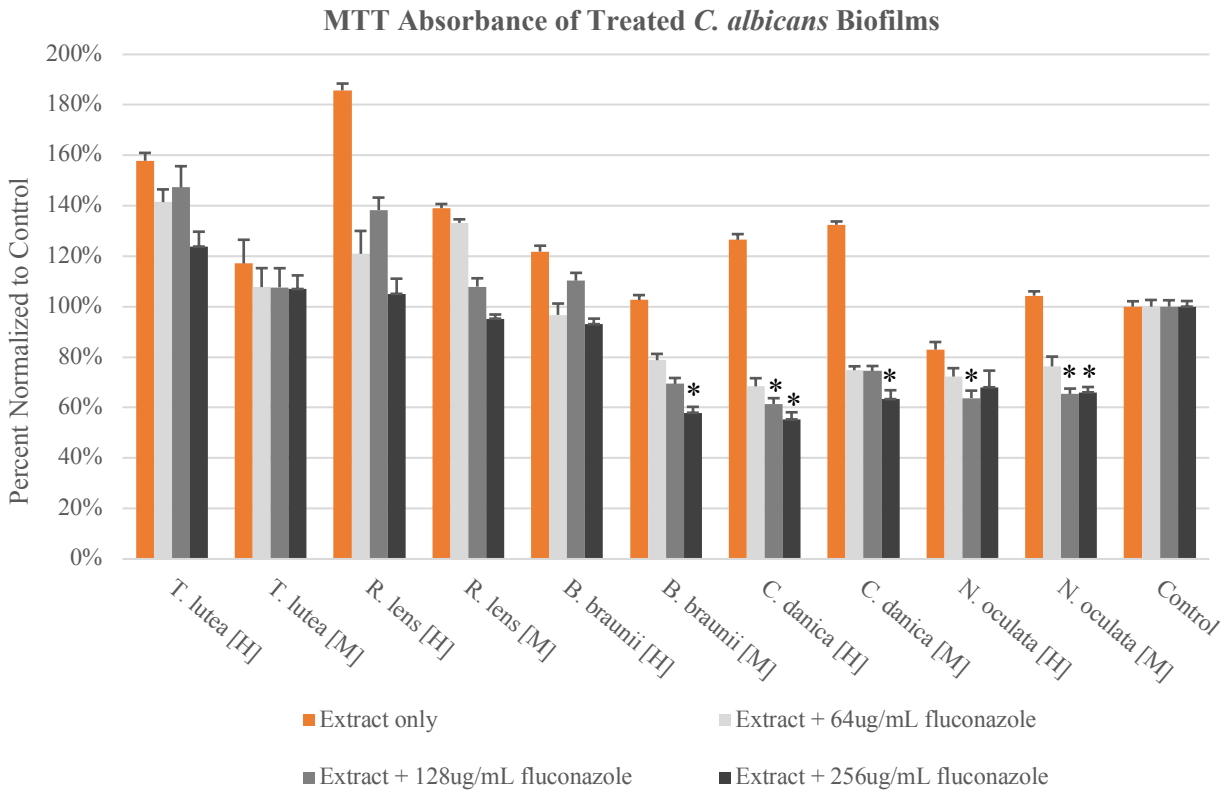

Figure 22. Cell viability of *C. albicans* in established biofilms treated with algal extract and fluconazole. Measurements are based on MTT absorbances and are normalized to their respective control groups. For combined treatments, control groups were treated with acetonitrile or fluconazole only.

The majority of treatments had little effect on cell viability, with many extracts only treatments increasing cell viability. However, there were a few extracts that showed a statistically significant reduction with higher concentrations of fluconazole. For treatments with extract and 128µg/mL of fluconazole, *C. danica* [H] ($p = 0.003$), *N. oculata* [H] ($p = 0.006$), *N. oculata* [M] ($p = 0.009$). For treatments with extract and 256µg/mL of fluconazole, *B. braunii* [M] ($p = 0.004$), *C. danica* [H] ($p = 0.002$), *C. danica* [M] ($p = 0.021$), *N. oculata* [M] ($p = 0.042$).

Summary of Antimicrobial Effects

A summary of the antimicrobial effects by algal extracts observed in this study is presented in Table 6. While the extracts did not exhibit strong growth inhibitory or bactericidal activity as single agents, the extracts in both solvent systems from *B. braunii*, *C. Danica*, and *N. oculata* caused significant decreases in *S. maltophilia* cell viability in biofilms when combined with either ceftriaxone or aztreonam. Extracts from these species also decreased cell viability in *Candida* biofilms when combined with fluconazole. Extracts from *T. lutea* and *R. lens* demonstrated limited impacts on either biofilm formation or antibiotic sensitivity of established biofilms in *S. maltophilia* or *C. albicans*.

***Table 6*. Summarization of Antimicrobial Effects**

			B. braunii [H]	*B. braunii* [M]	*C. danica* [H]	*C. danica* [M]	*N. oculata* [H]	*N. oculata* [M]
S. maltophilia	Planktonic Growth		-	+	+	-	+	+
	Biofilm Formation	CV	-	-	-	-	-	-
	Extract Only	CV	-	++	+	+	-	-
		MTT	+++	+++	++	++	+++	++
	Extract + Aztreonam	CV	-	+	+	+	-	-
		MTT	+++	+++	++	++	+++	+++
	Extract + Ceftriaxone	CV	+	+	+	-	-	-
		MTT	+	+++	++	++	+++	+++
C. albicans	Extract Only	CV	-	-	-	-	-	-
		MTT	-	-	-	-	-	-
	Extract + 64µg/mL fluconazole	CV	-	-	-	-	-	-
		MTT	-	-	+	-	+	-
	Extract + 128µg/mL fluconazole	CV	-	-	-	-	-	-
		MTT	-	+	+	+	+	+
	Extract + 256µg/mL fluconazole	CV	-	-	-	-	-	-
		MTT	-	+	+	+	+	+

Summarization of data for raw algal extracts. Only extracts with previous statistical significance are shown. A scale was used to present a visualization of performance across each test: '+' = reduction of 25%, '++' = reduction of 50%, and '+++' = reduction of 75% or more. For planktonic growth, the greatest reduction percentage was used.

The grouped scatter plot in Figure 17 shows a pairwise ratio of CV and MTT per extract based on extraction solvent and antibiotic combination for treatment. The overall distribution for the extraction solvent per treatment is similar, with the most noticeable difference of no antibiotic

(extract only). A statistical comparison of algal extracts based on extraction solvent using a Mann-Whitney U test was performed for each treatment condition in respect to CV and MTT percentages. Two scenarios were found to have been statistically different based on solvent: *B. braunii* CV for extract only treatment ($p = 0.045$) and *C. danica* CV for "extract + Aztreonam" ($p = 0.030$). All other instances showed no statistical significance between solvents per algae species and antibiotic.

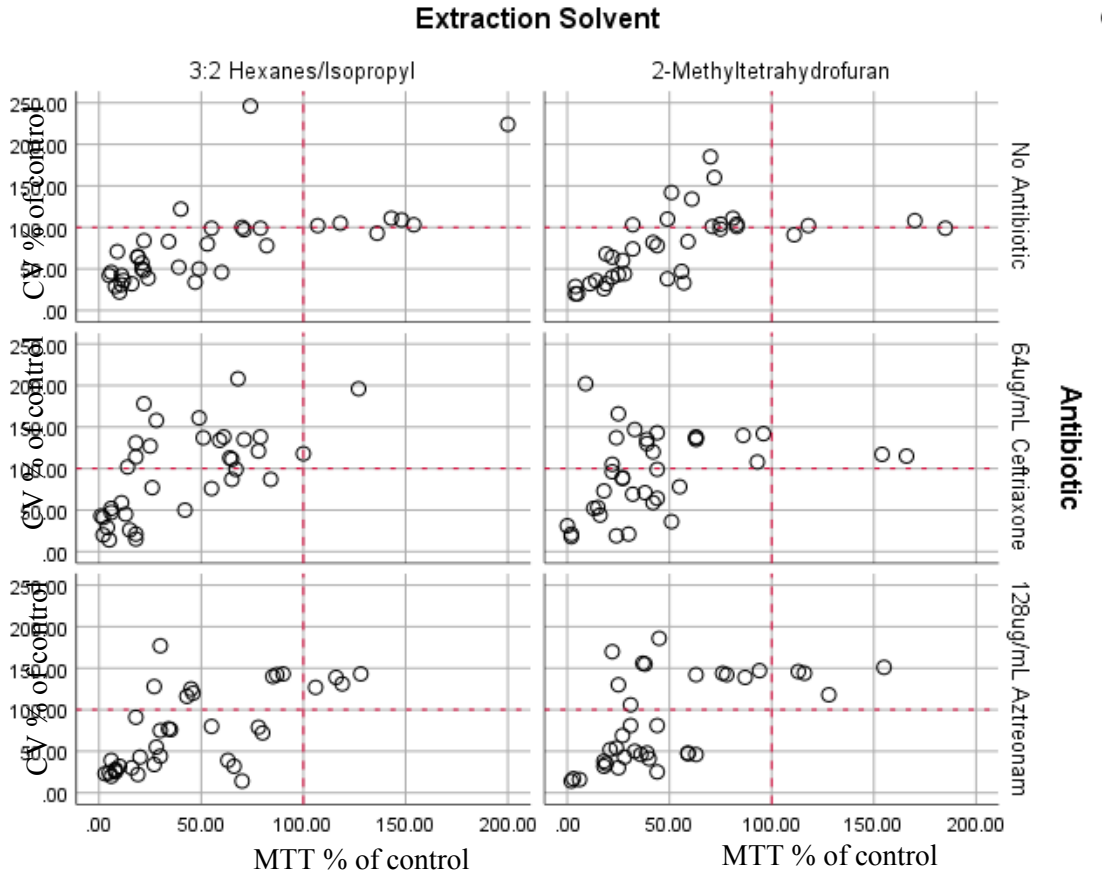

Figure 23. Solvent scatter plot of extract CV and MTT pairwise mean percentages grouped by antibiotic. Dotted red lines indicate control group to which each series is normalized.

Discussion and Conclusion

Extraction Solvents

Commercialized chemical processes require large amounts of solvents. With modern safety regulations, many processes are being re-evaluated with a focus on reducing environmental impact and increasing efficiency. Dichloromethane, diethyl ether, 1-ethyl-3-methylimidazolium methyl sulfate, benzene, petroleum ether, acetone, and chloroform have all been used as solvents and cosolvents to extract natural components, but most of these solvents are highly toxic to wildlife and pose serious health risks to humans.[140–144] Alternative solvents that are safer and more renewably sourced such as 2-MTHF provide and attractive alternative to traditional extraction solvents.

In the current study, two solvents were used in extraction of algal components, HIPA (3:1) and 2-MTHF. Extracted residue masses varied by species and solvent with the largest extracted masses seen with 2-MTHF from *C. danica* (14.6ug/mL), and the smallest extracted mass with HIPA from *R. lens* (3.6ug/mL). Based on mass, 2-MTHF produced generally higher yields than the HIPA except in the case of *N. oculata* (5.4ug/mL [M] and 5.6ug/mL [H]). As predicted, the higher yields of 2-MTHF was also associated with greater polyphenol concentration ($p = 0.008$). Improving polyphenol yield is important factor in future natural products research because their antimicrobial potential. These experiments demonstrate that the performance of 2-MTHF extracts in the antimicrobial assays described is comparable to the performance of the more traditional HIPA extracts.

Impacts on *S. maltophilia* Biofilm Formation and Cell Viability in Established Biofilms

Biofilm protection of nosocomial pathogens is responsible for many resistant hospital infections originating from implanted medical devices. Currently, the only way to treat such

infections is to remove the affected device because the film cannot be disrupted or penetrated with chemotherapy. For this reason, agents that can inhibit biofilm formation, alter biofilm metabolism, or increase sensitivity of biofilms to existing antimicrobial agents will constitute an important step in effectively treating biofilm-related infections. Reports describing anti-biofilm strategies have increased in frequency, indicating that this type of intervention will continue to be of interest amid escalations in resistant microbial infections.[145,146]

Biofilm formation by *S. maltophilia* was not significantly impacted by the crude algal extracts tested (Table 5). However, biofilms treated with extracts from the oleaginous algae, *B. braunii* and *N. oculata*, and the heterotrophic algae, *C. danica*, showed significant decreases in cell viability relative to the untreated control (acetonitrile only). This effect was observed with extracts from both solvent systems and was essentially the same whether the extracts were used as single agents or in combination with aztreonam and ceftriaxone. Taken together, these results suggest that (i) the algal extracts did not significantly impact the access or interactions of the selected antibiotics with biofilm cells; and (ii) likely components of the extracts such as polyphenols, quorum signaling molecules, neutral lipids, and some classes of fatty acids, have bactericidal or bacteriostatic effects.[23,68,70,118]

These data may also point to the involvement of *S. maltophilia* biofilm hydrophobicity in the observed effects of crude algal extracts on cell viability. Localized hydrophobic and hydrophilic regions are found in established bacterial biofilms.[101] The hydrophobicity may be linked to discrete purposes such as water storage. Hydrophobic regions are more likely to interact with many of the organic components of the algal extracts such as fatty acids and less polar polyphenols. On the other hand, the antibiotics, aztreonam and ceftriaxone, while amphipathic overall, have high polar surface area (PSA) meaning they are more likely to interact

with hydrophilic regions. It's possible that *S. maltophilia* produces largely hydrophobic ECM, allowing penetrance of the organic components but not the antibiotics.

Impacts on *C. albicans* Cell Viability in Established Biofilms.

C. albicans forms robust films that are difficult to remove or degrade. These yeast films have been shown to be highly hydrophobic due to the production, secretion, and branching of β-1,3 glucan throughout the ECM.[106] The branched polymers increase hydrophobicity which limits antifungal drug penetrance. Fluconazole it often used as a first line treatment for fungal infections, but biofilm associated *C. albicans* becomes exceedingly resistant. As reviewed by Desai et al., the biofilm is the single largest factor in resistance seen in *C. albicans*.[28]

Algal extracts showed in this study demonstrated almost no effect on biofilm mass as measured by CV staining, on their own or combined with fluconazole. However, some of the extracts were effective in reducing cell viability in *C. albicans* biofilms. Interestingly, the extracts that reduced cell viability in *S. maltophilia* biofilms, namely those from *B. braunii, N. oculata*, and *C. danica*, were also effective in decreasing the cell viability of fluconazole-treated C. albicans biofilms. Extracts did not significantly impact cell viability of C. albicans when used as single agents but did increase sensitivity to fluconazole at higher concentrations (128 μg/mL and 256 μg/mL). These findings may strengthen the argument for hydrophobic interactions of extract components driving changes at the film surface, since the less polar structure of fluconazole (relative to ceftriaxone and aztreonam) could render it more interactive with nonpolar and amphipathic components of the extracts and thereby provide improved access for fluconazole to filmed cells in the hydrophobic ECM of a C. albicans biofilm.

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APPENDIX A. DNA Isolation and 16S rRNA Profiling of Algal Cultures

Algal cultures were evaluated for bacterial constituency at harvest by pelleting a 2 mL volume of culture and storing the pellet at -20°C for DNA isolation and PCR using amplification of 16S rRNA according to the method of Krohn-Molt.[147] The results of this analysis for cultures extracted as described in these studies are given in Table AA1.

***Table A1*. Bacterial Constituency in Algal Cultures for Extraction.**

Algal Culture	Amplification of 16S rRNA Indicating the Presence of Bacteria
T. lutea	positive
R. lens	positive
B. braunii	positive
C. danica	positive
N. oculata	positive

APPENDIX B. Supplementary Data

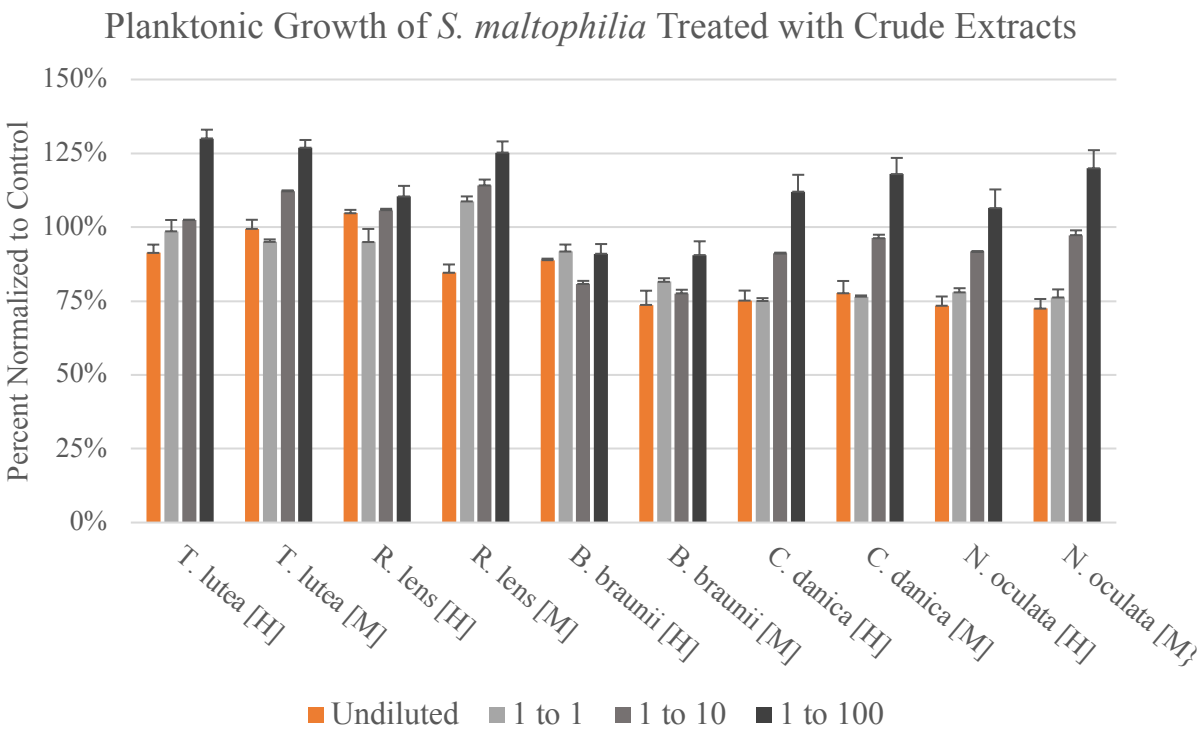

Figure B1. Impact of crude extracts on planktonic growth of *S. maltophilia*. All growth percentages are normalized to untreated control group. Error bars represent standard deviation.

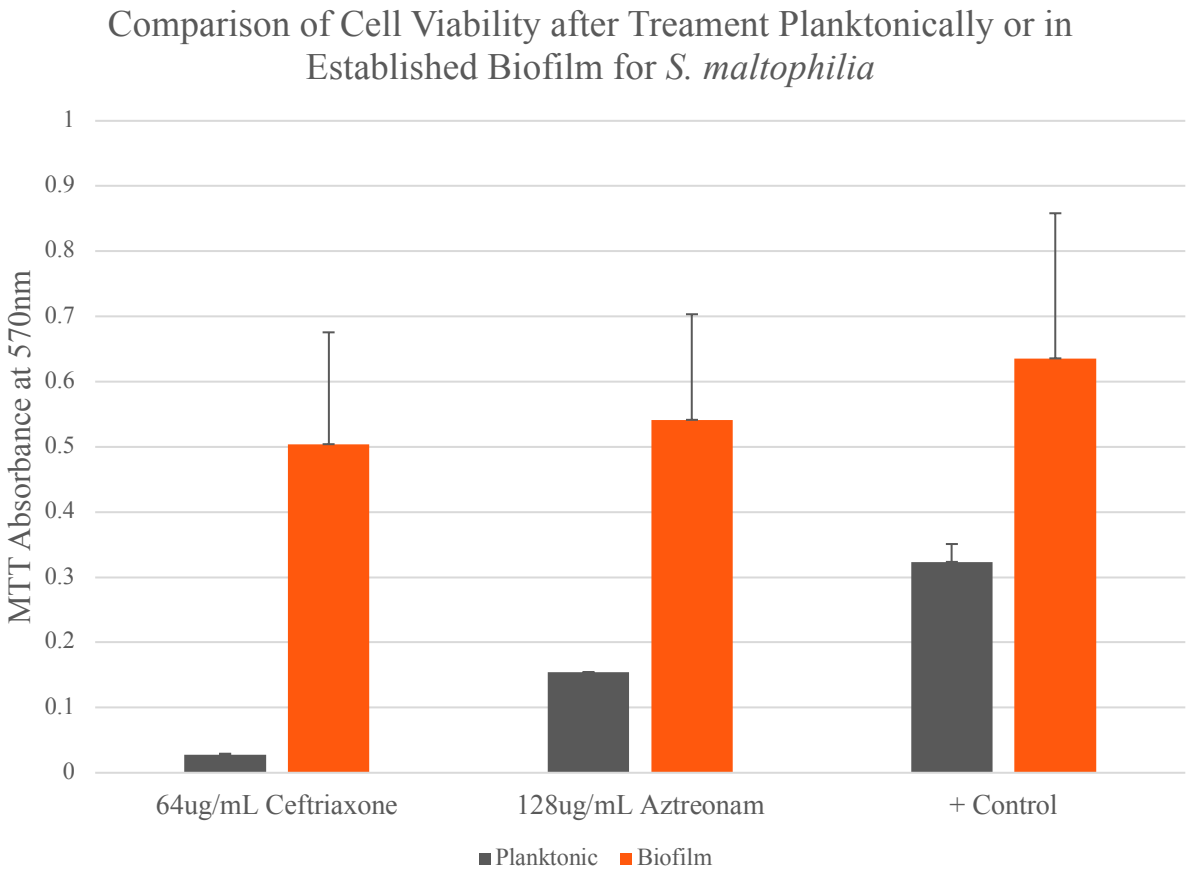

Figure B2. Comparison of sensitivity to antibiotic treatment planktonically vs. in an established biofilm. Error bars represent standard deviation.

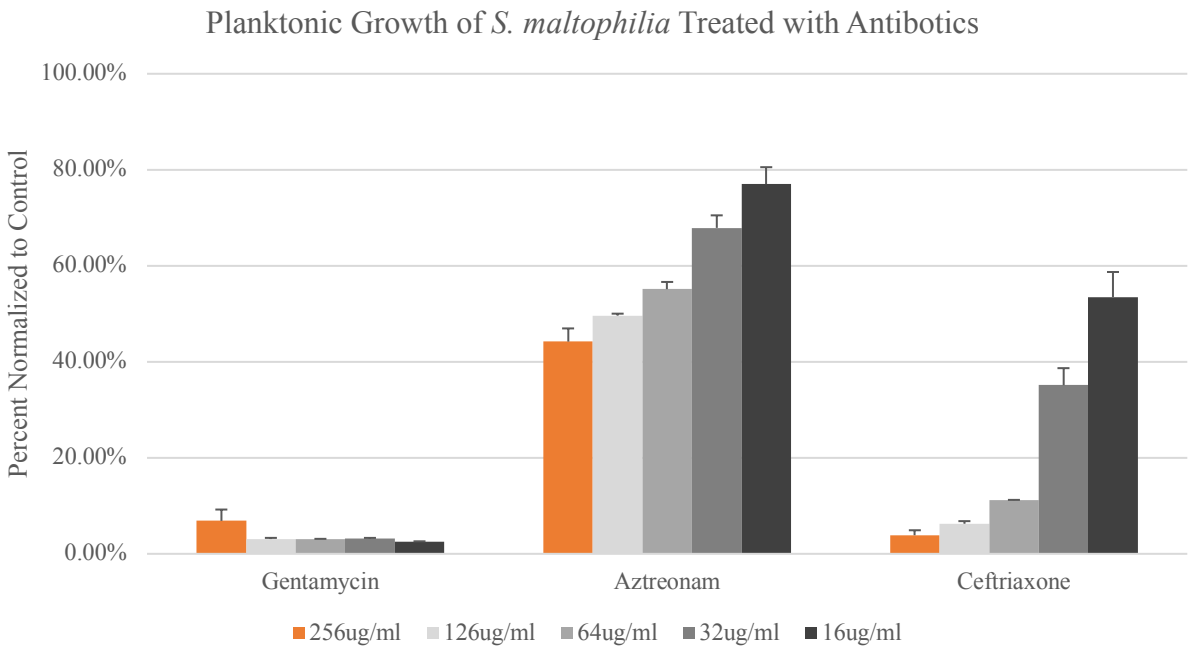

Figure B3. Impact of antibiotics on planktonic growth of *S. maltophilia*. All growth percentages are normalized to an untreated control group. Error bars represent standard deviation.

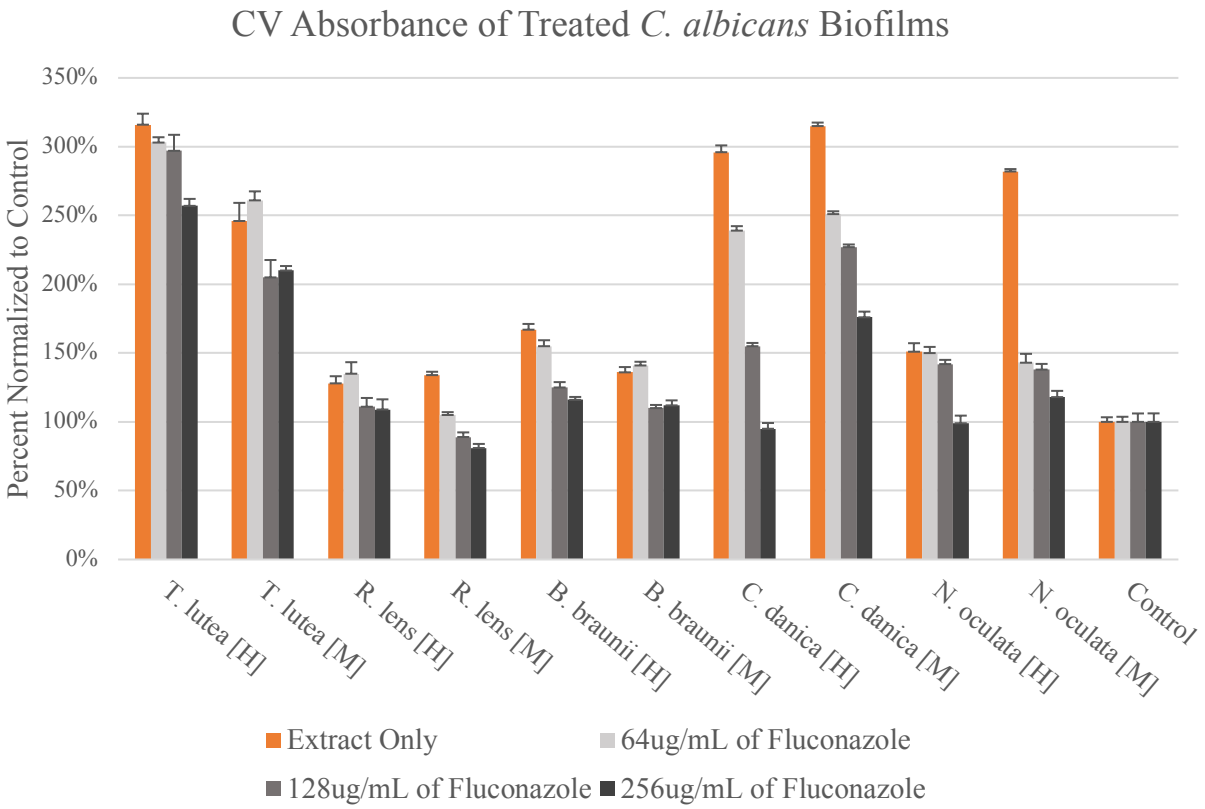

Figure B4. Measured biomass of established *C. albicans* films treated with algal extracts and combined treatment with fluconazole. Measurements are based on CV absorbance and are normalized to their respective control groups. For combined treatments, control groups were treated with acetonitrile or fluconazole only.

APPENDIX C. Statistical Analyses

Table C1. **Kruskal-Wallis analysis of solvent by treatment, extract only versus extracts + 128ug/mL of aztreonam.**

Test Statistics[a,b]

Algae	Solvent		CV	MTT
B. braunii	HIPA	Kruskal-Wallis H	.006	.058
		df	1	1
		Asymp. Sig.	.936	.810
	2-MTHF	Kruskal-Wallis H	1.452	.161
		df	1	1
		Asymp. Sig.	.228	.688
C. danica	HIPA	Kruskal-Wallis H	5.026	.026
		df	1	1
		Asymp. Sig.	.025	.873
	2-MTHF	Kruskal-Wallis H	.643	.103
		df	1	1
		Asymp. Sig.	.423	.748
N. oculata	HIPA	Kruskal-Wallis H	.521	.162
		df	1	1
		Asymp. Sig.	.470	.687
	2-MTHF	Kruskal-Wallis H	2.084	.026
		df	1	1
		Asymp. Sig.	.149	.872

a. Kruskal Wallis Test

b. Grouping Variable: Antibiotic

Table C2. **Kruskal-Wallis analysis of solvent by treatment, extract +128ug/mL versus extract + 64ug/mL of ceftriaxone**.

Test Statistics[a,b]

Algae	Solvent		CV	MTT
B. braunii	HIPA	Kruskal-Wallis H	.006	.161
		df	1	1
		Asymp. Sig.	.936	.688
	2-MTHF	Kruskal-Wallis H	1.641	.940
		df	1	1
		Asymp. Sig.	.200	.332
C. danica	HIPA	Kruskal-Wallis H	6.564	.231
		df	1	1
		Asymp. Sig.	.010	.631
	2-MTHF	Kruskal-Wallis H	8.337	.234
		df	1	1
		Asymp. Sig.	.004	.629
N. oculata	HIPA	Kruskal-Wallis H	2.564	.058
		df	1	1
		Asymp. Sig.	.109	.810
	2-MTHF	Kruskal-Wallis H	2.084	.412
		df	1	1
		Asymp. Sig.	.149	.521

a. Kruskal Wallis Test

b. Grouping Variable: Antibiotic

***Table C3*. Kruskal-Wallis analysis of solvent by treatment, extract only versus extract + 64ug/mL of ceftriaxone.**

Test Statistics[a,b]

Algae	Solvent		CV	MTT
B. braunii	HIPA	Kruskal-Wallis H	.006	.006
		df	1	1
		Asymp. Sig.	.936	.936
	2-MTHF	Kruskal-Wallis H	.232	.104
		df	1	1
		Asymp. Sig.	.630	.747
C. danica	HIPA	Kruskal-Wallis H	.103	.316
		df	1	1
		Asymp. Sig.	.749	.574
	2-MTHF	Kruskal-Wallis H	2.837	.231
		df	1	1
		Asymp. Sig.	.092	.631
N. oculata	HIPA	Kruskal-Wallis H	1.859	.412
		df	1	1
		Asymp. Sig.	.173	.521
	2-MTHF	Kruskal-Wallis H	.641	.234
		df	1	1
		Asymp. Sig.	.423	.629

a. Kruskal Wallis Test

b. Grouping Variable: Antibiotic

Table C4. **ANOVA post-hoc Dunnett test comparing corresponding control groups to extracts and extracts with treatment against *S. maltophilia*.**

Multiple Comparisons

Dunnett t (<control)[a]

Solvent	Treatment	Dependent Variable	Algae	Comparison Control	Mean Difference	Sig.	95% (CI) Upper Bound
HIPA	Extract Only	CV	T. lutea	Control	21.16667	.983	71.3824
			R. lens	Control	36.50000	.998	86.7157
			B. braunii	Control	-40.00000	.124	10.2157
			C. danica	Control	-45.83333	.075	4.3824
			N. oculata	Control	-36.16667	.167	14.0491
		MTT	T. lutea	Control	-31.66667	.109	6.5945
			R. lens	Control	-12.83333	.518	25.4278
			B. braunii	Control	-82.16667*	.000	-43.9055
			C. danica	Control	-69.33333*	.000	-31.0722
			N. oculata	Control	-83.83333*	.000	-45.5722
	with Aztreonam (128ug/mL)	CV	T. lutea	Control	5.83333	.906	52.3670
			R. lens	Control	-16.66667	.493	29.8670
			B. braunii	Control	-33.50000	.167	13.0337
			C. danica	Control	-67.50000*	.004	-20.9663
			N. oculata	Control	-48.66667*	.040	-2.1330
		MTT	T. lutea	Control	-32.33333	.056	.8336
			R. lens	Control	-36.50000*	.030	-3.3331
			B. braunii	Control	-77.50000*	.000	-44.3331
			C. danica	Control	-69.00000*	.000	-35.8331
			N. oculata	Control	-82.33333*	.000	-49.1664
	with Ceftriaxone (64ug/mL)	CV	T. lutea	Control	16.83333	.953	82.3827
			R. lens	Control	-31.33333	.368	34.2161
			B. braunii	Control	-9.33333	.717	56.2161
			C. danica	Control	-41.83333	.225	23.7161
			N. oculata	Control	-9.16667	.719	56.3827
		MTT	T. lutea	Control	-44.50000*	.029	-4.3707
			R. lens	Control	-51.00000*	.012	-10.8707
			B. braunii	Control	-61.33333*	.003	-21.2040
			C. danica	Control	-68.33333*	.001	-28.2040
			N. oculata	Control	-86.00000*	.000	-45.8707

2-MTHF	Extract Only	CV	T. lutea	Control	41.33333	.998	100.0748
			R. lens	Control	49.33333	.999	108.0748
			B. braunii	Control	-64.83333*	.030	-6.0919
			C. danica	Control	-48.33333	.111	10.4081
			N. oculata	Control	.16667	.835	58.9081
		MTT	T. lutea	Control	-26.83333	.174	10.9762
			R. lens	Control	-24.16667	.224	13.6429
			B. braunii	Control	-89.83333*	.000	-52.0238
			C. danica	Control	-61.83333*	.001	-24.0238
			N. oculata	Control	-68.16667*	.000	-30.3571
	with Aztreonam (128ug/mL)	CV	T. lutea	Control	33.83333	.999	76.9529
			R. lens	Control	-9.00000	.652	34.1195
			B. braunii	Control	-71.50000*	.001	-28.3805
			C. danica	Control	-56.83333*	.009	-13.7138
			N. oculata	Control	-30.50000	.176	12.6195
		MTT	T. lutea	Control	-29.83333	.156	10.5578
			R. lens	Control	-15.83333	.457	24.5578
			B. braunii	Control	-81.83333*	.000	-41.4422
			C. danica	Control	-62.16667*	.003	-21.7755
			N. oculata	Control	-70.16667*	.001	-29.7755
	with Ceftriaxone (64ug/mL)	CV	T. lutea	Control	42.83333	.999	91.6920
			R. lens	Control	-28.66667	.267	20.1920
			B. braunii	Control	-51.50000*	.038	-2.6413
			C. danica	Control	-28.66667	.267	20.1920
			N. oculata	Control	19.00000	.979	67.8587
		MTT	T. lutea	Control	-49.33333*	.023	-6.6864
			R. lens	Control	-22.16667	.327	20.4802
			B. braunii	Control	-87.66667*	.000	-45.0198
			C. danica	Control	-60.33333*	.005	-17.6864
			N. oculata	Control	-70.16667*	.001	-27.5198

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

***Table C5*. Mann-Whitney U comparison of component concentration by extraction solvent.**

Test Statistics[a]

	Antioxidant	Polyphenols	Flavonoid
Mann-Whitney U	6.000	0.000	12.000
Wilcoxon W	21.000	15.000	27.000
Z	-1.358	-2.611	-0.104
Asymp. Sig. (2-tailed)	0.175	0.009	0.917
Exact Sig. [2*(1-tailed Sig.)]	0.222[b]	0.008	1.000[b]

a. Grouping Variable: Solvent

b. Not corrected for ties.

***Table C6*. ANOVA post-hoc Dunnett test comparing control group to extracts with 64ug/mL of fluconazole against *C. albicans*.**

	Extract	Comparison	Mean Difference	Sig.	95% (CI) Upper Bound
Dunnett t (<control)[b]	*T. lutea* [H]	Control	0.4727	1.000	0.74492
	T. lutea [M]	Control	0.0775	0.986	0.34876
	R. lens [H]	Control	0.3831	1.000	0.65540
	R. lens [M]	Control	0.7923	0.987	0.35104
	B. braunii [H]	Control	1.0309*	0.994	0.37530
	B. braunii [M]	Control	-3.0607	0.024	-0.03385
	C. danica [H]	Control	-0.3855*	0.003	-0.11330
	C. danica [M]	Control	-0.2550	0.071	0.01716
	N. oculata [H]	Control	-0.3619*	0.006	-0.08977
	N. oculata [M]	Control	-0.3457*	0.009	-0.07356

*The mean difference is significant at the 0.05 level.

b. the Dunnett t-tests treat one group as a control and compare all other groups against it.

Table C7. **ANOVA post-hoc Dunnett test comparing control group to extracts with 128ug/mL of fluconazole against *C. albicans*.**

	Extract	Comparison	Mean Difference	Sig.	95% (CI) Upper Bound
Dunnett t (<control)[b]	*T. lutea* [H]	Control	0.3692	1.000	0.74492
	T. lutea [M]	Control	0.1832	1.000	0.34876
	R. lens [H]	Control	0.1620	0.999	0.65540
	R. lens [M]	Control	0.0530	0.975	0.35104
	B. braunii [H]	Control	0.0291	0.954	0.37530
	B. braunii [M]	Control	-0.3592*	0.004	-0.03385
	C. danica [H]	Control	-0.3883*	0.002	-0.11330
	C. danica [M]	Control	-0.2995*	0.021	0.01716
	N. oculata [H]	Control	-0.2475	0.067	-0.08977
	N. oculata [M]	Control	-0.2696*	0.042	-0.07356

*The mean difference is significant at the 0.05 level.

b. the Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table C8. C. albicans **MTT ANOVA test by treatment, grouped by extraction solvent.**

ANOVA

Species	Solvent		Sum of Squares	df	Mean Square	F	Sig.
Control	Control	Between Groups	.009	2	.004	.113	.893
		Within Groups	.936	24	.039		
		Total	.945	26			
T. lutea	HIPA	Between Groups	.011	2	.006	4.630	.061
		Within Groups	.007	6	.001		
		Total	.018	8			
	2-MTHF	Between Groups	.045	2	.023	3.520	.097
		Within Groups	.038	6	.006		
		Total	.084	8			
R. lens	HIPA	Between Groups	.005	2	.002	1.373	.323
		Within Groups	.010	6	.002		
		Total	.014	8			
	2-MTHF	Between Groups	.000	2	.000	.034	.967
		Within Groups	.011	6	.002		
		Total	.011	8			
B. braunii	HIPA	Between Groups	.000	2	.000	4.009	.078
		Within Groups	.000	6	.000		
		Total	.000	8			
	2-MTHF	Between Groups	.000	2	.000	2.298	.182
		Within Groups	.000	6	.000		
		Total	.000	8			
C. danica	HIPA	Between Groups	.001	2	.000	1.961	.221
		Within Groups	.001	6	.000		
		Total	.002	8			
	2-MTHF	Between Groups	.002	2	.001	1.767	.249
		Within Groups	.004	6	.001		
		Total	.006	8			
N. oculata	HIPA	Between Groups	.000	2	.000	.497	.631
		Within Groups	.000	6	.000		
		Total	.001	8			
	2-MTHF	Between Groups	.004	2	.002	9.786	.013
		Within Groups	.001	6	.000		
		Total	.005	8			

***Table C9*. Multiple Comparison analysis using Tukey HSD and Dunnett test for *S. maltophilia* cell viability**.

Species	Solvent		(I) Treatment	(J) Comparison	Mean Difference (I-J)	Std. Error	Sig.	Upper Bound
Control	Control	Tukey HSD	No Antibiotic	Aztreonam	.03268888	.09310048	.934	.2651875
				Ceftriaxone	.04223332	.09310048	.893	.2747319
			Aztreonam	No Antibiotic	-.03268888	.09310048	.934	.1998097
				Ceftriaxone	.00954444	.09310048	.994	.2420431
			Ceftriaxone	No Antibiotic	-.04223332	.09310048	.893	.1902653
				Aztreonam	-.00954444	.09310048	.994	.2229542
		Dunnett T3	No Antibiotic	Aztreonam	.03268888	.09706698	.981	.2908266
				Ceftriaxone	.04223332	.09562820	.959	.2969348
			Aztreonam	No Antibiotic	-.03268888	.09706698	.981	.2254489
				Ceftriaxone	.00954444	.08623426	.999	.2381008
			Ceftriaxone	No Antibiotic	-.04223332	.09562820	.959	.2124682
				Aztreonam	-.00954444	.08623426	.999	.2190119
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.03268888	.09310048	.914	.1860421
			Ceftriaxone	No Antibiotic	-.04223332	.09310048	.863	.1764977
T. lutea	HIPA	Tukey HSD	No Antibiotic	Aztreonam	-.06550001	.02832428	.130	.0214067
				Ceftriaxone	-.08126667	.02832428	.064	.0056400
			Aztreonam	No Antibiotic	.06550001	.02832428	.130	.1524067
				Ceftriaxone	-.01576666	.02832428	.847	.0711400
			Ceftriaxone	No Antibiotic	.08126667	.02832428	.064	.1681733
				Aztreonam	.01576666	.02832428	.847	.1026733
		Dunnett T3	No Antibiotic	Aztreonam	-.06550001	.03319070	.305	.0785623
				Ceftriaxone	-.08126667	.03084062	.204	.0773878
			Aztreonam	No Antibiotic	.06550001	.03319070	.305	.2095623
				Ceftriaxone	-.01576666	.01881562	.792	.0613290
			Ceftriaxone	No Antibiotic	.08126667	.03084062	.204	.2399211
				Aztreonam	.01576666	.01881562	.792	.0928623
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	.06550001	.02832428	.103	.1465853
			Ceftriaxone	No Antibiotic	.08126667*	.02832428	.050	.1623520
	2-MTHF	Tukey HSD	No Antibiotic	Aztreonam	.14376667	.06540351	.150	.3444426
				Ceftriaxone	.15606667	.06540351	.118	.3567426
			Aztreonam	No Antibiotic	-.14376667	.06540351	.150	.0569092
				Ceftriaxone	.01229999	.06540351	.981	.2129759
			Ceftriaxone	No Antibiotic	-.15606667	.06540351	.118	.0446092

				Aztreonam	-.01229999	.06540351	.981	.1883759
		Dunnett T3	No Antibiotic	Aztreonam	.14376667	.07994726	.409	.6391663
				Ceftriaxone	.15606667	.07968616	.367	.6569375
			Aztreonam	No Antibiotic	-.14376667	.07994726	.409	.3516330
				Ceftriaxone	.01229999	.00956068	.559	.0520184
			Ceftriaxone	No Antibiotic	-.15606667	.07968616	.367	.3448041
				Aztreonam	-.01229999	.00956068	.559	.0274184
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.14376667	.06540351	.119	.0434672
			Ceftriaxone	No Antibiotic	-.15606667	.06540351	.093	.0311672
R. lens	HIPA	Tukey HSD	No Antibiotic	Aztreonam	.04476667	.03318984	.422	.1466022
				Ceftriaxone	.05006667	.03318984	.352	.1519022
			Aztreonam	No Antibiotic	-.04476667	.03318984	.422	.0570689
				Ceftriaxone	.00530000	.03318984	.986	.1071355
			Ceftriaxone	No Antibiotic	-.05006667	.03318984	.352	.0517689
				Aztreonam	-.00530000	.03318984	.986	.0965355
		Dunnett T3	No Antibiotic	Aztreonam	.04476667	.04059443	.673	.2958170
				Ceftriaxone	.05006667	.04041899	.613	.3047969
			Aztreonam	No Antibiotic	-.04476667	.04059443	.673	.2062836
				Ceftriaxone	.00530000	.00480555	.658	.0270941
			Ceftriaxone	No Antibiotic	-.05006667	.04041899	.613	.2046636
				Aztreonam	-.00530000	.00480555	.658	.0164941
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.04476667	.03318984	.360	.0502476
			Ceftriaxone	No Antibiotic	-.05006667	.03318984	.295	.0449476
	2-MTHF	Tukey HSD	No Antibiotic	Aztreonam	.00400000	.03495912	.993	.1112642
				Ceftriaxone	.00906667	.03495912	.964	.1163308
			Aztreonam	No Antibiotic	-.00400000	.03495912	.993	.1032642
				Ceftriaxone	.00506666	.03495912	.989	.1123308
			Ceftriaxone	No Antibiotic	-.00906667	.03495912	.964	.0981975
				Aztreonam	-.00506666	.03495912	.989	.1021975
		Dunnett T3	No Antibiotic	Aztreonam	.00400000	.03911431	.999	.1748729
				Ceftriaxone	.00906667	.03862196	.992	.1818146
			Aztreonam	No Antibiotic	-.00400000	.03911431	.999	.1668729
				Ceftriaxone	.00506666	.02539361	.995	.1003199
			Ceftriaxone	No Antibiotic	-.00906667	.03862196	.992	.1636813
				Aztreonam	-.00506666	.02539361	.995	.0901865
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.00400000	.03495912	.990	.0960792
			Ceftriaxone	No Antibiotic	-.00906667	.03495912	.952	.0910126
	HIPA	Tukey HSD		Aztreonam	.00103334	.00438558	.970	.0144895

B. braunii			No Antibiotic	Ceftriaxone	.01123333	.00438558	.094	.0246895
			Aztreonam	No Antibiotic	-.00103334	.00438558	.970	.0124228
				Ceftriaxone	.01020000	.00438558	.127	.0236562
			Ceftriaxone	No Antibiotic	-.01123333	.00438558	.094	.0022228
				Aztreonam	-.01020000	.00438558	.127	.0032562
		Dunnett T3	No Antibiotic	Aztreonam	.00103334	.00465140	.993	.0236994
				Ceftriaxone	.01123333	.00507959	.236	.0321905
			Aztreonam	No Antibiotic	-.00103334	.00465140	.993	.0216327
				Ceftriaxone	.01020000	.00320347	.098	.0232217
			Ceftriaxone	No Antibiotic	-.01123333	.00507959	.236	.0097239
				Aztreonam	-.01020000	.00320347	.098	.0028217
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.00103334	.00438558	.960	.0115215
			Ceftriaxone	No Antibiotic	-.01123333	.00438558	.074	.0013215
	2-MTHF	Tukey HSD	No Antibiotic	Aztreonam	-.00080000	.00461142	.984	.0133491
				Ceftriaxone	.00813333	.00461142	.259	.0222824
			Aztreonam	No Antibiotic	.00080000	.00461142	.984	.0149491
				Ceftriaxone	.00893334	.00461142	.209	.0230824
			Ceftriaxone	No Antibiotic	-.00813333	.00461142	.259	.0060158
				Aztreonam	-.00893334	.00461142	.209	.0052158
		Dunnett T3	No Antibiotic	Aztreonam	-.00080000	.00528646	.998	.0322611
				Ceftriaxone	.00813333	.00203087	.105	.0199117
			Aztreonam	No Antibiotic	.00080000	.00528646	.998	.0338611
				Ceftriaxone	.00893334	.00563245	.452	.0370869
			Ceftriaxone	No Antibiotic	-.00813333	.00203087	.105	.0036451
				Aztreonam	-.00893334	.00563245	.452	.0192202
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	.00080000	.00461142	.978	.0140013
			Ceftriaxone	No Antibiotic	-.00813333	.00461142	.212	.0050680
C. danica	HIPA	Tukey HSD	No Antibiotic	Aztreonam	-.00580000	.01281622	.895	.0335237
				Ceftriaxone	.01850000	.01281622	.379	.0578237
			Aztreonam	No Antibiotic	.00580000	.01281622	.895	.0451237
				Ceftriaxone	.02430000	.01281622	.220	.0636237
			Ceftriaxone	No Antibiotic	-.01850000	.01281622	.379	.0208237
				Aztreonam	-.02430000	.01281622	.220	.0150237
		Dunnett T3	No Antibiotic	Aztreonam	-.00580000	.01494334	.968	.0737926
				Ceftriaxone	.01850000	.00651869	.115	.0429993
			Aztreonam	No Antibiotic	.00580000	.01494334	.968	.0853926
				Ceftriaxone	.02430000	.01506553	.446	.1025038

			Ceftriaxone	No Antibiotic	-.01850000	.00651869	.115	.0059993
				Aztreonam	-.02430000	.01506553	.446	.0539038
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	.00580000	.01281622	.866	.0424897
			Ceftriaxone	No Antibiotic	-.01850000	.01281622	.320	.0181897
	2-MTHF	Tukey HSD	No Antibiotic	Aztreonam	-.01906666	.01973566	.623	.0414878
				Ceftriaxone	.01803334	.01973566	.652	.0785878
			Aztreonam	No Antibiotic	.01906666	.01973566	.623	.0796211
				Ceftriaxone	.03710000	.01973566	.224	.0976544
			Ceftriaxone	No Antibiotic	-.01803334	.01973566	.652	.0425211
				Aztreonam	-.03710000	.01973566	.224	.0234544
		Dunnett T3	No Antibiotic	Aztreonam	-.01906666	.02353298	.806	.0753809
				Ceftriaxone	.01803334	.01427974	.583	.0867445
			Aztreonam	No Antibiotic	.01906666	.02353298	.806	.1135142
				Ceftriaxone	.03710000	.02026763	.383	.1475928
			Ceftriaxone	No Antibiotic	-.01803334	.01427974	.583	.0506778
				Aztreonam	-.03710000	.02026763	.383	.0733928
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	.01906666	.01973566	.558	.0755649
			Ceftriaxone	No Antibiotic	-.01803334	.01973566	.589	.0384649
N. oculata	HIPA	Tukey HSD	No Antibiotic	Aztreonam	.00600000	.00725243	.701	.0282525
				Ceftriaxone	.00650000	.00725243	.662	.0287525
			Aztreonam	No Antibiotic	-.00600000	.00725243	.701	.0162525
				Ceftriaxone	.00050000	.00725243	.997	.0227525
			Ceftriaxone	No Antibiotic	-.00650000	.00725243	.662	.0157525
				Aztreonam	-.00050000	.00725243	.997	.0217525
		Dunnett T3	No Antibiotic	Aztreonam	.00600000	.00613732	.721	.0323935
				Ceftriaxone	.00650000	.00836381	.823	.0383162
			Aztreonam	No Antibiotic	-.00600000	.00613732	.721	.0203935
				Ceftriaxone	.00050000	.00708331	1.000	.0332197
			Ceftriaxone	No Antibiotic	-.00650000	.00836381	.823	.0253162
				Aztreonam	-.00050000	.00708331	1.000	.0322197
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	-.00600000	.00725243	.642	.0147619
			Ceftriaxone	No Antibiotic	-.00650000	.00725243	.600	.0142619
	2-MTHF	Tukey HSD	No Antibiotic	Aztreonam	-.02160000	.01165965	.232	.0141750
				Ceftriaxone	-.05136667*	.01165965	.011	-.0155917
			Aztreonam	No Antibiotic	.02160000	.01165965	.232	.0573750
				Ceftriaxone	-.02976667	.01165965	.095	.0060083
			Ceftriaxone	No Antibiotic	.05136667*	.01165965	.011	.0871417
				Aztreonam	.02976667	.01165965	.095	.0655417

		Dunnett T3	No Antibiotic	Aztreonam	-.02160000	.01062811	.267	.0197654
				Ceftriaxone	-.05136667*	.01147422	.036	-.0051707
			Aztreonam	No Antibiotic	.02160000	.01062811	.267	.0629654
				Ceftriaxone	-.02976667	.01277606	.191	.0183843
			Ceftriaxone	No Antibiotic	.05136667*	.01147422	.036	.0975626
				Aztreonam	.02976667	.01277606	.191	.0779177
		Dunnett t (2-sided)[a]	Aztreonam	No Antibiotic	.02160000	.01165965	.189	.0549787
			Ceftriaxone	No Antibiotic	.05136667*	.01165965	.008	.0847453

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.