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Environmental Factors and Host Microbiomes Shape Host-Pathogen Dynamics

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Abstract

Microorganisms are increasingly recognized as ecosystem-relevant components because they affect population dynamics of hosts. Functioning at the interface of the host and pathogen, skin and gut microbiomes are vital components of immunity. Recent work reveals a strong influence of biotic and abiotic environmental factors (including the environmental microbiome) on disease dynamics, yet the importance of the host-host microbiome-pathogen-environment interaction has been poorly reflected in theory. We use amphibians and the disease chytridiomycosis caused by the fungal pathogen *Batrachochytrium dendrobatidis* to show how interactions between host, host microbiome, pathogen and the environment all affect disease outcome. Our review provides new perspectives that improve our understanding of disease dynamics and ecology by incorporating environmental factors and microbiomes into disease theory.

33 **From Disease Triangle to Disease Pyramid**

34 Directly transmissible and vector-borne pathogens, which are affected by global change, are
35 increasingly seen as risks to humans and wildlife [1,2]. Despite considerable research effort,
36 human malaria continues to be a major health issue for millions of people and predicting
37 future range expansion with expected climate changes has proved problematic; yet, for other
38 diseases, predictions of how climate changes may alter dynamics is improving [1,2]. For
39 example, the tiger mosquito *Aedes albopictus*, transmitting the Chikungunya viral disease, is
40 currently expanding its geographic range across Europe and the Americas, putting millions of
41 humans at risk. In livestock, the vector-borne bluetongue disease, has emerged in northern
42 Europe in response to climate change, and is causing the death of millions of animals at
43 massive financial costs [3].

44 A clear indication that host-pathogen interactions are responding to global change is supported
45 by the global increase of **emerging** and **re-emerging infectious diseases** (see Glossary) of
46 human, wildlife and plant hosts posing threats to biodiversity and public health [4]. Impacts of
47 the environment on host-pathogen interactions can be subtle and can vary across years. In
48 2010, a thermal anomaly occurred in Curaçao, causing a dramatic increase in white plague
49 disease and ciliate infection in Caribbean coral *Diploria labyrinthiformis* [1]. In a vastly
50 different system, Clare and colleagues demonstrated that seasonality, in particular the timing
51 of spring ice-thaw of a Pyrenean montane lake, affects the susceptibility of host amphibians to
52 infection by the emerging pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) [5]. These
53 examples illustrate how disease risk and dynamics may result from (at least) three factors: the
54 host, the pathogen and the environment.

55 In disease ecological theory, a ‘disease triangle’ is a concept that illustrates the outcome
56 of the dynamic interactions consisting of hosts, pathogens, and the environment [6] (see also
57 [7]) (Box 1). It was formalized in plant pathology because plants cannot move to escape
58 unfavorable environmental conditions. We believe the concept is useful to better understand
59 disease dynamics in human and wildlife diseases in a rapidly changing world. It considers the
60 environment in its large sense, including abiotic conditions (e.g. temperature, moisture, pH,
61 soil composition, solar radiation, seasonality, water chemistry), as well as biotic conditions
62 (e.g. social environment, community composition, sex ratio, vegetation cover, abundance and
63 distribution of vectors and intermediate hosts).

64 In spite of its explanatory power, the disease triangle represents an oversimplified view of the
65 dynamics of infectious diseases; and thus, we must broaden the original concept to include an
66 additional and often neglected aspect of the complex system: the diverse **microbiomes** of the
67 environment and the host. The advent of next generation sequencing has provided cost-
68 effective ways to describe the highly diverse world of **microorganisms**. We are only
69 beginning to appreciate the distribution, abundance, and diversity of microbial communities,
70 the full extent of which, however, remains highly controversial [8]. The total number of
71 bacteria and archaea are estimated as $\sim 10^{30}$, the atmosphere contains $\sim 10^{22}$ microbial cells, and
72 terrestrial and marine environments each comprise $\sim 10^{29}$ microorganisms [2]. A human body
73 harbors at least 10^{14} microbial cells and 10^{15} viruses [9], with the human gut microbiome
74 alone containing ~ 1000 bacterial species, but only 150 to 170 predominating in any one
75 person [10]. New data are helping to describe their functional and ecological capacities, as
76 well as the nature of their physiological interactions with hosts [2,9] (Figure 1).

77 **Microbiota** and microbiome research has identified microbial communities as
78 important contributors to ecosystem, wildlife and human health [2,9,11]. We now understand
79 that microbial communities maintain ecosystem health by influencing the global food web,
80 including agriculture, nutrient cycling, nitrogen retention and CO₂ sequestration [2] (Figure 1).
81 Further, by maintaining ecosystem health, they also contribute to plant, wildlife and human
82 health. Host microbial communities (i.e. **host microbiome**) can be considered part of the
83 extended phenotype of host defenses. The gut microbiome is involved in energy harvest and
84 storage, brain functioning, development of the intestine and immune system and susceptibility
85 to disease [9,11]. Not only does the gut microbiome act as a barrier to infection by defending
86 the host against colonization by pathogens, it also interacts with the host immune system,
87 providing signals to promote maturation of immune cells [9]. The properties of the host
88 microbiome have led to the concept of **holobiont** in which the host and its microbial partners
89 are merged into a symbiotic superorganism [12], and later to the concept of **pathobiome** to
90 further consider microbiome communities in disease dynamics [13,14].

91 In amphibians, as well as in other vertebrates, the skin microbiome is an integral component of
92 the immune system, acting as a barrier to infection (Box 2). For example, the amphibian
93 chytridiomycosis, a disease caused by the chytrid fungi *Batrachochytrium dendrobatidis*
94 (hereafter *Bd*) and *Batrachochytrium salamandrivorans*, is not simply occurring when the
95 pathogen is present and infects the skin of susceptible species but depends also on the
96 composition of skin microbial communities [15–17]. Generally, a better understanding of

97 microbiomes in disease dynamics and their appreciation in disease ecology will contribute to
98 disease mitigation.

99 Here, we review and illustrate the dynamic interaction between the host, the host microbiome,
100 the *Bd* pathogen, and the environment (including the environmental microbiome) (Figure 2,
101 Key Figure) using the well-studied case of the disease chytridiomycosis caused by *Bd*. This
102 four-way interaction highlights the need to adopt a holistic approach to understand disease
103 dynamics and ecology. Only when considering microbiomes in such a holistic approach, will
104 we be able to evaluate the impact of a changing environment on ecosystem, wildlife and
105 human health and overcome limitations of current concepts.

106

107 **Variety and Roles of Microorganisms**

108 In 1546, Fracastorius suggested that infection is contagious and transmitted by ‘particules’.
109 Microscopic organisms were discovered a century later, by Hooke and van Leeuwenhoek who
110 described respectively the microfungus *Mucor*, and ‘animalcules’ (i.e. protozoa and bacteria).
111 In 1857, Pasteur postulated that infectious diseases are caused by a variety of ‘germs’ that are
112 everywhere, even in the air. Microorganisms can be found in all habitats (soil, water, air), in
113 intestines of hosts, as well as on the exterior of organisms [18]. Host-associated
114 microorganisms can be pathogenic, but also can be commensal or beneficial symbionts.

115 Microorganisms play important ecological roles. Bacteria help controlling host populations as
116 they can be pathogenic, benign, or beneficial to hosts (e.g. helping in the digestion of food and
117 the protection of hosts against infections) [19]. Archaea provide major pathways for ammonia
118 oxidation in the environment, in methanogenesis, in sulphur oxidation and in nitrification [20].
119 Many fungi are also microscopic and are important in nutrient cycling, decomposition and
120 growth control of other organisms [21,22]. Parasitic and pathogenic fungi cause mycoses,
121 which are increasingly seen as threats to biodiversity [4].

122 The community of microorganisms is usually described by marker genes and in rarer cases
123 their full genome, i.e. the microbiome, a term usually used when they are associated to a host
124 individual or species [23]. The microbiome composition may vary between species, body
125 areas and geographic regions. The microbiome performs some main functions, such as disease
126 mitigation [15–17,24], digestion, and has an influence on host behavior, development and
127 reproduction [25–27] (Figure 1). The microbial taxa shared between the majority of

128 individuals within an animal species is called core microbiome, and it is probably the main
129 responsible component for performing these essential functions [28]. Perturbations in the
130 microbiome composition and function (known as dysbiosis) could interrupt its regular
131 functions and are suggested as a cause for some diseases [14].

132

133 **The Model System: Amphibian hosts and *Batrachochytrium dendrobatidis***

134 In a world where a quarter of all species are threatened with extinction [29], amphibians are
135 the most threatened vertebrates, with rapid population declines detected in 43% of the >8100
136 amphibians species described (<https://amphibiaweb.org>) [4,30]. Loss of amphibians has
137 cascading effects throughout food webs and may alter environmental balance (e.g. water
138 quality and pest control) [31,32]. In some systems (e.g. North American arboreal forests)
139 amphibians are the most abundant terrestrial vertebrates and they facilitate important carbon
140 cycle functions such as leaf litter retention and carbon capture [33], functions that are tied to
141 global cycles.

142 Together with habitat loss and overexploitation, emerging infectious diseases are the
143 major threats to amphibians. The fungus *Bd* is considered the most destructive pathogen for
144 wildlife, implicated in the decline of > 500 frog species worldwide [34], and the extirpation of
145 populations and entire species [35]. *Bd* pathogenicity is based on its ability to infect a vital
146 organ, amphibian skin, which is permeable and maintains homeostasis. *Bd* disrupts these
147 fundamental functions, ultimately resulting in cardiac arrest [36]. It infects hosts as a motile
148 zoospore form in water and colonizes a keratinized or keratinizing part of the host body [37].
149 In the host skin, *Bd* zoosporangia produce new zoospores that are released in the environment
150 and can either re-infect the same host or infect a new host. Many susceptible amphibians are
151 infected by *Bd* at the host larval stage and die during host metamorphosis, when host
152 immunity is suppressed and keratinized skin cells spread over the body. *Bd* can establish itself
153 in various environments and has been detected on all the continents that amphibians are
154 known to occupy [35]. Moreover, amphibians being ectotherms, their physiology, including
155 their immunity, is environment-dependent. Finally, the amphibian skin microbiome has
156 attracted considerable attention compared to other wildlife microbiomes, and therefore a large
157 body of literature is available on the interactions between amphibian hosts, the pathogen *Bd*,
158 skin microbiome and the environment.

159

160 **Amphibian Microbiome**

161 In amphibians, external (skin) and internal (gut) microbiomes have been described. The
162 amphibian gut microbiome includes protists and bacteria which stimulate growth [38]. The
163 amphibian skin microbiome is composed of different groups, such as bacteria, fungi and other
164 micro-eukaryotes [39,40]. The origin of amphibian gut and skin microbiome is not yet clear.
165 There is some evidence that the amphibian microbiomes may result from both species-specific
166 self-acquisition of microbes in the habitat, as well as social transmission, followed by
167 selection of rare environmental microbiome taxa [15,41–47] (Table 1). Transmission of
168 microorganisms, in this case bacteria, between individual hosts can be vertical or horizontal
169 (for fungi and other micro-eukaryotes the process is still unknown). Vertical transmission
170 occurs especially in species with parental care, when part of their microbiome is transferred by
171 hosts to eggs and protects them [48]. Horizontal transmission is likely to occur during mating
172 and during congregations of conspecifics or other species [49]. The environmental
173 microbiome also contributes to the host microbiome. Individuals kept in enclosed habitat are
174 exposed to a poorer environment compared to specimens that live in natural habitats,
175 translating in poorer skin microbiomes [50,51] (Table 1). Salamander larvae experimentally
176 transferred from stream to pond had a modified skin microbial community, likely due to
177 microbes gathering in their habitat [43]. In addition to environmental impacts on the
178 microbiome, species-specificity of microbiomes has also been described [16,52,53].

179 Gut and skin microbiomes can be influenced by many host factors, including genetics,
180 sex, behavior and diet [15,53,55] (Table 1). Captive individuals of the Red-eyed tree frog
181 (*Agalychnis callidryas*) that received a diet rich in carotenoid have greater species richness
182 and abundance of skin bacteria compared to those with a carotenoid-free diet [54]. Host health
183 status has been shown to influence host microbiome, which in turn can influence disease
184 dynamics [55–57]. Composition of the skin microbiome also varies with life stage: micro-
185 eukaryotes are significantly more diverse and abundant in adults and sub-adults as compared
186 to metamorphs and tadpoles, with a dominance marked by fungal taxa [58,59] (Table 1).
187 During metamorphosis, amphibians undergo many changes: body shape, diet, habitat (from
188 aquatic to more terrestrial) and skin cell composition. During that period, their immune system
189 is suppressed, which apparently creates open niches, inducing a higher bacterial diversity [58]
190 and facilitating the proliferation of, and infection by, opportunistic pathogens.

191

192 **Environmental Impacts on Amphibian Immunity**

193 In amphibians, as in all vertebrates, the first protection against pathogens is the physical,
194 chemical and biological barriers of the epithelium (Box 2). Amphibians have a permeable skin
195 that allows exchange of gases, water and electrolytes. The resident microbiome present on the
196 skin is a dynamic community that competes with exogenous microorganisms for space and
197 resources, and can prevent pathogens from adhering to the skin (Box 2). The host microbiome
198 can also actively defend the host by producing antimicrobial peptides or metabolites. For
199 example, *Janthinobacterium* spp., a symbiotic skin bacterium, produces the anti-fungal
200 metabolite violacein [17]. Violacein was isolated from amphibian skin and transferred to the
201 *Bd*-susceptible Mountain yellow-legged frog (*Rana muscosa*), resulting in a successful bio-
202 augmentation that increased host survival [60].

203 Many factors are known to affect amphibian immunity and thus host-pathogen interactions
204 (e.g. Table 1). Temperature is likely the most important environmental factor that affects
205 microbiomes. Since amphibians are ectotherms, all of their physiological responses are
206 temperature-dependent, including immunity. Several studies have found that *Bd* infection
207 probability and impacts of chytridiomycosis are higher at lower temperatures (e.g. [37,61,62]).
208 For that reason it could be suggested that climate change may be beneficial for hosts that
209 suffer from *Bd* infections. However, the interaction between the host, the pathogen and the
210 environment is complex. Climate change predictions suggest large increases in the variability
211 of climatic conditions that may favor chytridiomycosis [63,64]. Controlled-temperature
212 experiments on the Cuban treefrog (*Osteopilus septentrionalis*), revealed that the temperature-
213 dependent growth of *Bd* on hosts was greater at warmer temperatures than the pattern of *Bd*
214 growth in culture, emphasizing the importance of accounting for the host-pathogen interaction
215 when predicting climate-dependent disease dynamics [64]. Another striking result was the
216 demonstration that global warming is predicted to cause an increase in susceptibility to *Bd* in
217 some amphibian species due to earlier ice-thaw of montane lakes in spring [5].

218 Besides temperature, additional environmental parameters that may alter amphibian immune
219 responses include pH and chemicals in the environment. Studies have also shown that the
220 presence of heterospecifics, intra and inter-specific competition, predation, food availability,
221 and pond drying, can all cause stress in hosts that may suppress immune response [24]. In the
222 Pyrenees, presence of zooplankton in lake water also protects amphibians against *Bd*, since
223 zooplankton predation diminishes the population of the infectious stage (zoospores) of the
224 pathogenic fungus [65]. The list of factors is not exhaustive, and we believe additional impacts

225 of environmental conditions will be discovered as the impacts of human activities on the
226 environment continue to increase.

227

228 **Environmental Impacts on Host-Host Microbiome Interactions**

229 The skin and gut microbiome can be seen as a miniature ecosystem at equilibrium. Just like
230 any ecosystem, a stressor may alter the balance of the associated microbiome. As a response,
231 the richness and composition of the microbiome may either resist, or shift to a new stage. It
232 may stay permanently at this new equilibrium as an **acclimation response**, or it may exhibit
233 resilience and return to the former equilibrium. From the perspective of the host, when
234 environmental modifications occur, the microbiome may exhibit flexibility. This flexibility
235 might increase the host adaptive capacity by providing the host with higher **phenotypic**
236 **plasticity**, compared to the one acquired only through the host genome or through a static
237 gut/skin microbiome [66] (Figure 1). The bacteria *Serratia symbiotica*, for example, provides
238 a reproductive advantage (higher fecundity) to the insect pea aphid after an event of heat
239 stress, compared to control individuals [67]. Hence, the environment has the potential to
240 influence the outcome of host-microbiome interactions, because it can impact both the host
241 and the host microbiome.

242 The composition, richness and diversity of host microbiome can be modified directly by biotic
243 and abiotic environmental factors (Table 1). It can also be modified indirectly, through the
244 host physiology, as the host-microbiome interactions create the environment in which the
245 microbiome lives. A recent study analyzed samples from over 200 amphibian species (>2,300
246 individuals) across a broad biogeographic range to investigate how climatic variables
247 (temperature, precipitations and seasonality), elevation, latitude and microhabitat class relate
248 to skin microbial communities [68]. Cold winter temperatures and seasonality were the best
249 predictors of richness and composition of skin bacterial communities at the global scale.
250 Latitude (with a decreased richness at lower latitudes) was also important, likely due to an
251 inter-correlation between latitude and low temperatures [68]. Finally, skin bacterial richness
252 was also influenced by microhabitat. Bacterial richness on amphibian hosts appeared higher in
253 more seasonal environments, especially those with colder winters during which the amphibian
254 immunity is suppressed for longer periods of time. Thus, there might be selection for low
255 temperature resistance in the amphibian skin microbiome, counteracting low internal
256 immunity of amphibians [68].

257 Additional environmental stressors of importance for the host-microbiome interaction include
258 habitat degradation, ultraviolet radiation, environmental pollution, invasive species and
259 climate change [69–71]. In particular, climate change is predicted to impact many terrestrial
260 and aquatic host species and this may allow pathogens to spread into new geographic areas
261 under optimal temperatures [72,73]. Habitat degradation, such as deforestation and
262 fragmentation, decreases biodiversity in macro-organisms, and this pattern extends to their
263 associated microbiomes [74]. Habitat degradation thus impairs vertical and horizontal
264 transmission of microbiomes and induces lower microbiome skin diversity. Human activities
265 can also modify the communities of environmental microorganisms [74]. For example,
266 livestock grazing provides copious amounts of livestock feces, which can enrich the
267 environment for microorganisms, or, on the contrary, can deposit antibiotics and antifungal
268 compounds that degrade the habitat for natural microorganisms. Finally, other pathogens in
269 the environment may also change the outcome of host-microbiome-pathogen interactions
270 through alteration of the host immunity or condition, or through pathogen-pathogen
271 competition for hosts [24].

272

273 **Host-Host Microbiome-Pathogen Interactions**

274 Microorganisms in the environment, including potentially pathogenic microorganisms,
275 represent an important source of disturbance to the natural skin and gut microbiomes of hosts.
276 These potential pathogenic microorganisms may also be favored or inhibited by the
277 microbiome composition of the host and/or vector [75]. For example, the susceptibility of the
278 mosquito *Anopheles gambiae* to *Plasmodium falciparum* infection is inversely correlated to
279 the abundance of the bacteria *Enterobacteriaceae* in their gut [76]. In the *Bd*-amphibian
280 system, there is a significant relationship between *Bd* infection and the microbiome
281 composition of amphibian skin [e.g. 77–79] (Table 1). Hosts with greater microbiome
282 diversity are more resistant to this invasive pathogen, while populations that coexist with *Bd*
283 for a long time have a higher proportion of anti-*Bd* skin bacteria than populations which are
284 declining due to the disease and never had contact with *Bd* before [79,80]. This evidence
285 confirms that pathogens can influence and be influenced by the amphibian skin microbial
286 community.

287 Most research conducted so far on amphibian skin microbiome focused on bacteria (Table 1),
288 even though skin microbial communities also comprise eukaryotic microorganisms such as

289 protists and fungi [39,40]. Two recent studies suggest that fungi might be more efficient or
290 have a higher competition capacity against other invasive fungi [39,58], opening new avenues
291 on the role played by fungal communities in protecting amphibians against *Bd*. Bacterial
292 viruses (phage), as key drivers for mortality and diversity of bacterial communities, represent
293 another so far unexplored frontier in disease mitigation.

294 To our knowledge, research conducted on amphibian-host microbiome-pathogen interactions
295 has been almost exclusively conducted on *Bd*. So far, only a few studies explored this
296 interaction on different pathogens. A study on FV3-like ranavirus unravels the importance of
297 habitat-microbiome interactions on disease outcome in the amphibian host *Rana temporaria*,
298 and supports the idea that the amphibian skin microbiome likely also protects amphibians
299 against pathogens other than *Bd* [81]. In contrast, research on the newly discovered fungus *B.*
300 *salamandrivorans* suggests that skin microbiome of salamanders contains inhibitory bacteria
301 at numbers too low to confer sufficient protection against the pathogen and even constitutes a
302 source of opportunistic pathogens contributing to pathogenesis [82–84].

303

304 **Environmental Impacts on Host-Host Microbiome-Pathogen Interaction**

305 Due to recent technological breakthroughs, research on wildlife microbiomes is relatively
306 new, and thus, it is not surprising that the number of studies available to address the four-way-
307 interaction (the host-microbiome-pathogen-environment interaction) in disease ecology is very
308 limited. Our review reveals that most studies have only considered the geographic location as
309 a potential source of variation in the outcome of the host-microbiome-pathogen interaction
310 (Table 1). Regarding abiotic factors, studies have investigated the effects of elevational
311 gradient [85], water temperature [86], and pH, CaCO₃ and conductivity [87] on the skin
312 microbiome of amphibians. Few studies take biotic factors, such as land cover, forest type,
313 connectivity and habitat management into account when comparing skin microbiomes of
314 amphibians [87–90]. We expect future studies will include climatic variables, elevation,
315 latitude, and microhabitat characteristics at a large geographic scale as is seen in Kueneman et
316 al. [68]. Interestingly, to our knowledge, no study has considered the potential role
317 stochasticity may play in shaping the relationship between the host, the host microbiome, the
318 pathogen and the environment.

319 There is now substantial evidence that the host microbiome plays an important role in
320 modulating pathogen invasion and disease outcome. This is likely because host microbiomes

321 are located precisely at the interplay between host, pathogen, and environment. In some cases,
322 the microbiome is considered as an extension of the innate immune system of amphibian hosts
323 [24], and the host and its microbiome are fused into a holobiont [12]. As such the host
324 microbiome is considered a part of the host/holobiont when implementing the disease triangle
325 concept. Such a representation is restrictive, since it undermines the fundamental interaction
326 between two different entities (the host and its microbiome) and the fact that the environment
327 can affect both separately and independently, with various outcomes on disease dynamics.
328 Growing evidence from multiple studies demonstrates the importance of environmental
329 conditions in modulating the host-microbiome-pathogen interaction. Any environmental
330 condition is imposed simultaneously on the host, the microbiome and the pathogen; however,
331 all three factors may respond in different ways, independently of each other. We propose that
332 a holistic approach, in which the disease triangle is extended to a disease pyramid, can best
333 capture the complex nature of these interactions (Figure 2).

334 A disease triangle may allow the eventual linking of both single and multiple pathogen
335 systems as co-infections become more widespread. *Bd* and ranaviruses can affect the same
336 host population, requiring the immune system to act against two pathogenic targets
337 simultaneously. The pathobiome concept, representing a consortium of microbes acting
338 altogether as a pathogenic entity, is meant to capture the idea of co-infection [13,14]. In this
339 concept, the pathogen and either environmental or host microbe are assembled, leading to
340 similar issues raised for the holobiont. Because environmental microbiome and host
341 microbiome have different community composition, and different physiological and
342 ecological functions (Figure 1), we recommend considering them in a disease pyramid as two
343 separated factors, yet connected and interdependent, with microbes potentially moving from
344 one community to another. In our model, additional microbes acting with the pathogen can
345 either come from the environment (environmental microbiome), or already live on the host
346 (host microbiome). Generally speaking, the pathobiome concept makes it more difficult to
347 apprehend the pathogen at all its life stages. When the pathogen is colonizing the host, the
348 pathobiome is what we call the host microbiome (as at this stage, the pathogen is living on the
349 host). However, many pathogens have free life stages, outside the host, and it is unclear what
350 is the pathobiome at that moment (environmental microbiome or host microbiome?). Our
351 pyramid does consider the life of pathogens outside of the host and the various interactions it
352 might have.

353 We need to move from the reductive vision of a three-edged disease triangle to the broader
354 vision of a four-edged disease pyramid that considers the host, the microbiome, the pathogen,
355 and the environment as interdependent components that affect disease dynamics. Any
356 modification in any one of the vertices means a change in all other relationships; thus, the
357 disease pyramid takes into account the role of the environment without isolating the other
358 entities (host, microbiome, pathogen) from the whole system of which all are essential parts.
359 In addition, the theory developed for and applied to hosts in the disease pyramid is also
360 entirely valid for disease vectors such as, for example, mosquitos (see also [13] and Box 1).

361

362 **Concluding Remarks**

363 Human activities are profoundly changing the environment in numerous ways. Therefore, if
364 we want to maintain host health and control wildlife diseases, it is important that we develop a
365 research approach that includes both biotic and abiotic factors in studies of host-microbiome-
366 pathogen interactions (see Outstanding Questions). Research on the amphibian-*Bd* system
367 provides an opportunity to better understand the complex relationships between the host, the
368 microbiome, the pathogen, and the environment, depicted by the suggested disease pyramid.
369 Given the current threats pathogens pose to biodiversity, it is necessary to extend this research
370 to other wildlife pathogens. For amphibians, it means for example *B. salamandrivorans*,
371 ranaviruses, and herpesviruses. Harrison and colleagues studied the impact of different habitat
372 characteristics of garden ponds on the *Rana temporaria* microbiome-ranavirus interaction
373 [81]. They found that more diverse microbiomes were more resistant to the virus and that
374 individuals living in more complex habitats had lower rates of mortality.

375 Future research on wildlife pathogens should consider the disease pyramid as a foundation by
376 which to yield the necessary insights to understand and mitigate disease impacts and their
377 different components. The disease pyramid provides an overarching comprehensive
378 framework that can be applied to many hosts (animals, plants, humans), their microbiomes,
379 their pathogens, and can theoretically incorporate environmental conditions as well as varied
380 responses to environmental changes. Such a comprehensive framework will be increasingly
381 necessary in medical sciences and disease ecology in a rapidly changing world.

382

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391

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659 Glossary

660

661 **Acclimation response:** is a reversible change of a physiological trait in response to an
662 environmental change.

663

664 **Emerging infectious disease:** a disease that either has appeared and affected a population for
665 the first time, or has existed previously but is rapidly spreading, either in terms of the number
666 of individuals getting infected, or to new geographical areas. Emerging infectious diseases are
667 caused by pathogens that are increasing in their incidence, geographic or host range, and
668 virulence.

669

670 **Holobiont:** a symbiotic system, composed of the host and its microbial partners (bacteria,
671 archaea, viruses and eukaryotes).

672

673 **Host microbiome:** microbial communities living on (e.g. skin microbiome) or in (e.g. gut
674 microbiome) the host. Skin and gut microbiomes are currently better described, but other host
675 microbial communities exist, e.g. in the mouth, nose, pharynx, and respiratory and urogenital
676 tracts.

677
678 **Microbiome:** the communities of microorganisms including bacteria, yeast, fungi, protists and
679 archaea in combination with their genomes.

680
681 **Microbiota:** the collection of microorganisms that exists in a given environment, habitat, or
682 host (inside or on host).

683
684 **Microorganisms:** microscopic organisms that exist as single cells or cell clusters. They
685 include bacteria, protozoa, archaea, microscopic fungi, and microscopic algae. We include
686 here also viruses, which are microscopic but not cellular, although there is ongoing scientific
687 discussion regarding their characterization as “micro-organisms”.

688
689 **OTU:** “*Operational taxonomic unit*”, used to refer to a cluster of DNA sequences of
690 microorganisms, which are grouped by sequence similarity of a defined taxonomic marker
691 gene present in their DNA.

692
693 **Pathobiome:** Initially, the pathobiome concept was defined as the pathogenic agent integrated
694 within its microbial community (i.e. the pathogen interacting with the environmental
695 microbiome [13]. This concept has now evolved to host-associated microorganisms
696 (prokaryotes, eukaryotes and viruses) negatively impacting the health of the host, with the
697 interaction between the host and its pathobiome inevitably moderated by the environment
698 within the host and immediately surrounding it [14].

699
700 **Phenotypic plasticity:** the extent to which an organism can change its physiology, behavior,
701 morphology and/or development in response to environmental cues.

702
703 **Re-emerging infectious disease:** a known infectious disease increasing in incidence after a
704 period of reduced incidence, or even disappearance, of the disease.

705

706 **Box 1. The Disease Triangle and Former Disease Concepts**

707 The disease triangle concept was developed by Stevens [6] in the context of plant pathology.

708 Disease is the outcome of a dynamic interaction between the host, the pathogen and the
709 environment, and is illustrated using an equilateral triangle (Figure I) where the gradient of
710 host susceptibility (resistance and tolerance), pathogen pathogenicity (infectivity and
711 virulence), and environmental conditions affect the disease outcome at the center of the
712 triangle. The effect of disease is most intense at the center, where susceptibility, pathogenicity,
713 and environmental conditions favor the pathogen over the host. Disease outcome can vary
714 dramatically if environmental conditions, host resistance or tolerance, or the pathogen
715 pathogenicity change. Thus, a potentially infectious microorganism does not always invade a
716 host, and a ‘pathogen’ can be neutral or even a beneficial mutualist under other conditions.

717 The disease triangle is quite similar to the concept developed by Snieszko for fish diseases, in
718 which infectious disease occurs when a susceptible host is exposed to a virulent pathogen under
719 proper environmental conditions [7] (Figure I). While the environmental microbiome is
720 embedded in the ‘environment’ component of these models, they lack an important component of
721 disease dynamics: the host microbiome.

722 Recently, the importance of microorganisms in shaping disease dynamic gained more
723 attention and has been reflected in new theoretical models. Brucker and colleagues proposed
724 to illustrate the interactions between the host, the host microbiome and the pathogen with a
725 triangle of interactions (response or causation) [17] (Figure I). In this model, the environment
726 experienced by the host, the pathogen and the host microbiome was not considered. Finally,
727 the concept by Snieszko [7] between the host, the pathogen and the environment was extended
728 to include the microbial community of the pathogen, as the pathobiome concept arouse [13]
729 (Figure I). However, in this model, a clear distinction between the host microbiome, the
730 pathogen microbiome, and the environmental microbiome was not made. Interestingly, in this
731 model, the theories applying to hosts can also be applied to vectors of diseases, e.g. mosquitos.
732 In this case, the disease is the consequence of an interaction between the vector, the pathogen,
733 microbiome and the environment (Figure I).

734

735 **Figure I (in Box 1). Illustration of Former Models**

736

737 **Box 2. The Amphibian Immune System**

738 The immune system of amphibians is similar to other vertebrates [24] and is complex. It
739 includes three components: barriers (constitutive defenses), innate response and acquired
740 (adaptive) response (Figure I), whose functions partly overlap.

741 The first line of defense consists of (a) a physical barrier comprised of an epithelium, such as
742 skin or gut surface, covered by a mucus (a matrix of mucopolysaccharides secreted by the
743 mucous glands and complemented by antimicrobial peptides, AMPs, secreted by the granular
744 glands), (b) a biological barrier (i.e. skin and gut resident microbiomes), and (c) a chemical
745 barrier (e.g. the acidic pH of the gut, AMPs and lysozyme in the mucus, and antimicrobial
746 metabolites produced by the resident microbiome). Amphibians use their skin to breathe
747 (many species lack lungs) and to maintain homeostasis. Electrolytes and H₂O, O₂ and CO₂,
748 both actively and passively cross the amphibian epithelium surface and the mucus that act
749 together as a semi-porous physical barrier. This increases the importance of its chemical and
750 biological components when acting as a barrier against pathogens. Resident microbiomes,
751 naturally living on the host epithelium, compete for space and resources with pathogens,
752 presumably preventing them from adhering to the skin, proliferating and entering the host
753 [24]. Host microbiomes actively secrete antimicrobial metabolites, contributing to the
754 chemical barrier that the host creates.

755 Pathogens that cross those barriers face the innate and adaptive immune response. The innate
756 system includes lysozyme, complement lytic system, and AMPs. It also involves natural killer
757 cells (non-specifically attack and lyse infected cells), and dendritic and macrophage cells
758 (phagocyte infected cells, produce cytokines, and activate B and T lymphocytes) [24].

759 The most advanced response is the adaptive (acquired) response, preventing re-infection. After
760 first infection, this system provides a fast secondary response during subsequent exposure.
761 When stimulated by an antigen, B lymphocytes produce antibodies which neutralize
762 pathogens by agglutination, inducing complement activation, and tagging antigen for
763 destruction by phagocytes. T lymphocytes need direct contact with an infected cell to
764 eliminate it. They do not produce antibodies but regulate their production [24]. Several kinds
765 of T lymphocytes exist, including regulatory T cells, T helper cells (secret cytokines and
766 coordinate antibody production) and cytotoxic T cells (destroy pathogenic cells by physical
767 and chemical lysis). However, some pathogens, including *Bd*, can suppress the adaptive
768 response [91,92].

769 In larval amphibians (e.g. tadpole stage), the immune system is not as developed as it is in
770 fully metamorphosed individuals, but still competent [24].

771

772 **Figure I (in Box 2). The Three Components of the Amphibian and Vertebrate Immune**
773 **System - Barriers, Innate Response and Acquired (Adaptive) Response.**

774

775 **Figure 1. Importance of the Microbiome to Hosts and Ecosystems.** The host microbiome
776 allows the host to adjust to its environment, provides protection against pathogens, and
777 contributes to physiological functions (nutrition, growth and reproduction). Similarly, the
778 environmental microbiome promotes ecosystem stability and the maintenance of biodiversity
779 by preserving ecosystem health and contributing to important ecological functions (e.g.
780 nutrient cycling, energy uptake, carbon sequestration and nitrogen retention). Host and
781 environmental microbiomes are interconnected and regularly exchange microorganisms.

782
783 **Figure 2, Key Figure. The Disease Pyramid: A Four-Way Interaction between Host,**
784 **Microbiome, Pathogen and Environment.** The pyramid contains a gradient of host
785 susceptibility (from resistance and tolerance to high susceptibility), host microbiome
786 permeability to the pathogen (from a complete barrier to a fully porous barrier), pathogen
787 pathogenicity (from low to high infectivity and virulence), and environmental conditions
788 (from poorly to highly favorable to the disease) that each feed towards the center of the
789 pyramid to affect disease outcome. A permeable/porous host microbiome does not act as a
790 barrier to pathogens, it allows pathogen establishment, proliferation and invasion of the host.
791 A given environmental condition interacts simultaneously and independently with the host, the
792 host microbiome and the pathogen in various ways, modifying each as well as their
793 interactions with each other. The disease is more intense at the center, where the strongest
794 susceptibility, permeability, pathogenicity, as well as the most favorable environmental
795 conditions for the disease are found. Environmental conditions include biotic (e.g.
796 environmental microbiome) and abiotic factors. **(Figure 360)**

797

798 **Table 1: Overview of Recent Studies Focusing on Amphibian Skin Microbiome and Host-, Pathogen (*Bd*-), Environment-Related Variables.**

800

Host species	Sampling site	Molecular marker	Target microbial group	Interaction	Main findings	Ref.
Host-Microbiome						
<i>Anaxyrus boreas</i>	Colorado, USA	V4 region 16S rRNA gene	bacteria	life stages	skin communities changed across life stages	[59]
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i>	Panama	V4 region 16S rRNA gene	bacteria	year, date of sampling	skin communities differed across years on both species. Differences in relative abundance of key OTUs explained by rainfall	[47]
Host-Microbiome-Environment						
Acquisition/maintenance of microbiome						
<i>Cymops pyrrhogaster</i>	breeding facility and pond in Japan	V4 region 16S rRNA gene	bacteria	wild/captive conditions	wild individuals had more diverse communities, but similar OTUs richness	[50]
<i>Atelopus zeteki</i>	Panama	V4 region 16S rRNA gene	bacteria	wild/captive conditions	wild population had 3 times more unique OTUs	[51]
<i>Plethodon cirineus</i>	controlled environment	V4 region 16S rRNA gene	bacteria	environmental soil vs. sterile media	diversity across treatments and decreased in the sterile media	[42]

<i>Plethodon cirineus</i>	controlled environment	V4 region 16S rRNA gene	bacteria	cage with or without bacteria reservoir	bacteria related to antifungal isolates were more likely to persist on salamanders, regardless the environment	[93]
<i>Plethodon jordani</i>	North Carolina, USA	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	OTUs highly associated to salamanders tended to be absent/too rare in the environment	[94]
<i>Rana catesbeiana</i> , <i>Notophthalmus viridescens</i>	Virginia, USA	V2 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	relative abundance of OTUs shared by amphibians and environment was inversely related	[41]
<i>Atelopus certus</i> , <i>Craugastor fitzingeri</i> , <i>Colostethus panamansis</i> , <i>Espadarana prosoblepon</i> , <i>Strabomantis bufoniformis</i>	Serrania del Sapo, Panama	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	microbiome communities were enriched with rare environmental OTUs, and high percentage of OTUs shared between frogs and habitat	[44]
<i>Cryptobranchus alleganiensis</i> <i>alleganiensis</i>	Indiana, West Virginia, North California, Tennessee and Georgia, USA	V2 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	variation in community diversity among populations and in proportion of shared OTUs between animals and river	[95]

89 species of frogs	Madagascar	V4 region 16S rRNA gene	bacteria	host vs. environmental microbiomes	host microbial communities were different and less diverse than environmental ones	[96]
<i>Rana marina</i>	Puerto Rico and Costa Rica	V4 region 16S rRNA gene	bacteria	geographic location	significant environmental influence in composition, richness and abundance of microbiome taxa	[45]
genera <i>Ensatina</i> and <i>Batrachoseps</i>	California, USA	V3-V4 region 16S rRNA gene	bacteria	geographic location	strong site differences in bacterial communities	[46]
<i>Rana pipiens</i> , <i>Pseudacris</i> <i>triseriata</i> , <i>Ambystoma</i> <i>tigrinum</i>	Colorado, USA	V2 region 16S rRNA gene	bacteria	altitudinal gradient	host species community similarity	[52]
<i>Eleutherodactylus</i> <i>coqui</i>	Puerto Rico	V4 region 16S rRNA gene	bacteria	altitudinal gradient, intact/disturbed forest	diversity changed with site, elevation and land use	[97]
<i>Lissotriton</i> <i>boscai</i> , <i>Telmatobius</i> <i>marmoratus</i>	Galicia, Spain	V4 region 16S rRNA gene	bacteria	life stage, aquatic/terrestrial environment	terrestrial adults had more diverse and richer bacterial communities	[98]
<i>Anaxyrus boreas</i> , <i>Pseudacris</i> <i>regilla</i> , <i>Taricha</i> <i>torosa</i> , <i>Rana</i>	California, USA	V4 region 16S rRNA gene	bacteria	species, geographic location	amphibian skin identity was the strongest predictor of microbiome composition	[16]

<i>catesbeianus</i>						
<i>Plethodon glutinosus</i> , <i>P. cinereus</i> , <i>P. cylindraceus</i>	Central Appalachians, US	V3-V5 region 16S rRNA gene	bacteria	species, altitudinal gradient	diversity changed with elevation and co-occurring salamanders had similar microbiome structure	[99]
<i>Ensatina eschscholtzii xanthoptica</i>	San Francisco and Sierra Nevada, USA	V3-V4 region 16S rRNA gene	bacteria	life stage, sex, geographic location	isolated populations had similar communities, which were significantly different from the environment	[100]
Possible environmental stressors						
<i>Pelophylax perezii</i>	Portugal	nearly full length of the 16S rRNA gene	bacteria	metal contamination, salinity	low diversity and density on individuals from metal contaminated population	[101]
<i>Pseudacris crucifer</i>	controlled environment	V4 region 16S rRNA gene	bacteria	coal combustion waste	little impact from acute exposure to fly ash on the bacterial communities	[102]
205 amphibian species	13 countries	V4 region 16S rRNA gene	bacteria	thermal stability, habitat class (aquatic, terrestrial, arboreal), elevation	bacterial richness decreased in warmer and more stable environments, and in arboreal hosts	[68]
<i>Atelopus zeteki</i>	controlled environment	V4 region 16S rRNA gene	bacteria	microbiome, <i>Bd</i>	survival to <i>Bd</i> infection was related to initial composition of the skin bacterial community	[77]

12 amphibian species	Costa Rica	nearly full length of the 16S rRNA gene	bacteria	microbiome, <i>Bd</i>	11% of the bacterial isolates collected from the species exhibited <i>Bd</i> inhibition and 2,2% enhanced <i>Bd</i> growth	[78]
<i>Dendrobates sp.</i>	aquarium and animal care facility, USA	V4 region 16S rRNA gene + ITS	bacteria and fungi	<i>Bd</i> inhibition and enhancement	abundance of cutaneous fungi contributed more to <i>Bd</i> defense than bacteria; different	[39]
<i>Bombina orientalis</i>	controlled environment	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro-eukaryotes	life stage, microbiome, <i>Bd</i>	major change in community until 15 days after metamorphosis; richness diverged between aquatic and terrestrial stages	[103]
<i>Anaxyrus boreas</i>	Colorado, USA	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria	life stage, microbiome, <i>Bd</i>	life stage had the largest effect on microbiome; diversity of micro eukaryotes was lowest in tadpoles	[58]
<i>Rana sierra</i>	California, USA	V3-V4 region 16S rRNA gene	bacteria	life stage, microbiome, <i>Bd</i>	skin microbiome of highly infected juveniles had reduced richness and lower variation between individuals	[79]
<i>Rana cascadae</i>	Northern California, USA	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro-eukaryotes	life stage, microbiome, <i>Bd</i>	<i>Bd</i> was significantly lower on tadpoles and highest on subadults	[40]

<i>Lithobates yavapaiensis</i> , <i>Eleutherodactylus coqui</i>	Arizona, USA and Puerto Rico	V4 region 16S rRNA	bacteria	life stage, season, <i>Bd</i>	winter-sampled individuals exhibited higher diversity; hosts with higher bacterial diversity carried lower <i>Bd</i> loads	[104]
<i>Craugastor fitzingeri</i> , <i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i>	Panama	V4 region 16S rRNA gene	bacteria	different species, <i>Bd</i>	treefrogs had a significantly higher number of culturable <i>Bd</i> -inhibitory OTUs than terrestrial species	[105]
<i>Rana catesbeiana</i> , <i>Notophthalmus viridescens</i> , <i>Pseudacris crucifer</i> , <i>Anaxyrus americanus</i>	Virginia, USA	V4 region 16S rRNA gene	bacteria	different species, <i>Bd</i>	dominant bacteria had higher <i>Bd</i> inhibition in bullfrog and newt. Dominant and rare bacteria did not differ in inhibition in spring peeper and toad, in which <i>Bd</i> was lower	[106]
<i>Rana sierra</i>	California, USA	V1-V2 region 16S rRNA gene	bacteria	resistant/non-resistant population to <i>Bd</i> , <i>Bd</i>	different bacteria richness between resistant/non-resistant populations	[107]
<i>Rana sierra</i>	controlled environment	V1-V2 region 16S rRNA gene	bacteria	resistant/non-resistant population to <i>Bd</i> , <i>Bd</i>	frogs housed in water from resistant populations had greater bacterial richness than those housed in non-resistant population water	[56]
<i>Rana sierra</i>	California, USA	V1-V2 region 16S	bacteria	<i>Bd</i> (epizootic/enzootic)	100% mortality of post metamorphic frogs during <i>Bd</i> epizootic; several bacteria taxa showed the same	[109]

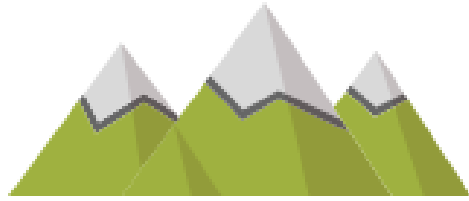
		rRNA gene			response to <i>Bd</i> across multiple field populations	
<i>Rana catesbeiana</i>	controlled environment	V4 region 16S rRNA gene	bacteria	before/after exposure to <i>Bd</i>	microbial community of frogs prior to <i>Bd</i> exposure influenced infection intensity	[110]
<i>Anaxyrus boreas</i>	controlled environment	V4 region 16S rRNA gene + V9 region 18S rRNA gene	bacteria and micro eukaryotes	four probiotic treatments, <i>Bd</i>	amphibians in captivity lost the <i>Bd</i> -inhibitory bacteria; inoculations of the <i>Bd</i> -inhibitory probiotic increased survival	[111]
Host-Microbiome-Pathogen-Environment						
<i>Bombina orientalis</i>	South Korea	V3 region 16S rRNA gene	bacteria	wild/captive conditions, <i>Bd</i>	<i>Bd</i> infection intensity was correlated neither with richness nor diversity indices; diversity was greater, and microbiome structure more complex in wild toads	[112]
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i> , <i>Craugastor fitzingeri</i>	Panama and USA	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , geographic location	no clustering of OTUs based on <i>Bd</i> infection status	[53]
<i>Agalychnis callidryas</i> , <i>Dendropsophus ebraccatus</i> , <i>Silverstoneia</i>	Panama and USA	16S rRNA gene database and ITS1	bacteria and fungi	species, <i>Bd</i> , geographic location	the host species was more important in determining microbiome composition than geographic location or <i>Bd</i> load	[113]

<i>flotator</i> , <i>Craugastor</i> <i>fitzingeri</i> , <i>Rana</i> <i>catesbeiana</i> , <i>Pseudacris</i> <i>crucifer</i> , <i>Notophthalmus</i> <i>viridescens</i> , <i>Anaxyrus</i> <i>americanus</i>						
<i>Silverstoneia</i> <i>flotator</i>	Panama	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , elevational gradient	similar skin communities across elevations; richness varied with <i>Bd</i> presence; severe outbreaks occurred at high elevation	[85]
<i>Rana</i> <i>sphenocephala</i>	controlled environment	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , controlled mesocosm	efforts to maintain a normal skin community using semi-natural mesocosms failed to provide long term protection	[88]
<i>Bolitoglossa</i> (3 spp.), <i>Pseudoeurycea</i> (3 spp.), <i>Plectrohyla</i> (4 spp.)	Mexico and Guatemala	V3-V4 region 16S rRNA	bacteria	<i>Bd</i> , forest type	phylogeny influence of diversity and structure of microbiome at higher taxonomic levels; the habitat predominated on lower scales	[89]
<i>Dendropsophus</i> . <i>minutus</i>	São Paulo and Rio Grande do Sul, Brazil	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , land cover, forest connectivity	bacterial diversity and <i>Bd</i> loads increased towards natural vegetation	[90]

<i>Ichthyosaura alpestris</i> , <i>Lissotriton vulgaris</i> , <i>Triturus cristatus</i>	Kleiwiesen and Elm, Germany	V4 region 16S rRNA gene	bacteria	<i>Bd</i> , water temperature	skin microbe fluctuations not correlated with fluctuations of pond microbiota; significant correlation between water temperature and newt bacterial community structure	[86]
<i>Acris blanchardi</i>	Ohio and Michigan, USA	V3 region 16S rRNA gene	bacteria	<i>Bd</i> , pH, CaCO ₃ , conductivity, natural/managed habitat	microbiome composition associated with water conductivity, ratio of natural to managed land, and latitude	[87]
<i>Craugastor fitzingeri</i>	Panama	metagenome	bacteria	<i>Bd</i> positive and negative sites	bacterial communities in positive sites were less diverse than in negative ones	[114]

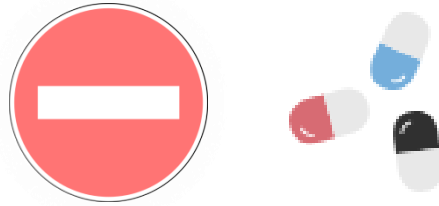
HOST

ADAPTATION TO THE ENVIRONMENT



- temperature tolerance
- short time adaptation to environmental changes

HOST HEALTH



- biological barrier, part of the immune system
- competition with pathogens
- disease mitigation

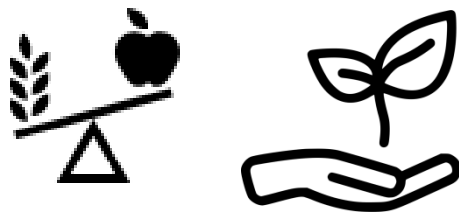
PHYSIOLOGICAL FUNCTIONS



- synthesis of vital nutrients
- energy uptake and growth
- influence on host behaviour and reproduction

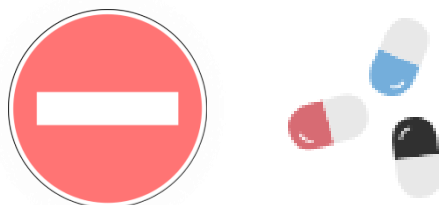
ECOSYSTEM

ECOSYSTEM STABILITY



- buffering against change
- maintenance of biodiversity
- maintenance of ecosystem processes

ECOSYSTEM HEALTH

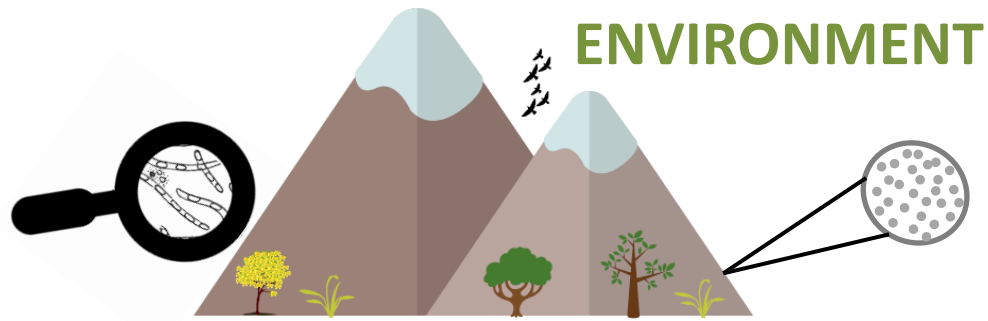


- environmental barrier against alien species
- competition with opportunists
- resistance, resilience or tolerance

ECOLOGICAL FUNCTIONS



- nutrient cycling
- energy flux
- CO₂ sequestration
- nitrogen retention



ENVIRONMENT



DISEASE

PATHOGEN



HOST



HOST
MICROBIOME

