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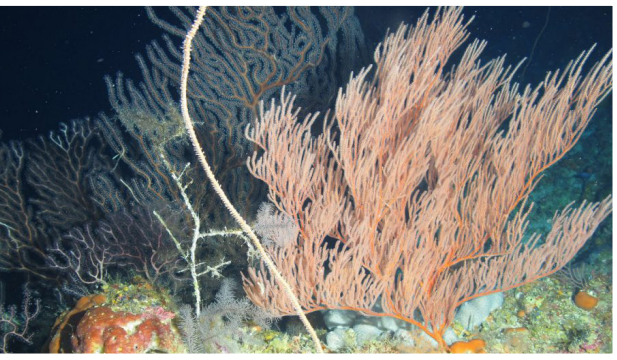
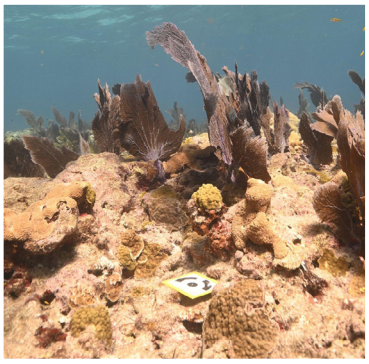
Amoxicillin Occurrence and Toxicity in the Aquatic Environment and Mechanisms of Antibiotic Resistance: A Review





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Amoxicillin Occurrence and Toxicity in the Aquatic Environment and Mechanisms of Antibiotic Resistance: A Review

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1 Introduction

Pharmaceuticals and personal care products (PPCPs) form a large group of substances that are considered contaminants of emerging concern. The U.S. Environmental Protection Agency (EPA) defines these contaminants as substances that are not regulated but have been discovered in natural waters with the potential to cause deleterious effects on aquatic life at environmental concentrations (U.S. Environmental Protection Agency 2008). Currently, PPCPs are not included in water quality monitoring programs in the U.S. resulting in potential risks to aquatic life and human health that go unmanaged. PPCPs include a wide range of synthetic and naturally occurring chemicals, many of which (e.g., pharmaceuticals) are designed to be biologically active. Examples of PPCPs include synthetic and natural hormones, anti-inflammatory drugs, antidepressants, antiepileptics, impotence drugs, tranquilizers, retinoids, preservatives, synthetic musks, sunscreen actives, antiseptics and disinfectants, antineoplastics, beta-blockers, and antibiotics (Kovalakova et al. 2020). PPCPs enter the environment through runoff (e.g., livestock production), mariculture, manufacturing plants, and wastewater treatment plant (WWTP) discharges. With their extensive use and continuous release into the aquatic environment, they are considered persistent or pseudo-persistent contaminants (Polianciuc et al. 2020).

Chronic exposure to PPCPs, especially the bioactive components can result in unintended consequences to non-target organisms, which are organisms that are not intentionally targeted by agents such as antibiotics, pharmaceuticals, personal care products, pesticides, herbicides, or biocides. Such exposures can result in behavioral, growth, development and/or reproductive changes, particularly when exposed during sensitive early life stages (Kovalakova et al. 2020). This ultimately can have population and ecosystem effects. Currently, there is a paucity of knowledge regarding the chronic effects of PPCPs on aquatic organisms, primarily because most studies have focused on acute (high-concentration) exposures (González-Pleiter et al. 2013; Broccoli et al. 2021). Thus, the risks posed to aquatic species from continuous exposure to low-level concentrations of PPCPs remains undefined (Elizalde-Velázquez et al. 2016).

Antibiotics are of particular concern because their biological activities not only affect target organisms but can adversely affect non-target organisms and disrupt natural microbiomes and biological cycling. Excessive, chronic antibiotic exposures also can adversely affect functions of natural microbial populations as well as promote antibiotic resistance that can threaten public and animal health. In 2018, a crisis state had been reached for Florida corals with a deadly coral disease, stony coral tissue loss disease (SCTLD). Over 20 stony coral species throughout Florida's Coral Reef and other parts of the Caribbean have been affected by SCTLD, causing rapid mortality for some species and significant population declines (Aeby et al. 2019; Neely et al. 2020; Aeby 2021; Shilling et al. 2021; Weil et al. 2019; Forrester et al. 2022; AGRRA 2023). The application of amoxicillin trihydrate (mixed with a carrier ointment) is the only treatment that has shown promise in halting or slowing tissue loss of affected corals in the laboratory (Miller et al. 2020) and in some field trials (Neely et al. 2020). However, the effectiveness of amoxicillin should not be interpreted to mean that bacterial agent(s) are responsible for SCTLD. Though several lines of investigation have explored potential bacterial, viral or environmental causes, the etiology of SCTLD remains unknown.

In an attempt to manage the spread of the deadly disease, the Florida Keys National Marine Sanctuary approved an emergency action that permitted field treatments of SCTLD-affected colonies with amoxicillin trihydrate incorporated into a proprietary coral ointment for drug delivery (i.e., vehicle) (CoralCure Ointment Base2B, aka CoreRx Base2B, Ocean Alchemists, Largo, FL). Base2B was specifically designed for direct underwater antibiotic application to SCTLD-affected corals (Favero and Balut 2019). However, there is currently no ecotoxicology information available for Base2B or Base2B mixed with amoxicillin and receptor organisms in proximity to its use. Identifying whether amoxicillin exposure causes adverse effects in protected coral reef species are of particular interest to coral reef managers needing to evaluate whether impacts could have occurred from the emergency permitting of its use in the field (Florida Keys National Marine Sanctuary, pers. comm.).

This review summarizes the available literature for the sources, environmental concentrations, and degradation of amoxicillin in the aquatic environment as well as reported ecotoxicological effects of amoxicillin on aquatic organisms. Finally, we present information regarding the various mechanisms by which antibiotic resistance can develop within microbial communities.

2 Amoxicillin Chemistry and Mode of Action

Amoxicillin (CAS number 26787-78-0, molecular weight 365.4; Fig. 1) is a broad-spectrum β-lactam antibiotic that targets a number of gram-positive and gram-negative bacteria, including *Staphylococcus, Streptococcus, Trueperella, Clostridium, Escherichia, Klebsiella, Shigella, Salmonella, Proteus* and *Pasteurella* that are associated with various types of infections (Elizalde-Velázquez et al. 2016; Litskas et al. 2018; Sodhi et al. 2021). It is a semi-synthetic β-lactam antibiotic that is considered bacterial autolytic or bactericidal. Its mode of action involves inhibiting bacterial cell wall synthesis that occurs when it is bound to multiple penicillin-binding proteins (PBPs) and disrupts the cross-linking of peptidoglycans. Amoxicillin also inhibits the transpeptidase enzyme, which is essential in the final stage of peptidoglycan synthesis, which is required for cell wall formation in gram-positive and gram-negative bacteria (Donowitz and Mandell 1988). The interference with peptidoglycan cross-linking leads to dysfunction and a general loss of bacterial cell integrity, resulting in cell lysis (Donowitz and Mandell 1988). Though effective in treating numerous gram-positive and gram-negative bacterial infections, amoxicillin is often augmented by co-administering clavulanic acid, a β-lactamase inhibitor that prevents the destruction of amoxicillin by bacterial β-lactamases (Geddes et al. 2007; Bush and Bradford 2016; Elizalde-Velázquez et al. 2016).

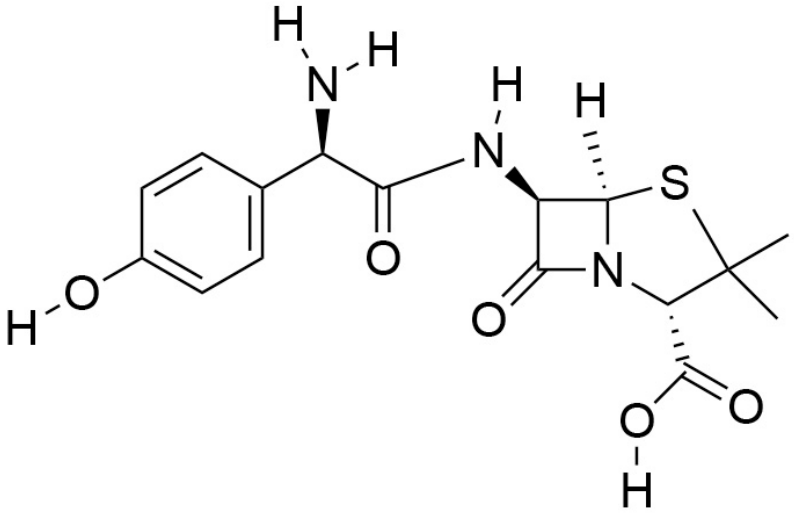

Figure 1. Molecular structure of amoxicillin, a semi-synthetic β-lactam antibiotic included in the penicillin class (chemical structure created using ChemDraw® software version 23.1).

3 Antibiotic Sources and Environmental Occurrence

3.1 Global use

Global antibiotic usage trends are used to estimate environmental levels, exposures to aquatic organisms, and generation of antibiotic resistant bacteria (ARB) (Bergman et al. 2006; Polianciuc et al. 2020, Shi et al. 2022). Between 2000 and 2018, the human consumption of antibiotics in over 200 countries increased by an estimated 46 % (Browne et al. 2021), with the penicillin group of antibiotics estimated to be 50-70 % of all antibiotics used in human medicine worldwide (Brogden et al. 1979; Längin et al. 2009). In 2022, the global amoxicillin (a penicillin group antibiotic) market was projected to generate 4,783.5 million U.S. Dollars (USD), with an expected increase to 5,812.3 million USD by 2028 (360 Research Reports 2022).

Interestingly on a global scale, the majority of antibiotics are administered to animals rather than people (Polianciuc et al. 2020). In the 1940s antibiotics were found to enhance growth and reduce mortality in livestock production (Graham et al. 2007). Since then, antibiotic use in livestock production has intensified,

becoming the largest sector of antibiotic consumption. Asia, the U.S., Brazil, India, and Germany have the highest antibiotic use in livestock production (Van Boeckel et al. 2015). Mulchandani et al. (2023) estimate that globally 99,502 tons of antibiotics are used in food-producing animals, which is projected to increase to 107,472 tons by 2030. The increasing need for additional animal protein has intensified aquaculture production and the concomitant increase in antibiotic use. Antibiotic use in aquaculture alone was estimated at 10,259 tons in 2017 and projected to increase by 33 % to 13,600 tons by 2030 (Shar et al. 2020).

Antibiotics consumed by either humans or animals are often metabolized in the liver and eliminated in urine and/or feces, which can be a mixture of the unchanged parent compound and its metabolites (Tran et al. 2018; Kovalakova et al. 2020). Although there is variation in how antibiotics are metabolized, the majority (70-80 %) of antibiotics detected in wastewater are in their unchanged form, with >70 % of amoxicillin excreted with an intact β-lactam ring in urine that is still biologically active (Dinh et al. 2017).

The estimated production and use values are indicators for the overuse and misuse of antibiotics. Many countries recognize this as being responsible for generating ARB (Durkin et al. 2018; Limmathurotsakul et al. 2019). In particular, the overuse of broad-spectrum antibiotics is thought to exacerbate antibiotic resistance (Liu and Wong 2013; Van Boeckel et al. 2015; Cižman and Srovin 2018). The accumulation of antibiotics in the aquatic environment has severe implications for ecosystem health that can manifest as dysregulation of environmental microbiomes, functional alterations in nutrient cycling, and increased pathogen loads (Shar et al. 2020).

3.2 Antibiotics in Agriculture

Agricultural use of antibiotics began in 1935, when Bayer Laboratories (Germany) marketed Prontosil (sulfochrysoidine) to treat gram-positive bacterial infections (Kirchhelle 2018). In 1948, Thomas Jukes discovered small doses of Aureomycin (chlortetracycline) had a “growth-promoting” effect on chickens, which helped fuel antibiotic use in livestock production (McKenna 2017). Further experiments by others confirmed that antibiotics not only facilitated growth in chickens but in other farmed animals (pigs, turkeys, and cattle) as well (McKenna 2017). By the 1960s, antibiotics were widely used across the globe to help increase animal protein production (Kirchhelle 2018). More recently, there has been marked growth in the aquaculture industry to meet the world-wide demand for animal protein. To enhance production, aquaculture operations routinely incorporate antibiotics, especially broad-spectrum antibiotics that target multiple bacterial types (Tacão et al. 2012). Frequently, amoxicillin is used as a prophylactic to guard against bacterial infections which threaten production yields (Romero et al. 2012; Chowdhury et al. 2022). These practices have facilitated what is now known as factory farming, an industry that presumably promotes the evolution of ARB (Anomaly 2015; Kirchhelle 2018).

Since the 1950s, antibiotics also have been used in cultivating field crops, though accounting for less than 1 % of total antibiotic use in the U.S. (McManus et al. 2002). Streptomycin and oxytetracycline are typically used for crop protection (McManus et al. 2002); however, other antibiotics, including amoxicillin, can enter the environment from associated practices for crop production. For instance, fertilizing crops with antibiotic laden manure (e.g., reclaimed water, WWTP biosolids or sludge) can enhance antibiotic resistance genes (ARGs) in agroecosystems (Smith et al. 2019). Further, Rahube et al. (2014) showed that harvested vegetable crops can carry ARGs and ARB.

4 Transport, Fate, and Potential Exposure in the Aquatic Environment

4.1 Transport of Amoxicillin in the Environment

Amoxicillin can enter waterways through various routes, including runoff from livestock operations, fields fertilized with manure or sludge, leachates from landfills, effluents from pharmaceutical industries, and effluents from WWTPs that include hospital and domestic sewage (Kovalakova et al. 2020; Aryee et al. 2022). While WWTPs are designed to remove various pollutants, effectiveness of removing antibiotic compounds varies. For example, amoxicillin has been detected in WWTP effluents in many countries (Tran et al. 2018). However, the detection of antibiotics in WWTP influent and/or effluent depends on many factors that include sample site, wastewater composition, treatment regimen, and the type of WWTP (Kovalakova et al. 2020). Currently, there

are no standards regarding the antibiotic concentrations in WWTP effluent in the U.S. or the European Union (Kovalakova et al. 2020).

While amoxicillin often has been detected in WWTP influent and effluent samples from Asian countries, detection in European and North American countries is reported less (Tran et al. 2018; Längin et al. 2009). It is unclear whether this is because it is not targeted in monitoring programs or the parent compound was not detected due to hydrolysis under environmental conditions (Elizalde-Velázquez et al. 2016). Table 1 captures environmental concentrations of amoxicillin that have been reported.

Table 1. Reported concentrations (ng L^{-1}) of amoxicillin detected in surface water, WWTP effluent, WWTP influent, and DWTP influent around the world. Abbreviation meanings are as follows; WWTP: wastewater treatment plant, DWTP: drinking water treatment plant, nd: not detected.

Location	Matrix	Concentration (ng L^{-1})	Reference
United States	Surface water (freshwater)	<10	Finnegan et al. 2010
Greece	Surface water (marine)	nd-127.8	Alygizakis et al. 2016
Ghana	Surface water (freshwater)	nd-2.7	Azanu et al. 2018
West Africa	Surface water (freshwater)	87-272,150	Ebele et al. 2020
Portugal	Surface water (estuarine)	29.33-83.14	Fonseca et al. 2021
South Wales, United Kingdom	Surface water (freshwater)	nd-622	Kasprzyk-Hordern et al. 2008
Australia	Surface water (freshwater, estuarine, marine)	nd-200	Watkinson et al. 2009
France	Surface water (freshwater)	5.0-21.0	Dinh et al. 2017
China	Surface water (estuarine)	34.60-786.40	Lu et al. 2020
Australia	WWTP effluent	50	Watkinson et al. 2009
Belgium	WWTP effluent	nd-17,500 (Brussels South) 29,200-116,400 (Brussels North)	Lorenzo et al. 2018
Ghana	WWTP effluent	nd-1.3	Azanu et al. 2018
Italy	WWTP effluent	7.40 (Cagliari) <1.80 (Cosenza) 120.35 (Palermo) 15.20 (Roma) 1.80 (Napoli) 4.74 (Torino) 4.68 (Varese Olona) 4.68 (Varese Lago)	Andreozzi et al. 2004
Iraq	DWTP influent	1500	Mahmood et al. 2019
Australia	WWTP influent	6940	Watkinson et al. 2009
Ghana	WWTP influent	2.0-6.0	Azanu et al. 2018
Belgium	WWTP influent	nd-33,800 (Brussels South) nd (Brussels North)	Lorenzo et al. 2018

4.2 Fate of Amoxicillin in the Aquatic Environment

Antibiotics are detected in relatively low concentrations in the environment ranging from nanograms to micrograms per liter (Sodhi et al. 2021). The fate and persistence of organic compounds in the aquatic environment depend on various natural processes, such as sorption and transformation (Kümmerer 2009; Elizalde-Velázquez et al. 2016). Amoxicillin contains an unstable β-lactam ring that is easily inactivated by the bacterial enzyme, β-lactamase (Elizalde-Velázquez et al. 2016). Ultraviolet light and heat also can degrade amoxicillin directly or indirectly in aquatic environments (Kovalakova et al. 2020; Aryee et al. 2022). Gozlan

et al. (2013) proposed a pH-dependent degradation pathway that begins with hydrolysis, which cleaves the β-lactam ring generating amoxicillin penicilloic acid. This intermediate can result in the formation of phenol hydroxypyrazine (most likely), or alternatively, it could yield either the diketopiperazine degradation product of amoxicillin at high pH (7 or 8) or amoxicillin penilloic acid at a relatively low pH (5) (Gozlan et al. 2013; Elizalde-Velázquez et al. 2016).

Under solar ultraviolet irradiation, amoxicillin can be degraded directly by photolysis. Andreozzi et al. (2004) determined the quantum yields of amoxicillin under solar irradiation and concluded that direct photolysis could be a viable method for removing amoxicillin from aquatic systems. Amoxicillin absorbs UV light at wavelengths longer than 290 nm, making it potentially susceptible to chemical changes or transformations upon exposure to UV radiation (Andreozzi et al. 2004). Solar irradiation also can generate reactive oxygen species (e.g., singlet oxygen, superoxide, peroxy and hydroxyl radicals) from nitrate ions and humic acids in natural aquatic systems, which can then react with amoxicillin and amplify its photodegradation potential (Andreozzi et al. 2004; Burns et al. 2012).

Although amoxicillin generally is thought to be prone to decomposition, Ecke et al. (2023) recently provided evidence that the process of amoxicillin hydrolysis can be influenced by the type of water. In this study, four types of water were investigated: ultrapure, tap water, canal surface water, and bottled mineral water. Interestingly, amoxicillin was most stable in ultrapure water in the dark at 4 °C for 60 days. Additionally, the stable end product of amoxicillin hydrolysis (3-(4-hydroxyphenyl)-pyrazine-2-ol) was observed to rapidly form in water containing copper and zinc ions, i.e., tap water and canal surface water (Ecke et al. 2023). Thus, the study concluded that amoxicillin hydrolysis is highly dependent on water type, presumably due to copper and zinc ion content with temperature and light exposures having a secondary influence.

The presence of specific iron species also may influence the degradation process of amoxicillin, including the abundance and types of intermediates produced. Trovo et al. (2011) conducted photo-Fenton experiments demonstrating amoxicillin degrades more rapidly in distilled water when the potassium ferrioxalate complex (FeOx) is present compared to ferrous sulfate, however, with fewer intermediate products. Trovo et al. (2011) also found that FeOx in distilled water degrades amoxicillin faster in light, but the associated degradation products have greater toxicity to *Daphnia magna*. This highlights the potential for unintended toxic effects if using iron to reduce amoxicillin concentrations in aquatic environments due to the presence of oxalate.

4.3 Bioaccumulation and Toxicity

Antibiotics introduced into aquatic systems have the potential to bioaccumulate in organisms and biomagnify through the food chain (Aryee et al. 2022). This has prompted studies investigating the bioaccumulation of antimicrobials in the tissues of aquatic organisms, which is influenced by the chemical characteristics of the substances, as well as the biological receptors and the surrounding environment (Zhu et al. 2022). The bioavailability and toxicity of a substance depends on the chemical structure, uptake ability, quantity that reaches and interacts with the active site(s), and the ability for an organism to detoxify and eliminate it (Boxall et al. 2004). To better understand and predict toxicity, physicochemical properties are first characterized. The octanol-water partition coefficient (log Kow) measures hydrophobicity/lipophilicity on a scale of –3 to +10 (very hydrophilic to hydrophobic) typically using a shake-flask method for predicting chemical distribution in the environment and living organisms, which can help determine toxicity and the propensity for bioaccumulation (Cumming and Rücker 2017). Additionally, the acid dissociation constant (pKa) can determine how much of the material will dissociate in water. Thus, if a particular substance has a higher pKa (weaker acid) and/or a higher log Kow (higher hydrophobicity and more fat-soluble), it will likely be toxic (Boxall et al. 2004). The pKa values of amoxicillin have been reported as 2.68, 7.49, and 9.63 (Aryee et al. 2022). This is due to amoxicillin existing in different forms in solution: cationic at a pH <2.68, zwitterionic at a pH 2.68-7.49, anionic at pH 7.49-9.63, and at a pH >9.63, it becomes more negatively charged (Aryee et al. 2022). Amoxicillin has better bioavailability due to its higher acid stability compared to other penicillins (Ecke et al. 2023). The log Kow of amoxicillin has been reported at 0.87, which indicates it may not easily partition into fatty substances, and therefore is less likely to bioconcentrate in living organisms. However, it has been reported that amoxicillin can still biomagnify (Aryee et al. 2022). This log Kow value may facilitate the transportation ability of amoxicillin, since it is somewhat soluble in water (Aryee et al. 2022).

5 Effects of Amoxicillin on Aquatic Organisms

Freshwater and marine organisms can experience acute and chronic exposures to amoxicillin and its stable degradation products, which can have serious consequences on non-target species such as algae, bivalves, zooplankton, fishes, and coral (Andreozzi et al. 2004; Vermeij et al. 2009; Matozzo et al. 2016a; Matozzo et al. 2016b; Souza et al. 2016). Specific antibiotics have been tested in studies conducted with various model organisms; however, there remains a knowledge gap concerning the effects of amoxicillin on many other, non-model species. Studies detailing toxicological benchmarks are summarized in Table 2. This table includes the lethal dose that causes death to half of the test organisms (LC_{50}), as well as sublethal endpoints represented by half maximal effect concentration (EC_{50}), reflecting concentrations that can lead to physiological effects with potential population consequences. Additionally, it presents the no observed effect concentration (NOEC), the highest concentration at which no adverse effects are observed, and the lowest observed effect concentration (LOEC), the lowest concentration at which adverse effects are observed. Sublethal endpoints may include growth, reproduction, behavior, development or various biochemical, physiological or molecular effects, which can signify lower fitness of an organism. For example, amoxicillin was reported to cause oxidative stress in carp and zebrafish, causing premature hatching of embryos (Oliveira et al. 2013; Yang et al. 2020). Other studies testing amoxicillin toxicity have reported sublethal endpoints such as genomic injury and developmental malformations (Elizalde-Velázquez et al. 2016).

Species sensitivity studies have shown that prokaryotic blue-green algae (cyanobacteria - autotrophic gram-negative bacteria) are the most sensitive taxa to amoxicillin. *Synechococcus leopoliensis* has a reported EC^{50} value of 0.00222 mg/L, while *Microsystis aeruginosa* ranges from 0.0037 – 0.00803 mg/L. EC^{50} values for various algal groups range from 10 mg/L to >1000 mg/L (Andreozzi et al. 2004; Kovalakova et al. 2020; Broccoli et al. 2021). Regarding eukaryotic species, crustaceans have a EC^{50} values >1000 mg/L, echinoderms have a reported NOEC of 250 mg/L, while mollusks have a NOEC greater than 1000 mg/L. Cnidarians exhibit a drastic difference between adults, with a NOEC of 0.01 mg/L, and larvae, with a NOEC greater than 500 mg/L. Fish embryos have a reported EC^{50} value of 132.40 mg/L (Lützhøft et al. 1999; Park and Choi 2008; Liu et al. 2012; González-Pleiter et al. 2013; Kovalakova et al. 2020; Broccoli et al. 2021; Zhong et al. 2021; see Table 2).

Cyanobacteria, as the most sensitive taxa, are large contributors to phytoplankton biomass and an essential group of prokaryotes. Marine phytoplankton contribute an estimated 48.5 % of the Earth's primary productivity and numerous ecosystem services, e.g., biogeochemical cycles, nutrient recycling, climate regulation, and food sources (Field et al. 1998; Naselli-Flores and Padisák 2023). In aquatic environments, cyanobacteria contribute to the total free oxygen and carbon dioxide fixation through photosynthesis, with various lineages able to fix atmospheric nitrogen (Sánchez-Baracaldo 2015; Välitalo et al. 2017). Nitrogen-fixing cyanobacteria perform a critical step in primary productivity cycling by not only exporting organic carbon to the deep ocean, but converting nitrogen gas into ammonium ions that are essential components for amino acid and protein production (Sánchez-Baracaldo 2015). The sensitivity of cyanobacteria to amoxicillin exposure at environmentally relevant concentrations, along with the basal roles this taxon plays in the aquatic food web, creates a vulnerability with the potential to disrupt the entire aquatic food web. Dysregulation of the growth and persistence of cyanobacteria can present immense challenges for higher trophic levels that rely on their energy supply and other vital ecosystem services.

6 Mechanisms of Antibiotic Resistance

Although amoxicillin is the focus of this review, it is important to realize that the development of antibiotic resistance in bacterial populations is a dynamic process. It is also important to realize there is not a one-to-one correspondence with the use of amoxicillin equaling a specific type of resistance mode of action, and should resistance occur, that it is limited to only this one drug. There are many factors that influence whether antibiotic resistance will arise in a bacterial population and the extent or prevalence of the resistance in a given organism or location (Andersson and Hughes 2012). Thus, this section was expanded from mechanisms of amoxicillin and β-lactam antibiotic resistance to include a range of mechanisms that can contribute to antibiotic resistance and multi-drug resistance.

A frequent misconception is that antibiotic resistance is a modern occurrence. This widespread perception equates the emergence of antibiotic resistance with the discovery and explosive use of antibiotics in medicine

Table 2. Toxicological dose descriptors for specific taxonomic groups subjected to amoxicillin for various lengths of time. All sources were obtained via the U.S. Environmental Protection Agency ECOTOX Knowledgebase (cfpub.epa.gov/ecotox/), except for unpublished data. NR: not reported.

Taxonomic Group	Scientific Name	Life Stage	EC^{50} (mg L^{-1})	LC^{50} (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})	Exposure Duration	Endpoint	Reference
Bacteria	*Vibrio fischeri* (proteobacteria, marine)	Cell culture	3597				15 min	Bacterial luminescence	Park and Choi 2008
	Microcystis aeruginosa (cyanobacteria, freshwater)	Exponential growth stage (log)	0.0037				7 days	Chlorophyll *a* concentration	Lützhøft et al. 1999
	Microcystis aeruginosa (cyanobacteria, freshwater)	Exponential growth stage (log)	0.00803				7 days	Growth inhibition	Liu et al. 2012
	Anabaena sp. strain CPB4337 (cyanobacteria, freshwater)	Exponential growth stage (log)	56.3				72 h	Growth inhibition	González-Pleiter et al. 2013
	Anabaena cylindrica (cyanobacteria, freshwater)	Exponential growth stage (log)	7.66			15	6 days	Growth inhibition	Zhong et al. 2021
	Synechococcus leopolensis (cyanobacteria; blue-green algae, marine)	Exponential growth stage (log)	0.00222		0.00078	0.00156	96 h	Growth inhibition	Andreozzi et al. 2004
Algae	*Rhodomonas salina* (red alga or cryptomonad, marine)	Exponential growth stage (log)	3108				72 h	Chlorophyll *a* concentration	Lützhøft et al. 1999
	Pseudokirchneriella (aka: Raphidocelis) subcapitata; Selenastrum capricornutum) (green alga, freshwater)	Exponential growth stage (log)	>1500				72 h	Growth inhibition	González-Pleiter et al. 2013
	Pseudokirchneriella subcapitata (aka: Raphidocelis) subcapitata; Selenastrum capricornutum) (green alga, freshwater)	Exponential growth stage (log)	10				72 h	Growth inhibition	Broccoli et al. 2021

Taxonomic Group	Scientific Name	Life Stage	EC^{50} (mg L^{-1})	LC^{50} (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})	Exposure Duration	Endpoint	Reference
Algae	*Pseudokirchneriella subcapitata (aka: Raphidocelis) subcapitata; Selenastrum capricornutum)* (green alga, freshwater)	Exponential growth stage (log)	213.14				72 h	Growth inhibition	Lee et al. 2021
	Phaeodactylum tricornutum (brown alga, marine)	Exponential growth stage (log)	30				72 h	Growth inhibition	Broccoli et al. 2021
	Phaeodactylum tricornutum (brown alga, marine)	Exponential growth stage (log)			250		96 h	Growth inhibition	De Orte et al. 2013
	Isochrysis galbana (brown alga, marine)	Exponential growth stage (log)			250		96 h	Growth inhibition	De Orte et al. 2013
	*Isochrysis galbana (*brown alga, marine)	Exponential growth stage (log)	30				72 h	Growth inhibition	Broccoli et al. 2021
	Chlorella pyrenoidosa (green alga, freshwater)	Exponential growth stage (log)	>2000		1000	2000	6 days	Growth inhibition	Zhong et al. 2021
	Chlorella sp. (green alga, freshwater)	Exponential growth stage (log)	853.54				96 h	Growth inhibition	Aubakirova et al. 2017
	Scenedesmus obliquus (green alga, freshwater)	Exponential growth stage (log)	30				72 h	Growth inhibition	Broccoli et al. 2021
	Tetraselmis suecica (green alga, marine)	Exponential growth stage (log)	20				72 h	Growth inhibition	Broccoli et al. 2021
	*Selenastrum capricornutum (*green algae*, aka: Pseudokirkneriella subcapitata,* freshwater)	Exponential growth stage (log)			>250		7 days	Growth inhibition	Lützhøft et al. 1999

Taxonomic Group	Scientific Name	Life Stage	EC^{50} (mg L^{-1})	LC^{50} (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})	Exposure Duration	Endpoint	Reference
Crustacean	*Daphnia magna* (fresh-water)	Juvenile	>1000				24 h	Immobile	Park and Choi 2008
	Daphnia magna (fresh-water)	Juvenile	>1000				48 h	Immobile	Park and Choi 2008
	Daphnia magna (fresh-water)	Adult			27.2		21 days	Reproduction	Lee et al. 2021
	Moina macrocopa (fresh-water)	NR	>1000				24 h	Immobile	Park and Choi 2008
	*Moina macrocopa (*fresh-water)	NR	>1000				48 h	Immobile	Park and Choi 2008
Invertebrate	*Paracentrotus lividus* (sea urchin, marine)	Egg				100	48 h	Development	Carballeira et al. 2012
	Arbacia lixula (sea urchin, marine)	Egg				100	72 h	Development	Carballeira et al. 2012
	Lytechinus variegatus (sea urchin, marine)	Embryo			250	500	48 h	Development	Woodley et al. (un-published)
	Crassostrea virginica (oyster, marine)	Embryo			>1000		48 h	Development	Woodley et al. (un-published)
	Hydra vulgaris (freshwater cnidarian)	Adult			0.01	0.1	7 days	General intoxication	Pascoe et al. 2003
	Hydra vulgaris (freshwater cnidarian)	Adult			10		17 days	Food consumption	Pascoe et al. 2003
	Acropora cervicornis (stony coral, marine)	Larvae				500	7 days	Settlement	Woodley et al. (un-published)
	Acropora cervicornis (stony coral, marine)	Larvae			>500		48 h	Mortality	Woodley et al. (un-published)

Taxonomic Group	Scientific Name	Life Stage	EC^{50} (mg L^{-1})	LC^{50} (mg L^{-1})	NOEC (mg L^{-1})	LOEC (mg L^{-1})	Exposure Duration	Endpoint	Reference
Fish	*Oryzias latipes* (medaka, freshwater)	Juvenile		>1000			48 h; 96 h	Mortality	Park and Choi 2008
	Oryzias latipes (medaka, freshwater)	Embryo			1.37		NR	Hatchability	Lee et al. 2021
	Oryzias latipes (medaka, freshwater)	Juvenile			21.8		40 days	Survival & growth	Lee et al. 2021
	Danio rerio (zebrafish, freshwater)	Embryo	132.4		128	221	48 h	Premature hatching of embryos	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Embryo			1125		72 h; 96 h	Hatching of embryos	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult			25	50	96 h	Catalase inhibition (head)	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult			10	25	96 h	Catalase inhibition (gill)	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult			100		96 h	Catalase inhibition (muscle and liver)	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult				1	96 h	Glutathione-S-transferase (GST) inhibition (head)	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult				1	96 h	Glutathione-S-transferase (GST) Increased activity (muscle)	Oliveira et al. 2013
	Danio rerio (zebrafish, freshwater)	Adult			1	10	96 h	Glutathione-S-transferase (GST) Increased activity (gill)	Oliveira et al. 2013

and agriculture, albeit less than 100 years ago. Multiple lines of evidence, however, have shown conclusively that antibiotic resistance is a natural occurrence. It has emerged from the environment as a result of microbes producing natural products, among which are antibiotics that played vital roles in the ecology and evolution of complex microbial communities (Waglechner et al. 2021; Shi et al. 2022). Further, antibiotic resistance, as we know it today, has ancient origins (reviewed by Perry et al. 2016). For example, multiple, viable bacterial strains across seven genera and carrying antibiotic resistance have been isolated from artic permafrost sediments with estimated ages ranging from 3000 to 3,000,000 years old (Mindlin et al. 2008). A metagenomic analysis of Beringian permafrost sediments show ARGs existing in nature at least 30,000 years ago (D'Costa et al. 2011). In samples collected from humans, ARGs also have been identified. Santiago-Rodriguez et al. (2015) found evidence of antibiotic genes including β-lactamases, penicillin-binding proteins, among others in the paleofeces of an 11th century pre-Columbian Andean mummy. In more recent history, but prior to the discovery and extensive use of antibiotics, the first bacterial isolate was submitted to the UK National Collection of Type Cultures in 1915 from a World War I soldier (Mather et al. 2014), and later genomic analyses identified this isolate as *Shigella flexeri* that carried resistance to penicillin and erythromycin with resistance genes similar to more modern isolates (Baker et al. 2014).

The ancient origins of antibiotic resistance do not diminish the societal impacts that modern antibiotics with targeted designs and widespread use have had on the pervasive rise in antibiotic resistance. Today, drugs are selected and purified from microbial natural products or designed and synthesized to attack specific features of bacterial structures or their metabolic processes. Multiple reviews have summarized mechanisms and modes of action for antibiotics as a) inhibiting cell wall synthesis, b) breaking down cell membrane structure or function, c) inhibiting nucleic acid functions, d) inhibiting protein synthesis, or e) blocking critical metabolic pathways (Etebu and Arikekpar 2016; Munita and Arias 2016; Hasan and Al-Harmoosh 2020; Mancuso et al. 2021; Uddin et al. 2021; Darby et al. 2023). Quorum sensing disruption is a new target for controlling pathogenic microbes by interfering with cell-to-cell communication among bacteria that express virulence factors and antibiotic resistance (Ghosh et al. 2020; Fan et al. 2022). Microorganisms, however, have optimized mechanisms to replicate, adapt and evolve quickly to changes in their environment to be able to persist. As microbes encounter impediments to their growth and dispersal, they are able to employ a plethora of mechanisms to evade the inhibitory or lethal effects of antibiotics (MacGowan and Macnaughton 2017; Uddin et al. 2021). With the increased selective pressure from the substantial overuse of antibiotics, coupled with the shortage of new drugs, microbe adaptations are outpacing the availability of effective drugs, resulting in less effective, straightforward therapies for many infectious diseases (Gajdács and Albericio 2019; Mancuso et al. 2021).

Antibiotic resistance within individual and communities of bacteria is rarely accomplished by single mechanisms, but frequently result from complex synergies between intrinsic and acquired resistance mechanisms. The particular mechanisms or combinations of interacting mechanisms that give rise to antibiotic resistance are influenced by multiple factors, that range from the particular bacterial strain or microbial community involved, their genetics and functional backgrounds, to the surrounding environmental conditions. The most common mechanisms of antibiotic resistance are reviewed below with emphasis on resistance mechanisms relevant to amoxicillin exposures.

6.1 Natural Resistance

The notion of natural resistance among bacteria is thought to originate among environmental microbes that naturally produce antibiotics as an ecological function whereby they have self-resistance determinants for protection against their natural products (i.e., producers) as well as being able to inhibit or kill susceptible microbes as a means of niche defense (Cox and Wright 2013; Peterson and Kaur 2018). Much of our knowledge of intrinsic resistance has been derived from studies of the phylum Actinobacteria (Barka et al. 2016). Collectively, the assortment of naturally occurring antibiotics that have been described affect most major microbial cell processes, including cell wall synthesis, protein synthesis, cell membrane function, and fatty acid biosynthesis. These targets for antibiotic action provide insights for the mechanisms that exist to resist their effects such as antibiotic modification or antibiotic target protection, antibiotic inactivation, sequestration and/or efflux (Mak et al. 2014; Peterson and Kaur 2018).

6.1.1 Intrinsic resistance

Intrinsic resistance is when a microbe is not affected by certain groups of antibiotics without prior antibiotic selection (Mancuso et al. 2021). This is largely due to their specific biochemistry and/or physiology (i.e., innate make-up, wildtype) (as reviewed in: McDermott et al. 2003; Blair et al. 2015; Hasan and Al-Harmoosh 2020; Darby et al. 2023) and intrinsic resistance can take on many different forms. The most general examples of intrinsic resistance include the type of bacterial outer membrane (e.g., gram-negative bacteria) or active efflux membrane pumps that are active against various chemical compounds. However, intrinsic resistance is more complex. Outer membrane permeability is restricted not only by the peptidoglycan and particle sizes able to traverse it, but also a unique composition of lipids and polysaccharides that reduce membrane fluidity and permeability (Mancuso et al. 2021). Certain bacteria have special transmembrane channel proteins called porins that can restrict compounds based on size and charge, including entry of some antibiotic classes (Nikaido et al. 1983; Bellido et al. 1992; Olesky et al. 2006). Other types of intrinsic antibiotic resistance can stem from the microbe lacking the antibiotic target. This can occur if the bacteria lack specific receptor or affecter molecules, e.g., mycobacteria that lack cell walls are unaffected by β-lactam antibiotics (Draper 1998). Some intrinsic resistance is due to reduced permeability of the cell envelope to particular antibiotics that require passage through the outer membrane of gram-negative cells (Draper 1998). In other cases, intrinsic resistance occurs when there are changes in the cytoplasmic membrane composition and structure (Mishra et al. 2012). For groups of antibiotics with intracellular targets that require a buildup of antibiotic concentration for efficacy, microbes equipped with membrane efflux pumps are able to actively export antibiotics conveying resistance to those drugs (Webber and Piddock 2003; Whittle et al. 2021).

6.1.2 Induction of resistance by antibiotic pressures

Intrinsic resistance also has been attributed to a network of genes related to a wide range of cellular functions (not acquired by horizontal gene transfer (HGT) or antibiotic selection), particularly proteins involved in cellular metabolism whose regulation can be influenced by metabolic state. These genes may be constitutively expressed or inducible. Frequently, resistance genes are tightly linked to antibiotic biosynthetic gene clusters, where they play a role in gene regulation of the antibiotic-producing cells or sibling non-antibiotic-producing cells (Fajardo et al. 2008; Cox and Wright 2013; Mak et al. 2014).

Peterson and Kaur (2018) provide a helpful overview of the variety of intrinsic self-resistance mechanisms that exist among microbes that are able to display single or multiple mechanisms to defend against antibiotics. A common mechanism that can render an antibiotic ineffective is antibiotic modification. This occurs in cells equipped with modifying enzymes able to create different types of chemical modifications, such as acetylation, phosphorylation, or adenylation, which inactivates the antibiotic. Enzymatic degradation is another mechanism of intrinsic resistance. A classic example is the β-lactamase superfamily of four related groups of hydrolytic proteins (A, B, C, D) which have similarities in sequence, active sites and mechanistic modes of action (King et al. 2016). Drug-binding proteins can sequester or prevent the antibiotic from either reaching or binding to intended targets as another form of intrinsic resistance. The sequestration essentially neutralizes the antibiotic until it can be degraded or transported from the cell (Sabnis et al. 2018).

The antibiotic target site(s) (e.g., penicillin binding proteins (PBPs) or ribosomal ribonucleic acid (RNA) methylation) are altered in some microbes to the point the microbe is intrinsically resistant. For example, in *Mycobacterium* spp*.,* the binding affinity for β-lactam antibiotics is very low, resulting in a resistance phenotype (Basu et al. 1996; Zapun et al. 2008; Ogawara 2015). In other cases, an over production of the PBPs or alteration of PBPs due to chromosomal mutations can result in different degrees of β-lactam antibiotic resistance (Malhotra-Kumar et al. 2016; Peterson and Kaur 2018). Changes in cell wall precursors (peptidoglycans) in some microbes are able to modify cell wall biosynthesis. These changes reduce the affinity of antibiotics for these modified peptidoglycans, allowing these microbes to evade the antibiotic (Binda et al. 2014). Other microbial defense mechanisms rescue protein synthesis from inhibition by modifying ribosomal RNA targets (e.g., methylation) or altering proteins that bind ribosomes. This adaptive response facilitates the removal of the antibiotic, thus rescuing bacterial cells from translational arrest (Buriánková et al. 2004; Galimand et al. 2011; Murina et al. 2018; Peterson and Kaur 2018). Some microbes have intriguing strategies that create conditions by which antibiotics bypass their target site(s) or create redundant biochemical functions that are unaffected by the antibiotic allowing them to persist despite antibiotic exposure. Metabolic pathways targeted by antibiotics also can be bypassed by overproducing the antibiotic target (Munita and Arias 2016).

Although these mechanisms have been presented in the context of intrinsic antibiotic resistance, there is increasing acceptance that acquired resistance, such as seen in clinical isolates, originated from environmental reservoirs of antibiotic resistance (Davies and Davies 2010). The spread of antibiotic resistance has been exacerbated by antibiotic pollution in the environment, which contributes to a rise in bacterial resistance, increased mobility of gene transfer, and hyper-mutable microbes (Davies and Davies 2010; Dantas and Sommer 2012).

6.2 Acquired Resistance

Acquired antibiotic resistance is an adaptive response and a strategy for survival that can manifest with antibiotic exposure. This can occur directly or indirectly either through mutations in genes associated with a particular antibiotic mode of action (e.g., cell wall synthesis, protein synthesis inhibition) or by acquiring external deoxyribonucleic acid (DNA) that encodes resistance factors, known as HGT (Li et al. 2019a).

6.2.1 Vertical gene transfer

Vertical gene transfer is the transfer of genetic information from one generation to the next with DNA replication and cell division (microbes reproduce asexually by cell fission or budding). In microbes, chromosomal mutations in genes or gene networks that are targets for antibiotic therapy also can arise *de novo* or from the SOS response, an error-prone DNA repair system. During replication, these mutations can become fixed in the genome and subsequently transferred to progeny bacteria (Händel et al. 2014; Munita and Arias 2016; Mancuso et al. 2021; Wang et al. 2020).

6.2.2 Horizontal gene transfer

Bacteria frequently acquire antibiotic resistance through HGT, which allows uptake of foreign DNA encoding resistance factors from the bacterial community at large. The transfer of genetic material can occur through transformation (direct transfer of cell-free DNA), transduction (transfer of DNA by bacteriophage), or conjugation (transfer of genes coding for resistance factors via plasmids) (Sabtu et al. 2015; Soucy et al. 2015; MacGowan and Macnaughton 2017; Larsson et al. 2022). Resistance factors are generally genetic determinants that are carried in the original host (described in section 6.1). Naïve bacteria uptake resistance genes by HGT or by bacterial transformation of environmentally available ARGs (Tao et al. 2022). Antibiotic resistance genes can be mobilized as extrachromosomal elements (e.g., from chromosomes to plasmids, bacteriophages, transposons, and integrons) or as naked DNA, existing extracellularly with the ability to move between cells (Peterson and Kaur 2018; Tao et al. 2022). Naked DNA can be released when cells lyse or may freely exist in the environment, where it can be taken up by bacterial cells through transformation and either incorporated into the genome or maintained on extrachromosomal DNA (e.g., plasmids) (Peterson and Kaur 2018). With the proliferation of ARGs and ARBs in the environment, they are now recognized as important emerging pollutants with direct implications for environmental and public health (Blair et al. 2015; Kovalakova et al. 2020).

6.3 Induction of Resistance by Non-Antibiotic Factors

There is a widespread assumption that the escalating antibiotic resistance reported today is solely because of antibiotic misuse, i.e., the reservoir hypothesis (Heinemann et al. 2000). However, except for production site waste and wastewater effluent, antibiotic concentrations in the environment are generally much lower than those needed for antibiotic selection in the laboratory (Bengtsson-Palme and Larsson 2016; Table 1), suggesting there are likely other agents able to enhance antibiotic resistance. A growing body of literature indicates that a wide range of non-antibiotic, anti-microbial (NAAMs) chemicals (e.g., non-antibiotic drugs, microplastics, organic and inorganic pollutants, disinfectants, etc.) can promote antibiotic resistance (Wang et al. 2017; Lu et al. 2018; Zhang et al. 2019; Wang et al. 2021; Yu et al. 2021; Ding et al. 2022; Jia et al. 2022; Xu et al. 2022; reviewed by Shi et al. 2022). Physical and chemical properties (e.g., nutrients, total organic carbon, contaminants, etc.) within a particular environment also have been shown to mediate the spread of ARBs and ARGs, directly or indirectly, by stimulating bacterial growth and bacterial synthesis processes (Zhang et al. 2016; Zheng et al. 2021; Shi et al. 2022). The action of NAAMs has a compounding effect on the spread of antibiotic resistance in the environment that differs from resistance due to excessive antibiotic exposure. NAAMs have varying modes of action; however, these generally include 1) oxidative stress, 2) increased cell membrane permeability, 3) induction of the SOS response to DNA damage, 4) enhancement of

HGT of ARGs, 5) stimulation of efflux pump production and activity, and 6) elevated mutation rates (Lu et al. 2018; Zhang et al. 2019; Wang et al. 2021; Yu et al. 2021; Ding et al. 2022; Xu et al. 2022).

Non-antibiotic drugs (e.g., non-steroidal anti-inflammatories, β-blockers, lipid-lowering, antidepressants) and non-nutritive sweeteners have been linked to enhancing antibiotic resistance and persistence primarily associated with reactive oxygen species (ROS) production (Wang et al. 2020; Yu et al. 2021; Li et al. 2022; Wang et al. 2023). Laboratory experiments have shown antidepressants can promote antibiotic resistance in *Escherichia coli* by enhancing conjugative HGT of environmental and clinical plasmids (Ding et al. 2022). Other studies have demonstrated that antidepressants can accelerate movement of antibiotic resistance by over production of ROS and ROS-induced mutations, SOS responses (error-prone DNA repair), increased membrane permeability, and enhanced efflux pump expression (Jin et al. 2018; Ding et al. 2022; Lu et al. 2022; Wang et al. 2023).

Non-antimicrobial chemicals have been linked to multi-drug resistance. For example, triclosan, a chemical commonly used in over 2,000 household and personal care products, has been shown to induce multi-drug resistance in wild-type *Escherichia coli* at environmentally relevant concentrations (measured concentrations up to 0.4 mg/L in aquatic environments) (Lu et al. 2018). Investigations by Lu et al. (2018) showed that triclosan induces oxidative stress causing multiple genetic mutations as well as playing a role in upregulating transcription of genes encoding β-lactamases and multi-drug efflux pumps. Broad spectrum disinfectants (e.g., surface cleaners and hand sanitizers) containing quaternary ammonium compounds (QACs) as active ingredients are used in medical, industrial and household products. These QAC compounds work by destroying or disrupting cell membranes. In an *E. coli* model, Jia et al. (2022) demonstrated that QAC-induced resistance resulted from *de novo* mutations that enhanced gene expression of efflux pumps, leading to increased antibiotic resistance. Heavy metals, particularly silver, zinc, and copper, are used as antimicrobials and work by inducing oxidative stress, depleting antioxidants, causing genotoxicity and other dysfunctions (Lemire et al. 2013). Despite the damaging effects of metal toxicity, Li et al. (2019b) showed that over time, exposure to sublethal concentrations of certain metals increased mutation rates in *E. coli* and increased *de novo* mutations, some of which occurred in genes that resulted in antibiotic resistance. Zhang et al. (2019) showed that nanoparticles of cuprous oxide and copper ions were able to stimulate HGT via conjugative transfer of multi-drug resistant genes at environmentally relevant concentrations (1-100 μmol/L). Similar to other studies, *E. coli* exposed to copper caused over-production of ROS, increased cell membrane permeability, and the upregulation of genes for pilus expression (Zhang et al. 2019).

7 Risk of Amoxicillin to the Marine Environment

Determinations of whether a chemical substance is a threat to the environment are usually carried out in laboratory experiments using accepted standard protocols (e.g., American Society for Testing and Materials, Organisation for Economic Co-operation and Development, and U.S. EPA) with targeted receptor species across multiple taxa. Establishing toxicity thresholds along with environmental concentrations (summarized in Table 2) are required for determining environmental or ecological risk assessments (e.g., U.S. Environmental Protection Agency 1998; European Commission 2003; European Medicines Agency 2006; Lee et al. 2021; Downs et al. 2022). A Risk (or hazard) Quotient (RQ) is the 'ratio of the potential exposure to a substance and the level at which no adverse effects are expected' (U.S. Environmental Protection Agency 1998; Downs et al. 2022). To calculate RQs, the actual measured or predicted environmental concentration (MEC) of the target analyte is compared to the predicted no-effect concentration (PNEC) using the equation:

$$RQ=(MEC)/(PNEC, NOEC, LC^{50} \text{ or } EC^{50}) \times (\text{uncertainty factor})$$

RQ=risk (or hazard) quotient
MEC=measured or predicted environmental concentration
PNEC=predicted no-effect concentration
NOEC=no observed effect concentration
LC^{50}= lethal dose that causes death to half of the test organisms
EC^{50}= half maximal effect concentration
Uncertainty factor=ranges from 10-1000

(European Commission 2003; European Medicines Agency 2006; Downs et al. 2022). Uncertainty factors usually range from 10-1000 depending on the data available (i.e., number of taxa with toxicity thresholds, acute exposures and/or chronic exposures) (Belanger et al. 2021). RQ values ≥1.00 signify an unacceptable risk for a potential toxic effect; RQ values 0.50-0.90 indicate a moderate threat of a potential toxic effect; and RQ values 0.10-0.49 represent a low risk of a toxic effect (Hernando et al. 2006).

Lee et al. (2021) created a RQ based on acute and chronic toxicity data and worldwide levels of antibiotic occurrence in freshwater environments reported from the literature, similar to Tables 1 and 2. The mean MEC (MECmean) for amoxicillin was 0.068 µg/L and a maximum MEC (MECmax) was identified as 1.654 µg/L. The most sensitive receptor was *Synechococcus leopoliensis* with a NOEC of 0.78 µg/L for growth and an uncertainty factor of 10 resulting in a PNEC of 0.078 µg/L. The derived RQ for amoxicillin for the MECmean was 0.87, suggesting a moderate level of concern. The MECmax, however, resulted in a RQ of 21.2, indicating that amoxicillin can cause potential ecological risks in hotspots of concentrated amoxicillin levels. Interestingly, if the MECmean was instead 0.078 vs. 0.068 µg/L, the RQ of 1.0 would indicate an unacceptable risk at locations with concentrations ≥0.078 µg/L of amoxicillin.

Unfortunately for coral reef environments in the Florida Keys where amoxicillin is being used to treat diseased corals, the measured or predicted environmental concentrations are unknown. Similarly, there are few toxicological studies with Caribbean reef organisms to confirm their toxicity reference values or sufficient data to determine a species sensitivity distribution to amoxicillin. Thus, it is not possible to assess the potential hazard of amoxicillin use on Caribbean reefs without these data. However, the available literature clearly indicates that the most sensitive taxa in the marine environment are the blue-green alga (cyanobacterium), *Synechococcus leopoliensus* (Broccoli et al. 2021). Available information indicates that amoxicillin should pose negligible risk to cnidarians and other invertebrates as well as fish, except in highly contaminated sites, since these sites have toxicity thresholds that are in the milligram to gram range (Table 2).

Antibiotic use poses another type of risk: the development of antimicrobial resistance (AMR). However, how to manage or mitigate this in the environment is only recently being recognized as a critical issue for human and environmental health. The ability to conduct an environmental risk assessment for antibiotic selection resulting in antibiotic resistance and its environmental impact will be critical for managing and mitigating the ongoing increase in antibiotic resistance. Murray et al. (2021) have provided recommendations for achieving a standardized novel methodology that aligns with current environmental risk assessment guidelines while incorporating concepts unique to a risk assessment for AMR. This approach promises to enable better stewardship of environmental and human health faced with the overuse of antibiotics.

8 Conclusions

- Amoxicillin is a broad-spectrum antibiotic in the penicillin group that is chemically characterized as having a β-lactam ring. This group of antibiotics is estimated to comprise up to 70 % of the global antibiotic use. The global amoxicillin market alone is estimated to be worth 4,783.5 million USD in 2022 and projected to increase to 5,812.3 million USD by 2028 (360 Research Reports 2022).

- With amoxicillin's considerable uses as a therapeutic in human and veterinary medicine and as a prophylactic and 'growth promoter' in agriculture, it enters aquatic environments primarily from wastewater treatment effluents and runoff from various agricultural practices.

- In the marine environment, prokaryotic blue-green algae (cyanobacteria) are reported as the most sensitive taxa, which is important because cyanobacteria are one of the largest contributors to the phytoplankton biomass and if reduced can adversely affect many ecosystem services, including primary productivity.

- Based on available information, amoxicillin should pose negligible toxicological risk to fish, cnidarians, and other invertebrates, except in highly contaminated sites where environmental concentrations reach the milligram to gram range.

- A major concern in public health is the development of antibiotic resistance. The most common perception is that it arises only in the presence of antibiotics and under antibiotic selection. However, resistance also can occur through exposure to agents other than antibiotics.

- It is important to recognize that antimicrobial resistance (AMR) can arise from exposures to non-antibiotic, anti-microbial (NAAM) chemicals. Such chemicals can promote AMR with varying modes of action with the most common being oxidative stress, increased cell membrane permeability, induction of an SOS response, stimulation of efflux pump production, and factors that increase mutation rates.

- An environmental risk assessment of AMR emerging from amoxicillin exposures in the environment, particularly with its use on reefs of the Caribbean afflicted with stony coral tissue loss disease, cannot be determined at this time because of insufficient data.

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