Antimicrobial activity of metals and metalloids

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Competition has been an integral part in the evolution of life. It is difficult to predict the beginning of life, but it is clear that the archaea, bacteria and bacteriophages were the earliest life forms to emerge on the primordial Earth (Clokie et al., 2011). Archaea and bacteria have always waged war with each other, competing for limited resources (Ghoul and Mitri, 2016).

Predator-prey relationships accelerated the rate of evolution and transition to more complex and larger life forms by 650 million years ago (Mya) (Narbonne, 2004). Reciprocal selection altered the biotic selective

environment of both predator and prey (Papkou et al., 2019). These prey-predator interactive networks are proposed to have accelerated the pace of evolution. In this evolutionary arms race, superior weapons such as metals and metalloids are essential for the predator, whereas superior defenses are essential for the prey. In this review, we focus primarily on copper (Cu) and arsenic (As). In terms of evolution, once a predator attacks a prey, survivors must have developed ways to defend themselves such as active efflux. Prey resistance in turn forces the predator to acquire new weapons, for example, using other toxic metals or antimicrobial peptides, leading to a new cycle of selective prey resistance. Therefore, both predator and prey evolve in parallel to avoid extinction. In Red Queen co-evolution (Nair et al., 2019), the Red Queen explained the looking glass land to Alice: *Now, here, you see, it takes all the running you can do, to keep in the same place.*

Life has been exposed to the toxic metalloid As (Fig. 1) and the toxic metal Cu (Fig. 2) since the rise of the first organisms, approximately 3.5 billion years ago (Bya), during the Archean Eon (4~2.5 Bya) (Chen et al., 2020; Chi Fru et al., 2016; Chi Fru et al., 2019; Zhu et al., 2014). The first bacteria not only adapted to survive in the presence of As but also adapted it as an offensive weapon in microbial warfare to gain a competitive advantage (Chen et al., 2019a). Many organisms from bacteria to vertebrates have genes for conversion of As into weapons and/or genes that

protect them from As toxicity. In bacteria, these genes are nearly all found in As resistance (*ars*) operons. We also briefly examine copper availability through the Earth's history and the factors that controlled its bioavailability, given that the evolution of life as a whole has always been linked to the bioavailability of essential metals (Ciscato et al., 2019; Moore et al., 2017; Robbins et al., 2016).

Arsenic dynamics throughout the history of the Earth

During the anoxic Archean Eon, geochemically-derived inorganic As would have existed primarily as trivalent As(III). About 2.4 Bya, the Earth's atmosphere and ocean surface became permanently oxygenated during the Great Oxygenation Event (GOE) (Fig. 1A), which oxidized inorganic arsenic (Lyons et al., 2014). Historical records of marine As sedimentary dynamics reconstructed from marine sedimentary iron formations and shales suggest that early oceans were rich in As. However, the dissolved concentrations would have been modulated by the high iron content, which would have acted as a potent sink for As removal from seawater (Fig. 1B). Iron formations occurred predominantly between 4.0-1.8 Bya and then re-appeared briefly towards the end of the Proterozoic Eon (0.5 Bya) in association with the termination of the Neoproterozoic global glaciations that occurred 0.720-0.635 Bya. This NOE rise of marine

As content coincided with the Neoproterozoic Oxygenation Event (NOE) that followed the glaciations (Fig. 1A-C). These glaciations and the earlier Huronian Snowball Earth glaciation that coincided with the GOE 2.4~2.1 Bya (Lyons et al., 2014) severely curtailed release of As into oceans because of ice house-suppressed weathering coupled to an inefficient hydrological cycle (Chi Fru et al., 2015).

As concentrations in marine sedimentary iron formations and shales suggest a high Archean As concentration with four critical peaks and three key depressions through Earth history (Fig. 1B). The high Archean As concentrations declined dramatically following the onset of the GOE and the associated Huronian Snowball Earth glaciations (Fig. 1B). Following deglaciation and return to a greenhouse state, the As concentrations increased again (Chi Fru et al., 2016). A major As spike occurred 1.4 Bya when atmospheric oxygen briefly rose (Large et al., 2019). Another spike followed the Marinoan Snowball Earth glaciation that ended 635 Mya. The post-Snowball increases have been linked to increased concentrations of As coming from continental bedrock erosion by the deglaciating ice sheets that delivered soluble As to the oceans (Chi Fru et al., 2016).

The highest extant As concentrations are found in shallow marine iron formations from the hydrothermal vent fields of Milos Island, Greece, where hydrothermal fluids contain greater than 3000-fold more As than seawater (Breuer and Pichler, 2013; Chi Fru et al., 2013). At this site, the

As efflux gene, *acr3* (Chen et al., 2020), is the most abundant As detoxifying gene found in microbial communities (Callac et al., 2017; Chi Fru et al., 2019). These modern shallow marine hydrothermal ecosystems are differentiated into iron oxide, sulfidic, anoxic, and oxic ecosystems similar to those that predominated the Precambrian world (Chi Fru et al., 2018; Poulton and Canfield, 2011a). Genes such as *ars3*, are also widespread in the volcanic As-rich ecosystems of the Andes Mountains, which are believed to be similar to the earliest oceans (Rascovan et al., 2016; Sancho-Tomás et al., 2018).

Early marine As concentrations would have been modulated by the large volume of iron-rich precipitates that formed vast iron formations (Fig. 1C). Nonetheless, a similar series of events was replicated when As is normalized to iron concentrations (Fig. 1C), as well as without normalization (Fig. 1B). This implies that As(III) was the dominant inorganic As species in the geobiosphere prior to 2.4 Bya due to its stability and high mobility in anoxic conditions. As(V) and various As sulfides became the prominent species following the GOE (Chi Fru et al., 2015; Chi Fru et al., 2019). This resultant shift in the oxidation state of As is thought to have triggered new adaptive responses in existing microbial communities (Chen et al., 2020; Chi Fru et al., 2019).

Copper throughout the Earth's history

A detailed examination of marine iron formations and shales suggests that long-term variations in sedimentary marine Cu concentrations in the geological record were generally small (Fig. 2). The data, however, reveal significant Cu burial in association with iron oxide-rich iron formations relative to iron oxide-poor marine shales that are predominantly a product of continental weathering (Fig. 2B). These observations insinuate that the reactive marine iron reservoir has controlled dissolved seawater Cu concentrations throughout Earth history (Chi Fru et al., 2016). Similar to As bioavailability, iron-rich ecosystems such as those that prevailed in the early oceans served as major sinks for dissolved Cu and recent evidence further points to seawater sulfide and organic matter content as powerful Cu sinks (Ciscato et al., 2019).

Thus, redox cycling of iron, sulfur and carbon would have played a major role in Cu bioavailability, especially after the GOE and the NOE. For example, there was a progressive reduction in seawater iron concentration across the Archean-Proterozoic boundary until about 0.58 Bya, when the deep oceans first became fully oxygenated (Poulton and Canfield, 2011b). This gradually reduction in the size of the ocean iron reservoir after the GOE, would have promoted an increase in dissolved surficial seawater Cu concentrations. These conditions would have enabled life in the iron-poor, open oxygenated ocean surface to flourish in greater

dissolved Cu conditions. On the other hand, sulfide-related Cu scavenging in the mid-depth near continental margin habitats where sulfide was prevalent and by the iron oxides that accumulated in the iron-rich deep ocean (Poulton and Canfield, 2011b), would have promoted low Cu bioavailability in these habitats. By allowing greater Cu bioavailability in the iron-deficient and sulfide-poor oxygen-rich surface oceans, this would have conferred a selective advantage for biological Cu utilization, including the potential for the development of Cu-containing biological weapons.

Arsenic-dependent biological warfare

One of the first enzymes in As biotransformation to have evolved was the ArsM As(III) *S*-adenosylmethionine methyltransferase, which can be traced back nearly 3.5 Bya by molecular clock reconstruction (Fig. 1A) (Chen et al., 2020). ArsM methylates inorganic As(III) into highly toxic MAs(III) (Fig. 3, A2) and DMAs(III) (Fig. 3, A4) and non-toxic volatile TMAs(III) gas (Fig. 3, A5). Only later did the Acr3 and ArsP, the efflux permeases evolve (Fig. 1A) to confer resistance to As(III) and MAs(III), respectively (Fig. 3, A1 and A3). While it may seem paradoxical that microbes would first make As more toxic before coming up with ways to tolerate it, one must consider that even the first microorganisms would

have been under selective pressure to outgrow each other, the origin of microbial warfare. Bacteria that innovated the ability to methylate inorganic As turned this unique adaptation into a potent weapon, bequeathing to them a powerful selective and competitive advantage against competitors.

In support of this novel hypothesis, in extant soil microbial communities, biogenic MAs(III) exhibits antimicrobial properties (Chen et al., 2019a). MAs(III) fits the classical definition of "antibiotic" introduced by Selman Waksman in the 1940s, as a toxic organic compound produced by one microbe to kill competitors (Waksman, 1947). DMAs(III) may also have antibiotic-like properties, but its lower stability compared with MAs(III) reduces its effectiveness as an antibiotic. Further methylation generates non-toxic volatile TMAs(III) gas, which may have functioned as a primitive self-protection mechanism in the producing microbe against the MAs(III) and DMAs(III) that it generates (Fig. 3, A5), especially before the evolution of more sophisticated and effective mechanisms such as ArsP. MAs(III) is very reactive and may have multiple targets in bacteria. Recently one bacterial target of trivalent organoarsenicals was identified (Garbinski et al., 2020). MAs(III), but not inorganic As(III), effectively inhibits MurA, the bacterial enzyme involved in the first step of peptidoglycan synthesis, suggesting that one mechanism of action of trivalent organoarsenical antimicrobials is inhibition of bacterial cell wall

synthesis (Fig. 3, D1).

The *arsM* gene is widespread in mainly the Bacterial Kingdom, where it is thought to have first emerged. However, as a result of lateral gene transfer, the *arsM* gene has been acquired by archaea and eukaryotes, including algae, fungi, protists, various animal lineages and as well as in humans as the *AS3MT* gene product (Chen et al., 2017).

The widespread distribution of the *arsM* gene raises the question of why methylated arsenicals are not abundant in the natural environment. For example, it is puzzling why most of the As present in seawater is not methylated and sequestered in marine biomass. Methylated arsenicals are the likely precursors of more complex organoarsenicals such as arsenosugars (Xue et al., 2019)((PMID: 30525501)), arsenolipids, arsenobetaine and related compounds that are sequestered by cyanobacteria and algae, resulting in bioaccumulation and biomagnification up the food chain. Since these complex organoarsenicals are essentially nontoxic, they likely represent an As detoxification mechanism (Taylor et al., 2017). These organoarsenicals are not easily biodegraded. For example, marine DMAs(V) has an 8.1 days turnover rate (Giovannoni et al., 2019). So, the biomass of dead marine organisms serves as an As sink in marine sediments.

196 In general, antibiotic producers are resistant to the antibiotics that they
197 produce, for example by removal from the cell using efflux pumps (Munita
198 and Arias, 2016). Acr3 and ArsP are efflux permeases for As(III) (Fig. 3,
199 A1) and MAs(III) (Fig. 3, A3) (Chen et al., 2019a), respectively. The
200 molecular fossil record is not entirely clear, but the *arsP* gene appears to
201 have evolved more recently than either the *arsM* or *acr3* genes and spread
202 through prokaryotes by horizontal gene transfer (HGT) as a mechanism for
203 MAs(III) resistance (Chen et al., 2020). However, the times of origin of
204 *arsM* and *arsP* overlap to some degree, so another possibility is that ArsP
205 evolved in parallel with ArsM to provide the producer with another way to
206 become resistant to its own product. Another pathway for MAs(III) efflux
207 is via bacterial aquaglyceroporins channels such as GlpF (Fig. 3, A3)
208 (Garbinski et al., 2019). GlpF facilitates As(III) uptake in *Escherichia coli*
209 (Sanders et al., 1997), and the human liver ortholog AQP9 is a bidirectional
210 facilitator of both As(III) and MAs(III) (Garbinski et al., 2019). These
211 channels move As(III) into cells down a concentration gradient from higher
212 extracellular to a lower intracellular levels. If As(III) is methylated inside
213 of bacterial cells, it could flow down its concentration gradient into the
214 extracellular milieu. In effect, bacterial GlpF orthologs exchange
215 extracellular As(III) for intracellular MAs(III), providing a pathway for
216 protecting MAs(III) producers from the bactericidal activity of MAs(III).
217 This speculation implies an early origin for the bacterial aquaglyceroporin

gene. However, these aquaglyceroporins are generalized channels for metalloids, including not only toxic As and antimony, but also boron and silicon, which have structural roles in plants (Mukhopadhyay et al., 2014) and might have had similar physiological functions in the first organisms. The major facilitator superfamily also has members that transport MAs(III) such as ArsK (Fig. 3, A3) (Shi et al., 2018). ArsK has lower selectivity than ArsP and confers resistance to not only MAs(III) but also inorganic As(III). When the *arsK* gene has emerged is unclear yet due to lack of molecular clock analyses.

As discussed above, MAs(III) may be been a primordial antibiotic. Some members of present-day anaerobic microbial communities produce MAs(III), but this is subsequently detoxified abiotically by oxidizing in air to MAs(V) (Fig. 3, A6). However, members of aerobic microbial communities reduce MAs(V) by as-yet unidentified pathways (Yoshinaga et al., 2011), taking advantage of the availability of microbially generated MAs(V) (Fig. 3, B1), producing a competitive advantage over As sensitive community members. Since this cycle of methylation, oxidation, reduction and resistance involves a number of bacterial species, these complex interactions are emergent properties of the entire microbial community (Chen et al., 2019a) . A hallmark of the battles that take place in microbial jungles is when one species produces an antibiotic, others acquire resistance mechanisms, as is the case for toxic biogenic MAs(III) (Fig. 3E).

Some sensitive bacteria acquired oxygen-independent resistance genes such as *arsP* by HGT (Fig. 3, E1), rendering them resistant to MAs(III). After the GOE, there were new opportunities for evolution of resistance mechanisms. First, microbial methylation of As(III) to MAs(III) by ArsM became a detoxification mechanism as MAs(III) was oxidized to MAs(V) in air (Fig. 3, A6). Second, the permanence of oxygen in the atmosphere provided a selective pressure for the evolution of new pathways of resistance using oxidative reactions (Yang and Rosen, 2016). Two oxygen-utilizing enzymes have been identified − ArsI and ArsH. ArsI is C-As bond lyase that confers resistance to MAs(III) by cleavage of the bond between the carbon and arsenic atoms, forming less toxic As(III) (Yoshinaga and Rosen, 2014) (Fig. 3, E2). ArsH is MAs(III) oxidase that catalyzes oxidation of MAs(III) to MAs(V), thus detoxifying it (Chen et al., 2015)((Fig. 3, E3). The MAs(III) resistance genes (*arsP, arsK, arsI* and *arsH*) are widely distributed in bacteria, which in turn supports our hypothesis that bacteria generating MAs(III), by either of inorganic arsenic methylation or MAs(V) reduction, utilize it for predation.

Aromatic arsenicals

Since Antoine Béchamp's synthesis and discovery of the first man-made aromatic arsenical atoxyl (also called *p*-arsinilic acid, *p*-

aminophenylarsenate or *p*ASA) in 1859 (Kritharis et al., 2013), a number of aromatic arsenicals have been synthesized and utilized in medicine (Gibaud and Jaouen, 2010), farming (Mangalgiri et al., 2015) and military (Radke et al., 2014). Many bacteria tolerate or metabolize synthetic organoarsenicals, showing their ability to rapidly adapt to new environmental stresses.

As is one of the oldest medicines, used in ancient Greece, Rome and China (Kritharis et al., 2013). Salvarsan, the first chemotherapeutic drug, is an aromatic arsenical (Wright et al., 2014). This “magic bullet”, the first effective anti-syphilis drug developed by Paul Ehrlich in 1910 was based on atoxyl, and it soon became the most world-widely prescribed drug and made significant contributions to improvement of public health until the advent of penicillin in the 1940’s. Synthetic aromatic arsenicals were next applied to animal husbandry, and for decades, have been mainly used as antiprotozoal growth promoters for poultry and swine production (Mangalgiri et al., 2015). Four pentavalent aromatic arsenicals – roxarsone (4-hydroxy-3-nitrophenylarsenate or Rox(V)), nitarsone (*p*-nitrophenylarsenate or Nit(V)), *p*-ASA and carbarsone (*N*-acetylated *p*-ASA) – were registered in the mid-1940’s and used extensively in the USA until banned in mid-2010, although they are still used in other countries. Those aromatic arsenicals are not highly accumulated in animals, with the majority of the drugs excreted unchanged. Although they are modified by

283 methylation, acetylation and other reactions, it is not clear whether those
284 modifications take place in the animals or their microbiomes or in the
285 excreted litter (Yang et al., 2016). Animal manure is used as fertilizer,
286 which has introduced massive amounts of aromatic arsenicals into the
287 environment over the past decades. It is estimated that nearly 900 tons of
288 the most widely used compound, roxarsone, was released into the
289 environment in the single year 2000 by the poultry industry in the US
290 (Rutherford et al., 2003). As is true for inorganic and methylated arsenicals,
291 aromatic arsenicals are more toxic in reduced trivalent forms compared
292 with their oxidized pentavalent counterparts (Garbinski et al., 2019). As
293 described below, soil bacteria have genes for roxarsone degradation
294 (Chen and Rosen, 2020; Chen et al., 2019b; Yan et al., 2019)(, so roxarsone
295 in animal manure is eventually recycled.

296 Paul Ehrlich predicted that "*resistance follows the drug like a familiar*
297 *shadow*", and resistance to salvarsan emerged in the 1930's (Stekel, 2018).
298 It was reasonable to predict that massive use of roxarsone and other
299 aromatic arsenicals would promote bacterial adaptation. Notably, the
300 nitrogen-fixing legume symbiont *Sinorhizobium meliloti* 1021 activates
301 Rox(V) by transforming it into trivalent 4-hydroxy-3-aminophenylarsite
302 (HAPA(III)) via two sequential steps: 1) reduction of the nitro group to an
303 amine by the NADPH-dependent nitroreductase MdaB, and 2) reduction
304 of the pentavalent As atom to trivalency by an unknown mechanism (Fig.

3, B2) (Yan et al., 2019). *S. meliloti* is also capable of reduction of pentavalent *p*-ASA to the bioactive form *p*-ASA(III), and also reduces MAs(V) to MAs(III) (Fig. 3, B1). *Pseudomonas putida* can also reduce the nitro group of roxarsone using the chromosomally-encoded *nfnB* gene product, another FMN-NADPH-dependent nitroreductase (Chen and Rosen, 2020). NfnB is not organoarsenical specific, and the gene is not in *ars* operons, but this nitroreduction confers resistance to roxarsone. However, among known MAs(V) reducers, only *S. meliloti* is capable of reducing both the nitro group and arsenic atom of aromatic arsenicals, presumably to utilize them as antimicrobials (Fig. 3, B2). Utilization of aromatic arsenicals as antimicrobials could provide the producers a major advantage over competitors in microenvironments. The MAs(III)-resistance genes *arsP*, *arsI*, *arsH* and *arsK* also confer resistance to trivalent aromatic arsenicals (Fig. 3E). Notably a novel *arsEFG* operon confers specific resistance to aromatic arsenicals has been recently identified in a number of obligate/facultative anaerobes (Chen et al., 2019b). ArsE and ArsF reduce the nitro group of Rox(III) or Nit(III) to amino group, generating HAPA(III) or *p*-ASA(III). ArsG extrudes the aromatic aminoarsenicals out of the cells, completing the resistance pathway (Fig. 3, E4). A unique feature of ArsEFG is that it confers resistance to aromatic arsenicals but not MAs(III).

Arsinothricin

Recently *Burkholderia gladioli* GSRB05, a bacterial isolate from the rhizosphere of rice grown in an As-contaminated site, was demonstrated to synthesize two novel organoarsenical compounds from inorganic arsenite As(III) (Fig. 3C) (Kuramata et al., 2016). The two new organoarsenicals were named arsinothricin ((2-amino-4-(hydroxymethylarsinoyl)butanoate, AST) and the unmethylated species hydroxy arsinothricin (AST-OH) due to their structural similarity with phosphinothricin (PT), the *Streptomyces*-produced phosphonate antibiotic, and the unmethylated species demethyl phosphinothricin (DMPT), an intermediate in the biosynthesis of PT. The mechanism of action of PT is competitive inhibition of bacterial glutamine synthetase (GS) that results in accumulation of toxic ammonia and lack of glutamine, leading to bacterial killing (Fig. 3, D5) (Nadar et al., 2019). The inhibitory activity of AST on bacterial GS is compatible to PT, but the antimicrobial activity of AST on several different bacteria is 15-fold greater than PT (Nadar et al., 2019), perhaps due to higher permeability of AST. AST effectively inhibits growth of both Gram-positive and Gram-negative bacteria, including pathogens such as *Myobacterium bovis* BCG, the etiological agent of bovine tuberculosis, and carbapenem-resistant *Enterobacter cloacae*, a WHO-designated critical priority pathogen, demonstrating that AST is a potent broad-spectrum antibiotic (Nadar et al., 2019). When *B. gladioli* was cultured with As(III), the amount of AST-OH

increased and then gradually decreased, and AST reciprocally increased, suggesting that AST-OH is the precursor of AST, just as DMPT is the precursor of PT (Kuramata et al., 2016).

AST is another demonstration that bacteria can utilize As as an antibiotic. As mentioned, pentavalent As species are much less toxic than trivalent species. The above-mentioned methyl/aromatic arsenite antimicrobials are in reduced trivalent form, achieving the potent antimicrobial effect through the robust affinity with thiols in essential enzymes for carbohydrate metabolism such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Fig. 4, D2) (Tokmina-Lukaszewska *et al* 2016, DOI: 10.1111/1462-2920.13615) and redox-regulating small proteins/molecules such as glutaredoxin/thioredoxin (Fig. 4, D3) and glutathione (Fig. 4, D4) , thus, their target molecules are rather broad than specific (Shen et al., 2013). In contrast AST contains pentavalent As and is as toxic as trivalent MAs(III) because it has a uniquely different mechanism of action than trivalent arsenicals (Nadar et al., 2019). Because it is a pentavalent arsenical, this As-based antibiotic likely emerged after GOE.

Bacterial resistance against AST is conferred by acetylation of the α-amino group catalyzed by ArsN1 (Nadar et al., 2019), an enzyme belonging to the GCN5-related *N*-acetyltransferases (GNAT) superfamily (Burckhardt and Escalante-Semerena, 2020). PpArsN1 encoded in the *ars*

operon from *P. putida* KT2440, is an AST-selective *N*-acetyltransferase.

Phosphonate natural products, represented by PT, are a rich source of antibiotics (Horsman and Zechel, 2017). AST is the arsonate counterpart of PT, and we predict that additional arsonate antibiotics exist. A second type of GNAT gene, *arsN2*, is found in bacterial *ars* operons (Nadar et al., 2019; Sharma, 2012). ArsN2 is more closely related to *N*-acetylglutamate synthetase (ArgA) that catalyzes *N*-acetylation of glutamate, the initial step in de novo arginine biosynthesis (Chauhan et al., 2009). No function has been identified for ArsN2, but we propose that it confers resistance against another as-yet unknown As-containing antibiotic.

Copper homeostasis: the need for a balance

Cu is an essential trace transition metal in most organisms (German et al., 2013; Ladomersky and Petris, 2015). Overall, more than 2/3 of all organisms are dependent on this metal (Ridge et al., 2008). However, excess Cu is toxic through mechanisms including ROS generation (Fig. 4, B6), displacement of iron from iron-sulfur clusters (Fig. 4, B7), thiol depletion in the glutathione pool (Fig. 4, B8), and/or mismetallation and inactivation of metalloproteins by replacing other metal cofactors (Fig. 4, B9). Consequently, all organisms have developed methods to respond to low and high Cu. These mechanisms involve i) active efflux by P_{1B}-type

ATPases, the resistance-nodulation-cell division (RND)-type transport systems and cation diffusion facilitators (CDF) (Fig. 4, B1) (Argüello et al., 2016; Delmar et al., 2014; Moraleda-Muñoz et al., 2010a, b; Nies, 2003); ii) cellular sequestration by metallochaperones (Fig. 4, B5) (Robinson and Winge, 2010); and iii) oxidation of Cu(I) to less toxic Cu(II) by multicopper oxidases (Fig. 4, B??) (Chandrangsu et al., 2017; Sánchez-Sutil et al., 2007). Intracellular Cu is controlled by metal-sensing regulatory transcription factors and signaling systems consisting of one-component systems, two-component systems, serine-threonine protein kinases, as well as extracytoplasmic function sigma factors (Lonetto et al., 2019; Moraleda-Muñoz et al., 2019; Rademacher and Masepohl, 2012). Although many organisms possess Cu exporters that can protect them against Cu uptake, there is little correlation between occurrence of Cu transporters and cuproproteins, suggesting that pathways of utilization and detoxification evolved independently (Ridge et al., 2008).

Role of copper in bacterial interactions

Transition metals, including iron, Cu, manganese (Mn) and zinc (Zn), are essential trace nutrients in virtually all biological systems. Cu distribution in soil is influenced by climatic, physic-chemical properties and possible exogenous inputs from volcanic eruptions, windblown dust and forest fires.

Soil Cu levels are increased by anthropogenic sources including leather processing, municipal refuse, waste from electroplating and iron and steel producers, and discarded Cu products from plumbing, wiring, mining, traffic and domestic heating (Cornu et al., 2017; Pal et al., 2017; Tella et al., 2016). Cu is also utilized as fungicides and herbicides for agricultural crops such as olive groves and vineyards (Ballabio et al., 2018). Cu is also used as a feed additive in animal husbandry and is excreted in animal manure (Seiler and Berendonk, 2012). In addition, Cu-containing products are used on hospital surfaces, in clinical surgery and in medicine (Lemire et al., 2013; Page et al., 2009; Schmidt et al., 2016; Vincent et al., 2018). In 2008, the US Environmental Protection Agency (EPA) recognized Cu and its alloys as the first effective metallic antimicrobial agent. Nevertheless, these activities have led to the emergence of Cu-tolerant microbes and the spread of resistance to other metals and antibiotics (Li et al., 2017; Pal et al., 2017; Rensing et al., 2018).

Copper as offensive weapon in bacterial interactions

Cu toxicity has been implicated in interactions between protozoa and bacteria, where eukaryotic organisms up-regulate genes in Cu handling and trafficking during the phagocytosis, inducing accumulation of Cu(I) in the phagosome to kill bacteria (German et al., 2013; Hao et al., 2016). In response, bacteria use mechanisms to survive inside of phagosomes such as digestion resistance and up-regulation of expression of genes involved

in Cu detoxification (Djoko et al., 2015; Espinoza-Vergara et al., 2020; Ladomersky and Petris, 2015; Sun et al., 2018).

Cu is utilized for predation by the soil bacterium *Cupriavidus necator* (Casida, 1987, 1988), a non-obligate predator that preys on a wide range of Gram-positive and Gram-negative bacteria (Makkar and Casida, 1987; Zeph and Casida, 1986). *C. necator* is not only resistant to Cu but requires high Cu concentrations for initial growth (but not subsequent growth). It produces a heat-stable Cu-binding peptide growth initiation factor, which is also used to kill its prey such as the actinomycete *Agromyces ramosus*. *A. ramosus* counterattacks by producing mycelia that lyses approximately one-third of the *C. necator* cells. However, the surviving *C. necator* cells lyse *A. ramosus* mycelia using the excess Cu delivered by Cu-binding peptide. Nevertheless, *C. necator* is unable to lyse the dormant rod cells that *A. ramosus* quickly forms and fragments from the mycelium. The dormant cells allow *A. ramosus* to grow again (Casida, 1987, 1988). *C. necator* also preys on *Bacillus subtilis*, and its predatory activity increases in the presence of Cu in a concentration-dependent manner. *C. necator,* in contrast to group predators, does not depend on outnumbering the prey nor does it require prey contact for predatory strategy, suggesting that *C. necator* kills prey using secreted extracellular factors (Seccareccia et al., 2016). *B. subtilis* forms spores to avoid predation by *C. necator* and other known Cu-using predatory bacterium such as *Myxococcus xanthus* (Müller

et al., 2014; Müller et al., 2015). A metabolically inactive state (*i.e.*, persister-like cell state) is sufficient for protection from *C. necator,* whereas an intact spore coat is required to resist predation by *M. xanthus* (Seccareccia et al., 2016), indicating that the Cu-dependent predatory system of the latter is more powerful than that of the former. *M. xanthus* exhibits a complex response to Cu (Pérez et al., 2018), which implies that numerous genes coding for structural elements are involved in efflux, complexation and oxidation of Cu (Moraleda-Muñoz et al., 2010a, b; Sánchez-Sutil et al., 2007). Expression of some genes increases after exposure to Cu but rapidly decreases to basal levels, allowing an immediate response to the metal, whereas expression of other genes slows after Cu addition and plateaus after 24–48 hours as a maintenance response (Moraleda-Muñoz et al., 2019). This hierarchical response of *M. xanthus* to Cu is controlled and coordinated by diverse and specific regulatory elements (Gómez-Santos et al., 2011; Marcos-Torres et al., 2016; Sánchez-Sutil et al., 2016; Sánchez-Sutil et al., 2013). Since *M. xanthus* is not specifically resistant to Cu compared with other bacteria, some elements have been proposed to be required for the multicellular lifestyle of *M. xanthus* (Contreras-Moreno et al., 2020) . Cu would be used as an arsenal for cooperative predation to kill prey in a similar way as used by eukaryotic predators, macrophages or highly-Cu resistant bacterial predators.

Unlike *C. necator*, *M. xanthus* requires cell-cell contact and close-

proximity for its predatory activity. This may be due to limited diffusion and/or the delivery mechanism used to lyse prey, and could involve the participation of outer membrane vesicles (OMVs). Bacterial extracellular OMVs emerge after fission from the secreting cell. OMVs contain diverse cargo, including nucleic acids, proteins, lipids, virulence factors and metabolites. A number of functions for OMVs has been demonstrated, including intercellular communication, procurement of nutrients, biofilm formation, modulation of host immune responses, delivery of toxins and virulence factors, and secretion of molecules (Bitto et al., 2017; Caruana and Walper, 2020; Chen et al., 2016; Deatherage and Cookson, 2012; Mashburn and Whiteley, 2005; Théry et al., 2009). Packaging within OMVs allows for a highly concentrated dose of molecules to be delivered to distant and inaccessible locations. Consequently, OMVs may enhance Cu toxicity in bacterial interactions by concentrating the metal and ensure a more focused transport and intervention of the metal in the predatory activity which would increase predation efficiency and reduce prey resistance (Fig. 4, B2).

Additionally, the predatory activity of *M. xanthus* has been recently demonstrated to involve Cu accumulation in the region where the predator collides with the prey *S. meliloti.* Cu accumulation consequently up-regulates expression of the P_{1B}-ATPase CopA, the multicopper oxidase CuoA and the CBA efflux pump Cus2 in the predator cells. Cu

accumulation also triggers overproduction at the predator-prey interface of Cu-inducible melanin by the prey, which protects it from predation (Fig. 4B10-12) (Contreras-Moreno et al., 2020).

Melanins are polymeric pigments found in all domains of life that play a wide variety of functions (Cordero and Casadevall, 2017). Melanins protect bacteria from environmental stress conditions, influencing bacterial interactions with other organisms (Pavan et al., 2020). Melanins have free radical scavenging potential, so these pigments can diminish oxidative bursts, protecting bacteria from oxidative stress (Fig. 4, B10) (Ahmad et al., 2016; Keith et al., 2007). Melanin production also has been proposed to help cope with high concentrations of heavy metals (Fig. 4, B11 and B12) (Pavan et al., 2015). A consequence derived from this result is that the utility of metals as antimicrobial drugs against melanin-producing organisms may be lower than that against non-melanin-producing microbes (Cordero and Casadevall, 2017). Importantly, melanins can also neutralize antibiotics, increasing the inhibitory dose of antibiotics and improving the viability of bacteria (Lin et al., 2005). Altogether, the protective role of melanins produced by the prey during the interaction with the predators might suppose a crucial element of protection against predation, both helping cope with reactive oxygen species associated to Cu potential toxicity and neutralizing the antibiotics released by the predator.

In the environment, Cu may interfere in microbial interactions,

523 modifying the activity of the antibiotics produced by interacting organisms,
524 creating a variety of outcomes ranging from hindrance to enhancement of
525 antibiotic activity (Poole, 2017). Cu may also modulate predator and prey
526 antibiotic activity. Thus, predators could increase the toxic facet of the
527 metal, using it to enhance the antimicrobial activity of their own antibiotics
528 (Fig. 4, B3) and/or to neutralize antimicrobials released by the prey (Fig.
529 4, B4).

530

531 Defensive prey responses to face copper toxicity

532 Interspecific interaction with the predator may prompt the prey to
533 experience structural adaptations that help to resist or escape predation by
534 the formation of a mechanical barrier, such as exopolysaccharide, mucus
535 or biofilms (Fig. 4, A3), involved in neutralizing or counteracting Cu
536 toxicity (DePas et al., 2014; Nair et al., 2019; Perez et al., 2014).

537 Bacterial biofilms confer resistance to antibiotics and to metals
538 (including Cu) (Harrison et al., 2004; Høiby et al., 2010; Teitzel and Parsek,
539 2003; Young et al., 2015). However, bacterial predators can use Cu to cause
540 an unspecific reduction of expression of biofilm matrix-promoting genes
541 of the prey. This results in changes in both the biofilm surface roughness
542 and wetting behavior, producing biofilms that are more susceptible to
543 treatment with aqueous antibiotic solutions (Dinh et al., 2019; Harris et al.,

544 2018). During their attack, consequently, bacterial predators may use not
545 only the inherent toxicity of Cu, but also the ability of this metal to prevent
546 biofilm formation by the prey and/or weaken the defensive features of
547 existing biofilms. This increases susceptibility of the prey population to the
548 arsenal of lytic products released by the predators. In fact, the dual role of
549 Cu and other metals as biofilm inhibitors and antimicrobial agents has been
550 widely explored (Dinh et al., 2019; Dupont et al., 2011; Hsueh et al., 2015;
551 Lemire et al., 2013; Sirelkhatim et al., 2015).

552 Nevertheless, biofilms not only exhibit a protective role against
553 metals, but their generation is induced by metals, as in the case of the plant
554 pathogen *Xylella fastidiosa* (Cobine et al., 2013). Cu selection of dormant
555 persisters has also been described in *X. fastidiosa.* The pretreatment of
556 biofilms with subinhibitory Cu concentration has been showed to increase
557 the number of persisters recovered following treatment with toxic Cu levels
558 (Muranaka et al., 2012). Similarly, metal-selected persisters in the biofilms
559 of *Pseudomonas aeruginosa* may be responsible for increased metal
560 tolerance after short-term exposure to Cu or Zn (Harrison et al., 2005).
561 Altogether these results support the hypothesis that metal selection of
562 persisters is responsible for biofilm tolerance to metals and, particularly, to
563 Cu (Fig. 4, A2). Cu has also been shown to induce so-called viable
564 nonculturable (VNC) cells, a stress-induced dormant state, in a variety of
565 Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, and *Salmonella*

566 *enterica serovar* Typhi (Aurass et al., 2011; Dwidjosiswojo et al., 2011;
567 Jiang, 2014). Additionally, as mentioned above for the interaction of *B.*
568 *subtilis* with *C. necator* or *M. xanthus*, it has also been described the
569 differentiation of prey vegetative cells in stress-resistant spores to avoid
570 predation (Fig. 4, A1) (Muller et al., 2014; Muller et al., 2015; Seccareccia
571 et al., 2016).

572 The bacterial differentiations listed above reflect diverse approaches
573 adopted by prey to manage natural or predator-induced Cu toxicity. Some
574 of these tactics may enable the establishment of a physical barrier to
575 prevent Cu accession to the prey, whereas other defensive methods hinge
576 on conversion of vegetative cells on cellular types exhibiting more
577 resistance to Cu and anticipation that metal concentrations be restored to
578 tolerable levels.

579

580 Protective role of chalkophores (and other metallophores) 581 against copper toxicity

582 An apparently surprising component of prey defensive equipment
583 against Cu are metallophores (Fig. 4, B5). Metallophores are considered
584 primarily in the context of their role in metal uptake and metal homeostasis,
585 but many appear to have a broad range of secondary roles, ranging from
586 regulatory functions (Kenney et al., 2016) to protection against toxicity

587 caused by metals (Xin et al., 2014) or reactive oxygen species (Choi et al.,
588 2008) to biomedically relevant antibiotic or therapeutic functions
589 (Johnstone and Nolan, 2015; Kraemer et al., 2015; Lichtmannegger et al.,
590 2016).

591 Although metallophores have been identified for diverse metals,
592 including Mn (Parker et al., 2014), nickel and cobalt (Ghssein et al., 2016),
593 Zn (Bobrov et al., 2014), gold (Johnston et al., 2013), or even molybdenum
594 and vanadium (Wichard et al., 2009), best characterized are siderophores,
595 small iron-binding natural products that are secreted from cells and bind
596 extracellular iron with high affinity (Lankford and Byers, 1973). Iron-
597 bound siderophores are then taken back up into the cell, where the iron is
598 liberated from the compound and incorporated into the cellular iron pool
599 (Raymond et al., 2015). Similar strategies to microbial active iron uptake
600 by using siderophores exist also in fungi and plants (Budějínský et al.,
601 1980; Haas et al., 2008).

602 Nevertheless, as indicated above, this strategy is not limited to iron.
603 Production and deployment of metallophores satisfies the need for other
604 metals, the metal deficiency, or even to defend against metal toxicity in a
605 number of bacteria (Johnstone and Nolan, 2015; Kraemer et al., 2015). The
606 best studied family of non-iron metallophores are chalkophores (chalko- is
607 derived from the Greek word for Cu), a family of Cu-binding natural
608 products which exhibit great affinity and specificity to this metal (Kenney

and Rosenzweig, 2018). The largest and best-understood group of chalkophores is methanobactin (Mbn). Mbns have an exceedingly high affinity for Cu and bind Cu from soluble or mineral sources upon secretion (Dassama et al., 2016; Kenney et al., 2018). Although Mbns were originally identified in methanotrophic bacteria, which require large amounts of Cu, there is genomic evidence for their production in a wider range of bacteria, spanning both Gram-negative and Gram-positive bacteria (Dassama et al., 2016; Kenney et al., 2018; Kenney and Rosenzweig, 2013), fungi and algae (Zhang et al., 2020).

Mbns may have an important role in bacterial interactions due to their ability of not just to bind Cu but to reductively bind Cu(II). This produces CuMbn which has oxidase, superoxide dismutase (SOD), and hydrogen peroxide reductase activity (Choi et al., 2008). Extracellular SOD activity of secreted CuMbns by prey may be biologically important and have a relevant defensive role against the oxidative stress associated to the offensive use of Cu by bacterial predators.

Yersiniabactin (Ybt), an iron-binding natural product produced by *Yersinia pestis*, binds Cu(II) competitively with Fe. Interestingly, Ybt is used for Cu uptake and as a mechanism to mitigate Cu-mediated damage in bacteria (Kenney and Rosenzweig, 2018; Nolan, 2017). Ybt has a

630 protective role from Cu toxicity during human infection by uropathogenic
631 *E. coli* (Chaturvedi et al., 2012). Under iron-limited conditions,
632 uropathogenic *E. coli* produces catecholate siderophores that are highly
633 efficient Fe chelators but are also responsible for catecholate-mediated
634 reduction of Cu(II) to more bactericidal form Cu(I). Nevertheless, Cu(II)
635 sequestration by Ybt protects from catecholate-mediated toxic Cu(I)
636 formation, so *E. coli* isolates that produce Ybt are more resistant to Cu. In
637 addition, isolates that do not produce Ybt but are supplemented with
638 purified Ybt regain resistance to toxic levels of Cu (Chaturvedi et al., 2012),
639 Like CuMbn, Cu-bound Ybt (CuYbt) exhibits SOD activity, potentially
640 providing protection against phagocytic killing (Chaturvedi et al., 2014).
641 Altogether, Ybt possesses the ability to protect *E. coli* from Cu toxicity and
642 redox-based phagocyte defenses, which distinguishes it from other
643 siderophores in *E. coli* (Koh and Henderson, 2015). These results lay out
644 the possibility that secreted Cu-binding molecules evolved in pathogens to
645 neutralize the antibacterial activity of Cu.

646 The siderophores pyochelin (Pch) and pyoverdine (Pvd), which are
647 produced by *P. aeruginosa,* are also capable of binding a range of divalent
648 metal ions, including Cu and Zn. These alter the dynamics and the
649 ecotoxicity of Cu in soil (Cornu et al., 2019). Additionally, as with Ybt,
650 Pch and Pvd may sequester Cu outside of the cell, playing a protective role
651 against Cu toxicity. Consequently, Cu binding that does not result in Cu

652 uptake may be a biologically relevant function of several siderophores
653 (Kenney et al., 2018) and may represent a defensive strategy of prey to face
654 the potential Cu toxicity employed by predators (Fig. 4, B5).

655 In the environment, metallophores produced by bacteria are sometimes
656 utilized by other nearby microbes such as fungi and other bacterial species
657 to promote their growth (Barber and Elde, 2015; Challenger et al., 1951;
658 Grinter et al., 2019; Mozzi et al., 2018; Traxler et al., 2012). Cu piracy has
659 also been speculated to occur in high-Cu demand methanotrophic
660 communities, where Mbs, in addition to binding Cu, also serve as
661 interspecies signaling molecules (Farhan Ul-Haque et al., 2015; Vorobev
662 et al., 2013). Further studies are necessary to determine if Cu competition
663 triggers induction of secondary metabolites synthesis or, even more
664 interestingly, induction of genes responsible for production of yet unknown
665 compounds involved in microbial interactions.

666

667 **References**

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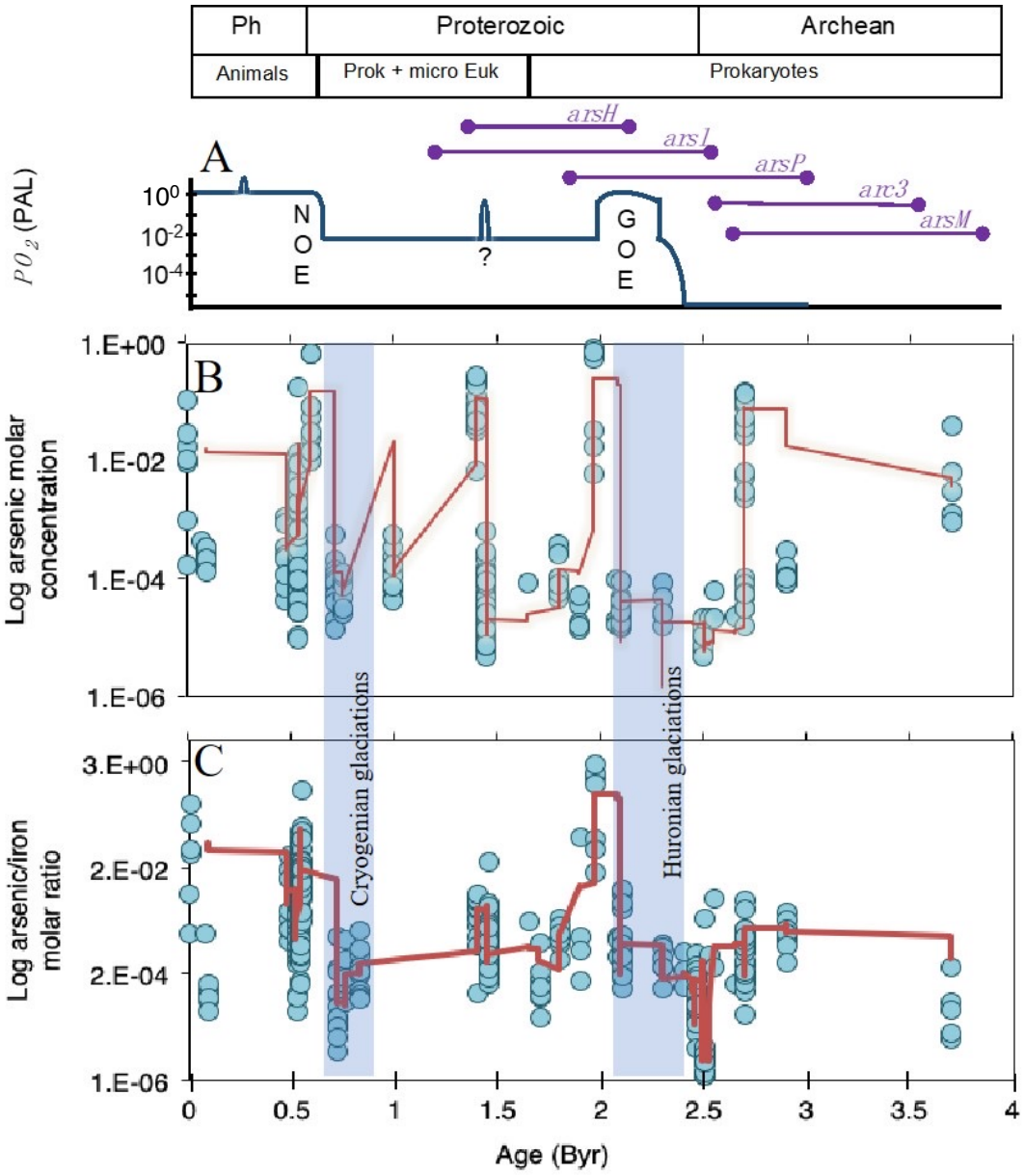

Fig. 1. Geological timeline for marine arsenic(As) evolution and the emergence of the As methylation (ArsM, Arsl ArsP, and ArsH) and the Arc3 As(III) efflux pump, and corresponding atmospheric oxygen trends. A) Emerging model for atmospheric oxygenation (see Lyons et al. (2014) and Large et al. (2020)). **B)** As concentrations in marine iron formations and shales (See Chi Fru et al. (2015). **C)** As concentrations in marine sediments normalized to the strong arsenic sink, iron. The red line in B and C represents the moving average. Ph=Phanerozoic. GOE=Great Oxidation Event. NOE= Neoproterozoic Oxygenation Event. PAL=Present day Atmospheric levels. ?=a proposed 1.4 Byr ago oxygenation event suggested by Diamond and Lyons, 2018. Also see Large, 2019.

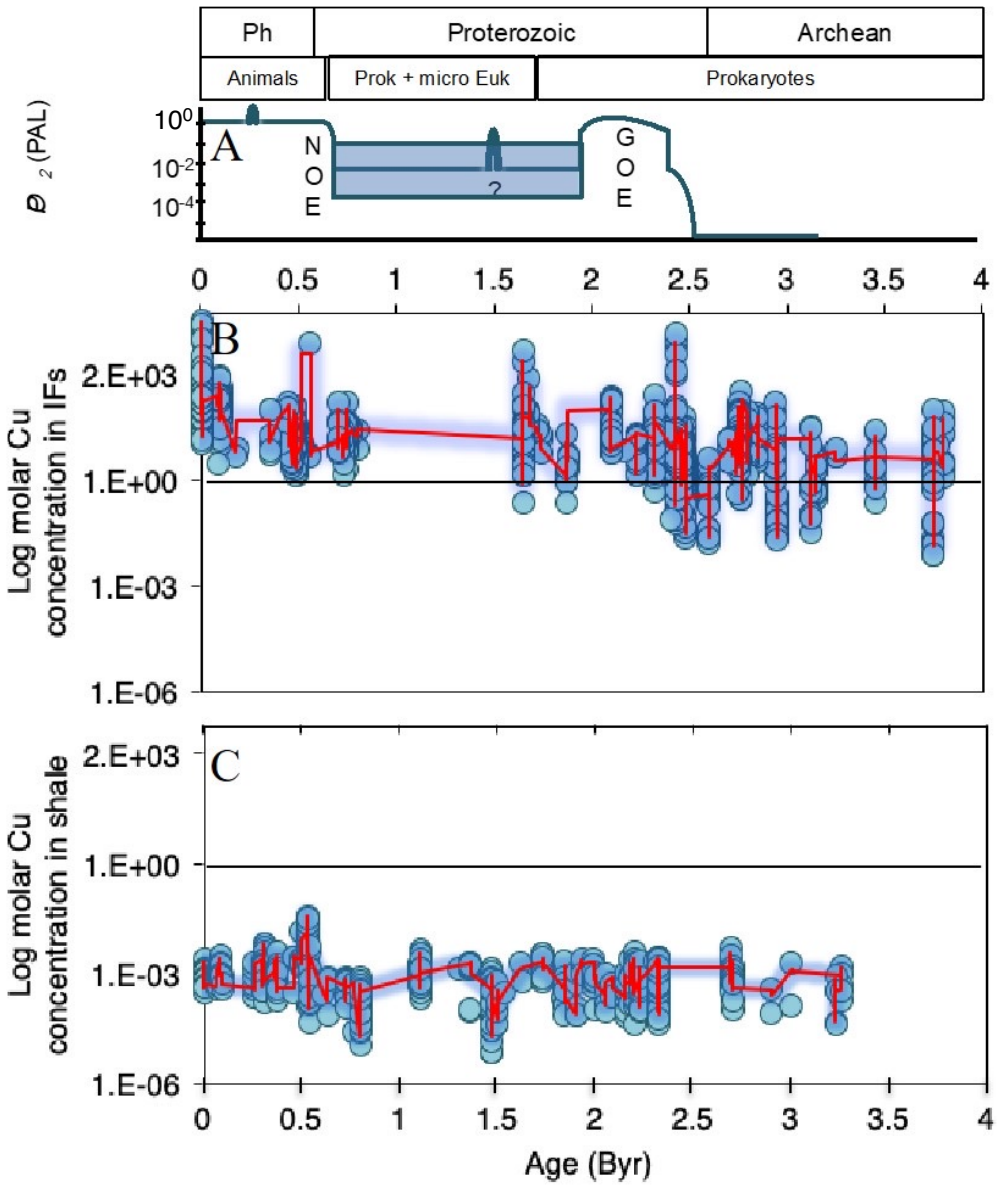

Fig. 2. Geological timeline for marine copper(Cu) evolution and corresponding atmospheric oxygen trends. A) Emerging models for atmospheric oxygenation (see Lyons et al. (2014) and Large et al. (2020)). **B)** Cu concentrations in marine iron formations (adapted from Chi Fru et al., 2016. **C)** Cu concentrations in marine shales (adapted from Chi Fru et al., 2016). The red line in B and C represent the moving average. Ph=Phanerozoic. GOE=Great Oxidation Event. NOE=Neoproterozoic Oxygenation Event. PAL=Present day Atmospheric levels. Prok=Prokaryotes. Micro Euk=Microeukaryotes.

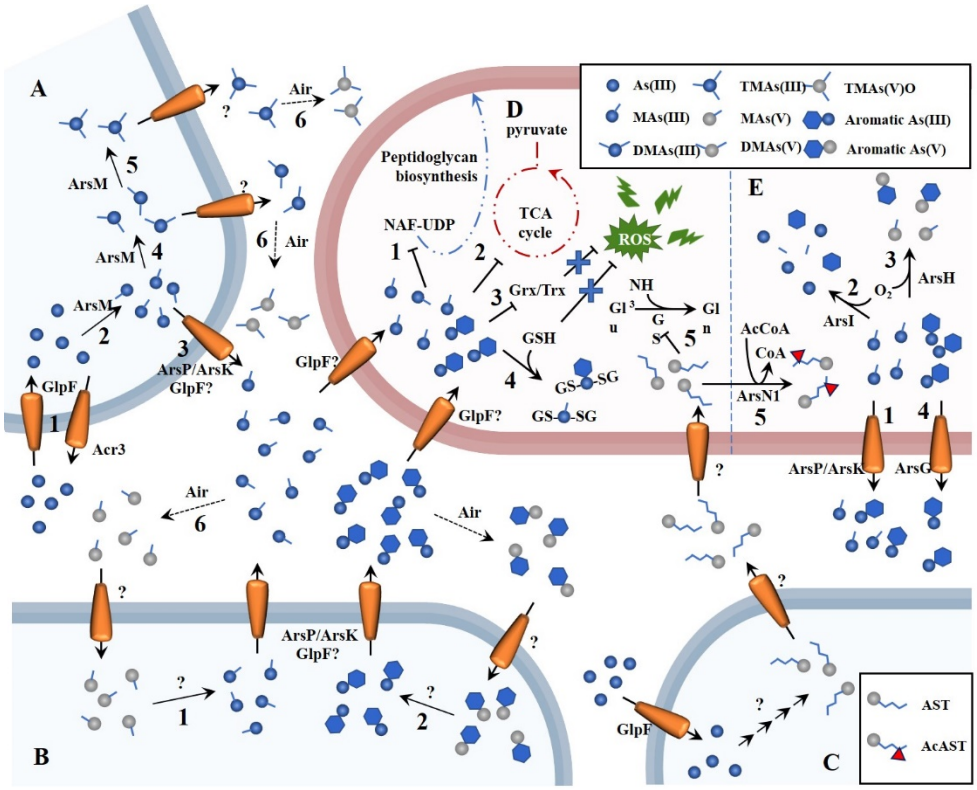

Figure 3. Bacterial warfare over arsenic(As) – Mechanisms of production (A-C), action (D) and resistance (E) of organoarsenical antimicrobials. A) MAs(III) production via methylation. As(III), which enters bacterial cells via aquaglyceroporins such as GlpF, is extruded via As(III) efflux permeases such as Acr3 **(1)**. Some bacteria methylate inorganic arsenite As(III) by ArsM, producing MAs(III) that has potent antimicrobial properties **(2)**. MAs(III) is secreted via selective efflux permeases (ArsP, ArsK) or potentially via channels such as GlpF or unknown pathways **(3)**. Some of the produced MAs(III) is further methylated by ArsM to di-methylated DMAs(III) **(4)**, which may also function as antibiotic. Additional methylation produces non-toxic volatile gas tri-methylated TMAs(III) **(5)**, which probably confers self-resistance against MAs(III)/DMAs(III), especially in anaerobic condition. In aerobic conditions, MAs(III), DMAs(III) and TMAs(III) are rapidly oxidized to non-toxic pentavalent counterparts in air **(6)**. **B)** MAs(III) production via reduction. Some aerobes acquired the ability to reduce non-toxic MAs(V) to MAs(III) **(1)**, utilizing it as antibiotic. Some of MAs(V)-reducing aerobes are also capable of reduction of aromatic arsenate to produce aromatic arsenite **(2)** that have potent antimicrobial activity. Molecular mechanisms for the organoarsenical reduction are yet unknown. **C)** AST production. Some bacteria have even evolved to biosynthesize arsinothricin (AST), a more complex organoarsenical antibiotic. The pathways for AST biosynthesis and efflux are yet unknown. **D)** Mechanisms of actions. MAs(III)

and aromatic As(III), taken up by neighboring cells probably via GlpF, inhibit various proteins involved in bacterial life-supporting processes such as peptidoglycan biosynthesis **(1)** and TCA cycle **(2)** by binding their cysteine residues. MAs(III) and aromatic As(III) also bind to and deplete small proteins/molecules for regulation of redox homeostasis such as glutaredoxin/thioredoxin **(3)** and glutathione **(4)**, leading damages from reactive oxygen species (ROS). AST, taken up by surrounding cells via unknown pathways, inhibits glutamine synthetase **(5)**, causing accumulation of toxic ammonia and depletion of glutamine that leads eventual bacterial death. **E)** Resistance mechanisms. Some bacteria have evolved resistance mechanisms against organoarsenical antibiotics for survival. ArsP and ArsK are specific efflux permeases that extrude MAs(III) and aromatic As(III) out of the cells, which confers resistance in an oxygen-independent manner **(1)**. In contrast, ArsI **(2)** and ArsH **(3)** detoxify MAs(III) and aromatic As(III) in an oxygen-dependent manner: ArsI is a dioxygense that degrades them into As(III) by incorporating dioxygen molecule into the C-As bond; ArsH is an oxidase that oxidizes them to non-toxic pentavalent counterparts. Some anaerobes have a resistance mechanism specific for aromatic As(III) but not for MAs(III), which completes the detoxification process by ArsG the aminoaromatic As(III) specific efflux permease **(4)**. ArsN1 **(5)** is the only known AST resistant mechanism, which detoxifies AST by acetylation.

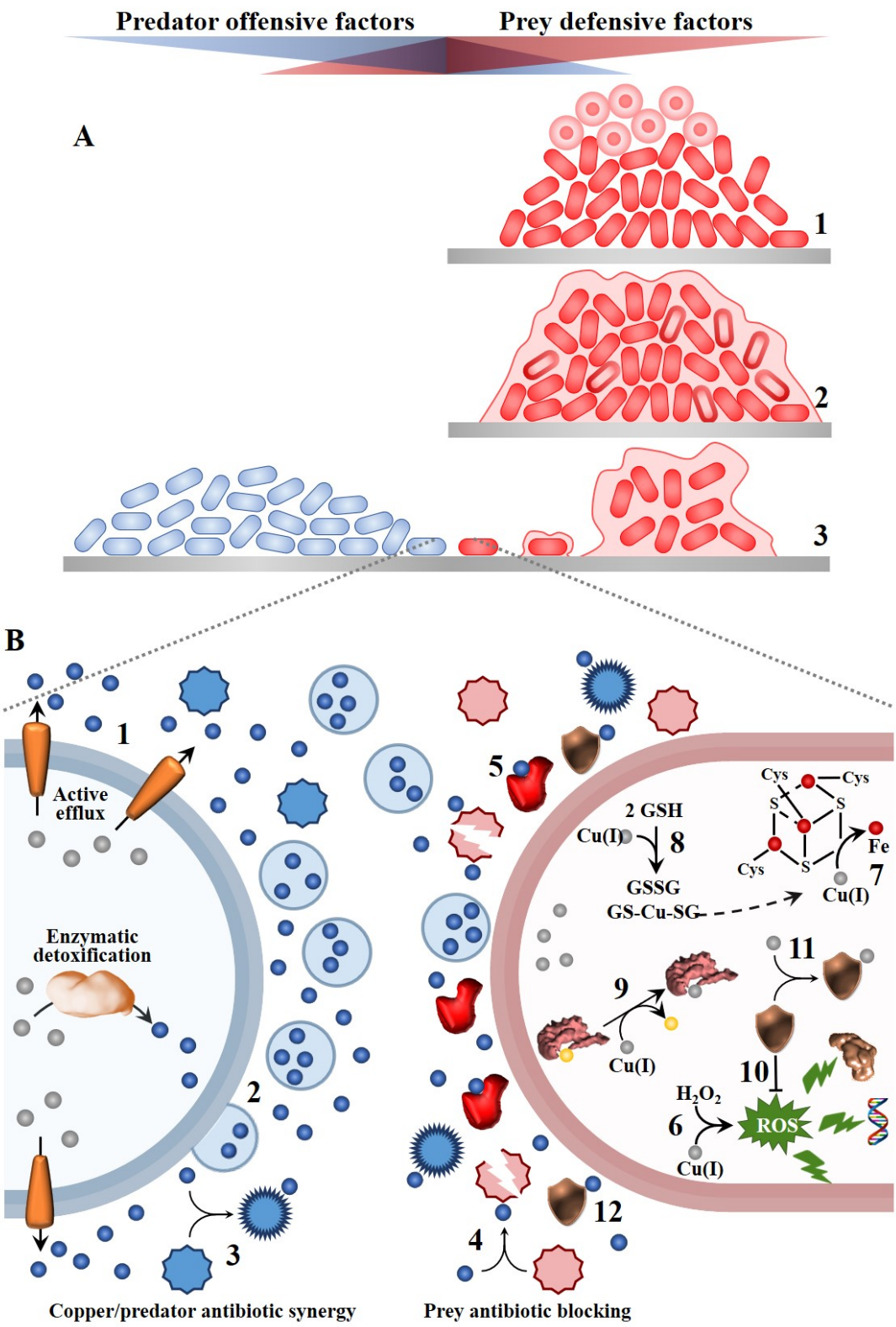

Figure 4. Copper(Cu) involvement in bacterial interactions. A) Prey differentiations to hamper Cu toxicity: **1)** generation of stress-resistant spores; **2)** conversion of vegetative cells on Cu-induced dormant persisters; **3)** Cu-induced exopolysaccharide and/or extrapolymeric substance production, and biofilm generation. **B)** Mechanisms of Cu toxicity and defensive prey responses. (**1)** Cu may be

pumped out by predator active efflux systems generating an increasing gradient of metal concentration towards predator-prey interface; (**2)** Cu can also be dispatched from the predator via OMVs; (**3)** complexation of predator antibiotics (ten-pointed blue star) with Cu can result in a synergycally increase in the antimicrobial capability of both compounds (32-pointed blue star). On the contrary, (**4)** interaction of Cu with prey antibiotics (ten-pointed pink star) can inactivate the antibiotic or reduce its activity (ripped ten-pointed pink star). (**5)** Cu(II) sequestration by metallophores (red molecule) protects from catecholate-mediated toxic Cu(I) formation. Once Cu reaches the reducing bacterial cytoplasm, metal can exerts toxicity through different processes: (**6)** Cu(I) can produce ROS participating in Fenton-type reactions; (**7)** Cu toxicity can also be performed via displacement of iron from iron-sulfur clusters by Cu(I), leading to loss of protein function; (**8)** Cu(I) can lead to thiol depletion in the glutathione pool; glutathione-Cu complexes (GS–Cu–SG) can act as Cu-donors for metalloenzymes under anaerobic conditions (dashed arrow); (**9)** replacement of other metal cofactors by Cu on several metalloproteins can promote mismetallation and inactivation of prey proteins. In order to protect from Cu toxicity, (**10)** prey melanins (brown shield) can diminish intracellular ROS burst triggered by Cu(I) and, also sequester internal, (**11)** and external, (**12**) Cu due to its metal affinity and high adsorption capacity.