Review

Patterns of Macroinvertebrate and Fish Diversity in Freshwater Sulphide Springs

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Abstract: Extreme environments are characterised by the presence of physicochemical stressors and provide unique study systems to address problems in evolutionary ecology research. Sulphide springs provide an example of extreme freshwater environments; because hydrogen sulphide's adverse physiological effects induce mortality in metazoans even at micromolar concentrations. Sulphide springs occur worldwide, but while microbial communities in sulphide springs have received broad attention, little is known about macroinvertebrates and fish inhabiting these toxic environments. We reviewed qualitative occurrence records of sulphide spring faunas on a global scale and present a quantitative case study comparing diversity patterns in sulphidic and adjacent non-sulphidic habitats across replicated river drainages in Southern Mexico. While detailed studies in most regions of the world remain scarce, available data suggests that sulphide spring faunas are characterised by low species richness. Dipterans (among macroinvertebrates) and cyprinodontiforms (among fishes) appear to dominate the communities in these habitats. At least in fish, there is evidence for the presence of highly endemic species and populations exclusively inhabiting sulphide springs. We provide a detailed discussion of traits that might predispose certain taxonomic groups to colonize sulphide springs, how colonizers subsequently adapt to cope with sulphide toxicity, and how adaptation may be linked to speciation processes.

1. Introduction

Freshwater ecosystems harbour a considerable proportion of Earth's biodiversity, even though they cover <2% of the land surface [1–3]. Most of this diversity is located in lakes and river systems, particularly in the tropics [4]. However, other freshwater environments that often occur at small spatial scales can harbour a significant portion of biodiversity, not necessarily because of their species richness, but because of high levels of endemism. Many spatially restricted habitats can be considered extreme environments that are characterised by the presence of physicochemical stressors lying outside the range normally experienced by a taxon and requiring costly adaptations absent in closely related taxa for the maintenance of homeostasis [5,6]. Such adaptations allow some organisms to thrive in places that are lethal for most others, giving rise to unique ecological communities. Prime examples of extreme freshwater environments include subterranean streams and lakes [7,8], desert springs [9,10], Antarctic lakes [11], and environments with rampant hypoxia [12]. Extreme environments have provided excellent study systems in ecology and evolution research, as they allow elucidating the effects of physicochemical stressors at multiple levels of biological organization [13,14], yet patterns of biodiversity in extreme environments remain relatively understudied.

Environments rich in naturally occurring hydrogen sulphide (H_2S) are one form of extreme habitat found in aquatic systems throughout the world. Because of its lipid solubility, H_2S freely penetrates biological membranes and readily invades organisms [15,16]. Like cyanide, it is an inhibitor of cytochrome c oxidase and blocks electron transport in aerobic respiration, thereby hampering the function of mitochondria and the production of ATP [17,18]. H_2S is also able to modify oxygen transport proteins [19] and inhibit about 20 other enzymes [15]. Consequently, H_2S is highly toxic for aerobic organisms even in micromolar concentrations [16,20,21].

Naturally occurring H_2S can be found in a variety of aquatic environments. It is produced in anoxic sediments of swamps, marshes, and cold seeps by bacterial metabolism of organic and inorganic carbon sources, and disturbance of sediments can result in high—but often temporally variable—H_2S concentrations in the water column [22–25]. High and sustained concentrations of H_2S can also be found in aquatic environments associated with oil deposits and geothermal activity [26,27]. The effects of H_2S on biodiversity, as well as the ecology and evolution of organisms inhabiting sulphidic environments, have mostly been studied in cold seeps and deep-sea hydrothermal vents [28–33]. Here, we focus on patterns of macroinvertebrate and fish biodiversity in freshwater springs that discharge water rich in H_2S. We first qualitatively review the global occurrence of such springs and the organisms that have been able to colonize them, focusing in part on specific adaptations to sulphide-rich environments, and then provide a quantitative comparison of diversity patterns between sulphidic and adjacent non-sulphidic habitats in Southern Mexico.

2. Freshwater Sulphide Springs: Occurrence and Environmental Variation

Freshwater springs discharging sulphide-rich waters can be found on all continents, with the exception of Antarctica (see Figure 1 for an overview). In general, the presence of sulphide is associated with either of two sources. First, sulphide springs can be associated with volcanic activity, where H_2S from geological origins is enriched in groundwater through similar processes as those occurring in deep sea hydrothermal vents [34,35]. During the interaction of the water with hot basaltic rock, a diversity of chemicals leach into solution, including sulphate and other sulphur species that are readily transformed into sulphide under the highly reductive conditions [36–38]. Second, sulphide springs can be associated with underground oil deposits, where mineral-rich groundwater containing sulphate mixes with hydrocarbons derived from fossil organic matter in the absence of oxygen [39–41]. As in marine cold seeps, sulphur-reducing bacteria then reduce sulphate to H_2S, while oxidizing organic compounds, during energy metabolism [22,42]. During this process, groundwater is being enriched in H_2S and ultimately discharged through surface springs [40,43].

Figure 1. (**A**) Political map of the world indicating the locations of H_2S-rich springs (blue dots). Red dots represent sulphide springs with records of fish. Locality information was assembled by reviewing previously published literature (see Table 1 and [35,40,41,44–59]). Additionally depicted are representative fish species from sulphide spring habitats: (**B**) *Cyprinodon bobmilleri* (Mexico); (**C**) *Limia sulphurophila* (Dominican Republic); (**D**) *Aphanius dispar* (Iran; photo by Azad Teimori); (**E**) *Gambusia eurystoma* (Mexico); (**F**) *Poecilia thermalis* (Mexico); and (**G**) *Xiphophorus hellerii* (Mexico).
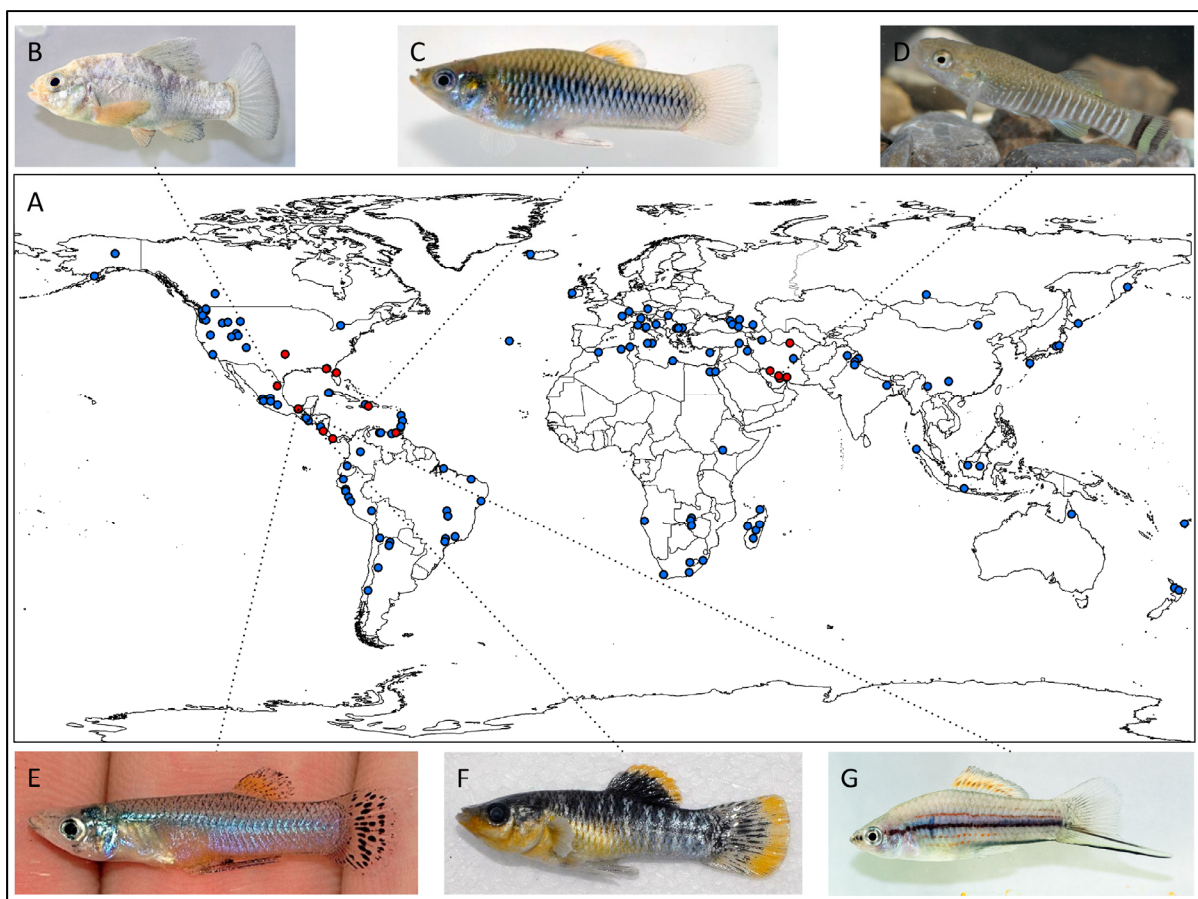
Table 1. Evolutionarily independent populations recorded from sulphide spring habitats, including specific localities and references.

Species	Locations	References
Synbranchidae		
Ophisternon aenigmaticum	El Azufre II (Rio Tacotalpa drainage) and La Gloria (Rio Pichucalco drainage) springs, Mexico	This study
Cyprinodontidae		
Cyprinodon bobmilleri [†]	Baños de San Ignacio (Rio San Fernando drainage), Mexico	[60]; Schlupp (personal communication)
Aphanius ginaonis [†]	Ginao spring (Hormuzgan drainage), Iran	[61,62]
Aphanius dispar *	Khurgu, Faryab, and Howba springs (Hormuzgan drainage), Dalaki and Mirahmad springs (Helleh drainage), Iran	[62,63]
Aphanius furcatus *	Khurgu and Faryab springs (Hormuzgan drainage), Iran	[62,63]
Poeciliidae		
Acanthophacelus reticulata	Poza Azufre (Rio San Juan drainage), Venezuela	[64,65]
Brachyrhaphis roseni	Spring near David (Rio David drainage), Panama	S. Ingley (personal communication)
Gambusia affinis	Vendome Well/Black Sulphur Springs (Red River drainage), Oklahoma	[66,67]
Gambusia eurystoma [†]	Baños del Azufre (Rio Pichucalco drainage), Mexico	[68,69]
Gambusia holbrooki *	Green (St. John's River drainage), Newport (Wakulla River drainage), and Panacea Mineral Springs (Dickerson Bay area), Florida	[70]
Gambusia sexradiata	Mogote del Puyacatengo (Puyacatengo drainage), Mexico	[71]
Limia sulphurophila *[†]	Balnearios La Zurza and La Zufrada (Lake Enriquillo basin), Dominican Republic	[72,73]
Poecilia formosa	Baños de San Ignacio (Río San Fernando drainage), México	Schlupp (personal communication)
Poecilia latipinna	Panacea Mineral Springs (Dickerson Bay area), Florida	[74]
Poecilia mexicana limantouri	Baños de San Ignacio (Río San Fernando drainage), México	Schlupp (personal communication)
Poecilia mexicana mexicana	El Azufre springs and Cueva del Azufre (Tacotalpa drainage), Mexico	[75,76]
Poecilia mexicana mexicana	La Lluvia and Puyacatengo springs (Puyacatengo drainage), Mexico	[76,77]
Poecilia sulphuraria [†],*	Baños del Azufre and La Gloria springs (Pichucalco drainage), Mexico	[76,77]
Poecilia thermalis [†]	La Esperanza springs (Ixtapangajoya drainage), Mexico	[77]
Poeciliopsis elongata	Spring near David (Rio David drainage), Panama	S. Ingley (personal communication)
Priapichthys annectens	Spring in Rincón de la Vieja National Park (Rio Colorado drainage), Costa Rica	J. Johnson (personal communication)
Priapichthys panamensis	Spring near David (Rio David drainage), Panama	S. Ingley (personal communication)
Pseudoxiphophorus bimaculatus	La Gloria springs (Pichucalco drainage), Mexico	This study
Xiphophorus hellerii *	La Lluvia (Puyacatengo drainage) and La Gloria springs (Pichucalco drainage), Mexico	This study

[†] Species that were described from and are endemic to sulphide springs. * Species that potentially include populations that have independently colonised sulphide spring environments.

H$_2$S concentrations found in freshwater sulphide springs can be highly variable across springs, although most springs tend to discharge relatively constant levels of H$_2$S [78]. Some springs classified as sulphidic contain as little as 20 μM of H$_2$S [76], but even such low concentrations are biologically relevant and considered toxic for most metazoans [16]. Conversely, peak concentrations of H$_2$S have been reported to exceed 7000 μM in some springs [79]. Currently available data suggest that H$_2$S concentrations in most springs lie in between these extremes, with a large number of springs having concentrations towards the lower end of the spectrum (*i.e.*, <2500 μM H$_2$S [40,41,44,47,76,78,80]).

Besides the presence of H$_2$S, sulphide springs also differ from adjacent, non-sulphidic habitats in a series of other biologically relevant environmental parameters. Similar to variation in H$_2$S concentrations, springs substantially vary in temperature, with reported values ranging from around 5 °C to over 100 °C [35,40,41,47,48,57,76,81]. Temperature variation appears to be related in part to the geographic location of springs as well as the ultimate sources of H$_2$S production. Sulphide springs are often also characterised by increased concentrations of bicarbonate, calcium sulphate, sodium chloride, and other ions (leading to substantial increases of specific conductivity [39]), and by lower pH likely caused by the presence of sulphuric acid from chemical and bacterial H$_2$S oxidation (although pH reductions are dependent on the buffering capacity of the water in the region) [57,76,82]. Finally, upon the discharge of sulphidic water at the surface, H$_2$S spontaneously oxidizes in water, causing and aggravating hypoxic conditions in aquatic systems [83,84]. Consequently, environmental conditions in sulphide springs are not only toxic for most metazoans, but variation in correlated environmental parameters may also affect the acid-base balance, osmoregulation, and constrain oxygen acquisition of aquatic organisms [16,21].

3. An Overview of Biodiversity in Sulphide Springs

H$_2$S-rich freshwater environments (Figure 2) have long captured the attention of microbiologists, because a wide variety of bacteria and archaea are associated with the natural sulphur cycle [85–87]. This not only includes sulphur reducing microbes, including the sulphate reducers involved in the production of H$_2$S mentioned above [22,88], but the presence of H$_2$S inevitably also supports a diversity of sulphur oxidizing bacteria [89,90]. The coexistence of sulphur reducers and oxidizers creates complex dynamics of sulphur cycling, where sulphate-reducing bacteria use sulphate as a terminal electron acceptor to metabolize a variety of carbon sources to produce sulphide as a by-product, which in turn is used by sulphide-oxidizing bacteria as an energy source to assimilate CO$_2$ and produce oxidised sulphur species as metabolic by-products [87,91]. Sulphur metabolizing microbes play a critical role in sulphidic ecosystems, because they contribute to primary production through chemoautotrophy [36,92–94] and can serve as a food source for consumers inhabiting sulphide springs. While biodiversity of microbes inhabiting sulphide-rich environments is relatively well understood at multiple levels of biological organization, few studies have addressed patterns of metazoan diversity.

Figure 2. Examples of the diversity of sulphide springs inhabited by fishes. (**A**) Green Springs, Florida (inhabited by *Gambusia holbrooki*; photo by Rüdiger Riesch); (**B**) La Zurza spring, Dominican Republic (*Limia sulphurophila*); (**C**) Baños de San Ignacio, Mexico (*Cyprinodon bobmilleri*, *Poecilia mexicana limantouri*, and *P. formosa*; photo by Ingo Schlupp); (**D**) El Azufre II, Mexico (sulphidic ecotype of *P. mexicana mexicana* and *Ophisternon aenigmaticum*); (**E**) Puyacatengo springs, Mexico (sulphidic ecotype of *P. m. mexicana*); (**F**) La Esperanza (big) springs, Mexico (*P. thermalis*); (**G**) La Gloria springs, Mexico (*P. sulphuraria*, *Pseudoxiphophorus bimaculata*, *Xiphophorus hellerii*, and *Ophisternon aenigmaticum*); (**H**) a sulphide spring near David, Panama (*Brachyraphis roseni*, *Poeciliopsis elongata*, and *Priapichthys panamensis*; photo by Spencer Ingley); and (**I**) Faryab springs, Iran (*Aphanius dispar* and *A. furcatus*; photo by Azad Teimori).
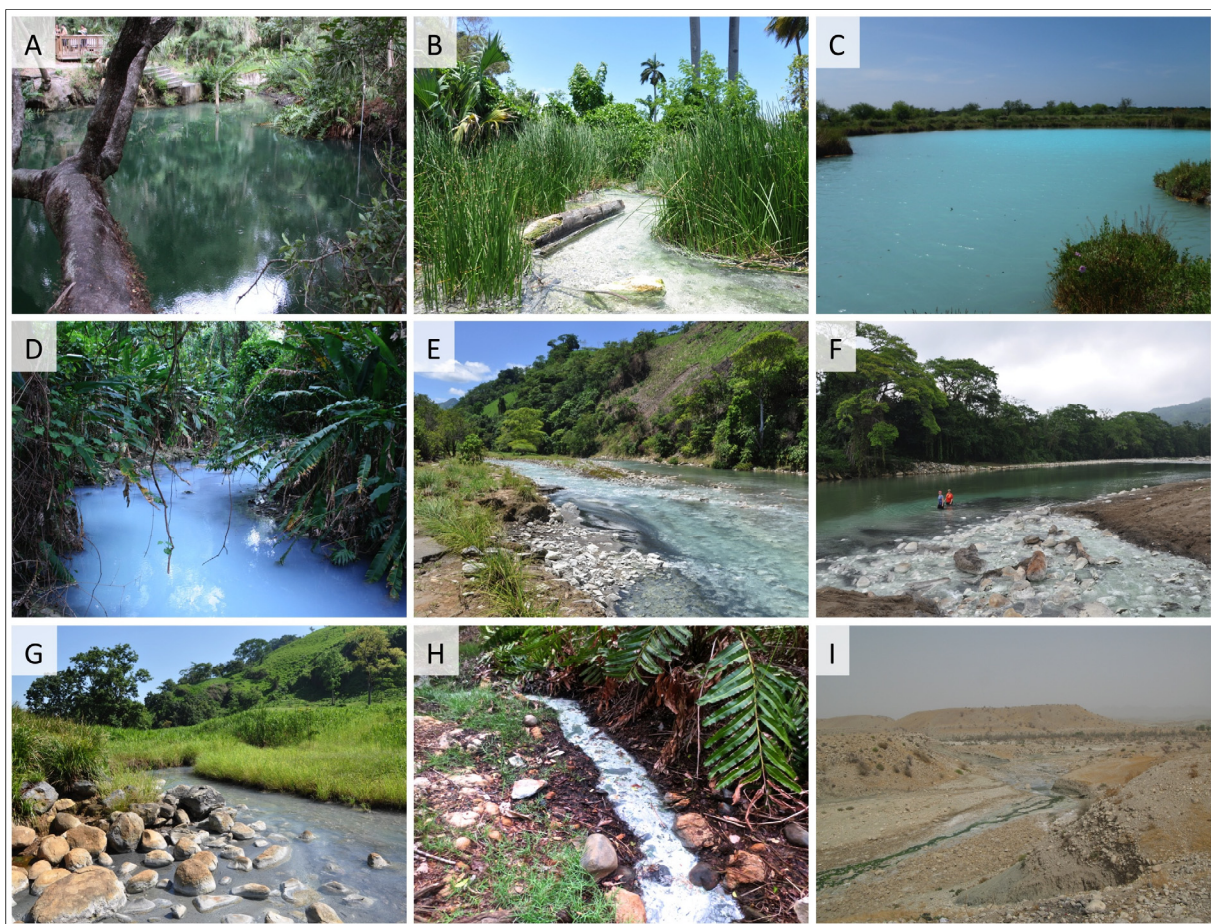
3.1. Macroinvertebrates in Sulphide Spring Environments

Macroinvertebrate diversity in environments with sulphurous waters has particularly been studied in hot mineral springs of Yellowstone National Park and other parts of the western United States, some of which contain elevated levels of H_2S (e.g., [95,96]). Records from sulphide springs in other parts of the world are relatively scarce and often anecdotal. Larvae of dipterans appear to dominate sulphide-rich spring environments both in terms of diversity and abundance. Multiple families of dipterans are represented in sulphide springs, including a species of Ceratopogonidae (*Bezzia setulosa*) in Yellowstone [97], multiple species of Chironomidae [97,98], a species of Culicidae (*Culiseta incidens*)

in springs of Western North America [99], two species of Ephydridae in California (*Thiomyia quatei*) and Yellowstone (*Ephydra thermophila*) [45,82,97], a species of Psychodidae (*Pericoma truncate*) in California [45], a species of Stratiomyidae (*Odontomyia* cf. *occidentalis*) in Yellowstone [82], as well as species of Syrphidae in Israel (*Eristalis* sp.) and the Russian Kamchatkan peninsula (*Eristalinus sepulchralis*) [46,100]. Dipteran larvae are mostly found living in and feeding on bacterial mats within sulphide springs, and ephydrid and pyschodid adult flies have been observed feeding and reproducing on these mats as well [45,46,82,101]. Besides dipterans, the only other insects reported from sulphide springs are trichopterans (based on the presence of larval casings in Yellowstone) [101] and hemipterans of the genus *Belostoma* in Mexico [102]. Finally, aquatic snails (Gastropoda) have also been recorded in sulphide springs. *Melanoides tuberculata* (a livebearing snail of the family Thiaridae) has been reported from a spring in Israel [100], *Physa gyrina* (Physidae) and *Helisoma trivolvis* (Planorbidae) in Oklahoma [66], and *Stagnicola palustris* (Lymnaeidae) in Yellowstone [96].

While records for the macroinvertebrate fauna in surface sulphide springs remain fragmentary, it is important to note that such springs are also present in a variety of subterranean habitats, including the Movile Cave (Romania) [93], Frasassi Cave and Grotta de Fiume Coperto (Italy) [103,104], Villa Luz Cave (Mexico) [105,106], Lower Kane Cave (Wyoming) [107], and the Edwards Aquifer (Texas) [108]. The faunal communities of these subterranean aquatic habitats with elevated H_2S concentrations have recently been reviewed by Summers Engel [109]. The assembled data indicate that approximately 40 invertebrate species (many of which remain to be identified or described) from eight phyla inhabit H_2S-rich waters, including members of the Oligohymenophorea, Rotifera, Platyhelminthes, Nematoda, Annelida, Mollusca, Crustacea, and Hexapoda. Most notably, some sulphidic caves – particularly Movile Cave–exhibit high levels of endemism [93,110]. It remains unclear whether differences in the reported biodiversity of surface and subterranean H_2S-rich habitats are due to sampling bias or whether the combination of the presence of H_2S or the absence of light causes differential persistence of populations in cave *vs.* surface habitats.

How macroinvertebrates living in freshwater sulphide springs cope with the toxic levels of H_2S has yet to be examined. Some taxa described above, such as hemipterans and the larvae of some dipteran groups, have the ability to breathe atmospheric air through respiratory siphons or tubes, which likely reduces their exposure to H_2S dissolved in water [111,112]. Adaptations to sulphide-rich conditions have received more attention in invertebrates from marine environments, and depending on the taxonomic group, elevated tolerances have been associated with a wide variety of behavioural, physiological, and morphological modifications, including structural barriers that exclude H_2S from the body, modification of molecular targets of H_2S that reduce binding, reliance on anaerobic metabolism, active detoxification mechanisms, and symbioses with sulphide metabolizing microbes (see [21,113] for reviews).

3.2. Fish in Sulphide Spring Environments

3.2.1. Overview

The majority of records of fish inhabiting sulphide springs stem from North America and the Neotropics, with few additional reports from the Middle East. Overall, 24–putatively evolutionarily independent–invasions of sulphide springs have been documented (Table 1 and references therein;

Figure 1). However, this number may be an underestimation even for documented populations, because the phylogenetic relationships of most species inhabiting multiple springs (sometimes in different drainages) remain to be studied. With one exception (*Ophisternon aenigmaticum*, Synbranchidae), all sulphide spring fishes belong to the order Cyprinodontiformes. Pupfish (Cyprinodontidae) have been recorded from H_2S-rich springs in Northern Mexico (*Cyprinodon bobmilleri*) and in Iran (three species in the genus *Aphanius*). Livebearers (Poeciliidae) from the genera *Acanthophacelus*, *Gambusia*, *Limia*, *Poecilia*, *Poeciliopsis*, *Pseudoxiphophorus*, and *Xiphophorus* have colonised sulphide springs in North, Central, and South America, as well as some Caribbean Islands. One population of *Poecilia mexicana* also occurs in a H_2S-rich cave in southern Mexico [106,114,115].

Overall, six described species are highly endemic; they have been described from and exclusively inhabit sulphide springs (Table 1). Even though not described as distinct species, several populations particularly in the family Poeciliidae also represent phenotypically divergent and genetically distinct ecotypes, indicating that they represent locally adapted populations restricted to H_2S-rich environments [67,70,76]. However, additional research is required to test for the presence of local adaptation in the Cyprinodontidae and Synbranchidae, as at least some populations may be temporary or represent sinks that solely persist in H_2S-rich environments because of continuous immigration from adjacent non-sulphidic habitats. For example, it is unlikely that *Ophisternon aenigmaticum*, which is occasionally collected in sulphide springs of Southern Mexico, maintains viable populations in toxic springs due to their extremely low abundance.

Although all sulphide springs inhabited by fish occur in geographic regions with considerable fish diversity, relatively few taxonomic groups have apparently managed to invade and persist in such extreme environments. This begs questions about what traits potentially characterize successful invaders. All groups found in sulphide springs share the presence of alternative respiratory strategies that facilitate oxygen acquisition in the hypoxic waters [116–118]. Synbranchids have aerial-respiratory surfaces in the mouth and branchial chambers, allowing them to extract oxygen directly from atmospheric air [119]. Similarly, cyprinodontiforms are adapted to conducting aquatic surface respiration, where fish skim the uppermost water level that is disproportionally oxygenated because of direct contact to air, when exposed to hypoxic conditions [120,121]. The presence of alternative respiratory strategies could be critical for survival under sulphidic conditions, both because oxygen to fuel metabolism is scarce [16] and H_2S detoxification is an oxygen consuming process [122]. In addition, all members of the subfamily Poeciliinae are livebearing [123]. Internal gestation likely shields developing embryos from adverse conditions in sulphide springs and allows females to select habitat patches with more suitable environmental parameters [124]. It remains unclear how egg-laying species persist in sulphide spring environments, especially considering the rampant hypoxia in benthic microhabitats.

3.2.2. Adaptation to Sulphide Spring Environments in the Family Poeciliidae

Detailed studies on mechanisms of adaptation to sulphide springs so far have been conducted in members of the family Poeciliidae, particularly in the genus *Poecilia*, where many sulphide spring populations diverged in a series of phenotypic traits from ancestral forms in non-sulphidic environments. Most phenotypic traits investigated show strong signals of convergent evolution in evolutionarily independent lineages of *Poecilia* as well as other poeciliid taxa investigated to date [71,74,76,77].

Sulphide spring *Poecilia* exhibit significantly elevated tolerances to sulphide [76]. Differences in tolerance likely arise from behavioural, morphological, and physiological modifications. Fish from sulphidic habitats readily engage in aquatic surface respiration upon exposure to H_2S [125], and the ability to engage in this compensatory behaviour determines survivability in the natural environment [126]. In addition, sulphide spring fish are characterised by increases in gill surface area and head size, which improves oxygen acquisition efficiency [75,76]. They also exhibit physiological modifications that allow for minimizing adverse effects of potentially elevated endogenous concentrations and for maintaining low endogenous concentrations despite the constant influx of environmental H_2S. At least some lineages of sulphide spring *Poecilia* exhibit an H_2S-resistant cytochrome c oxidase (the primary target of sulphide toxicity) that maintains functionality with increasing H_2S concentrations [127]. Furthermore, gene expression studies have indicated that enzymes involved in oxidative sulphide detoxification, including sulphide:quinone oxireductase [128], are consistently up-regulated in natural sulphide spring populations [129]. Interestingly, common-garden raised individuals from one sulphide spring lineage retain higher constitutive expression of genes involved in detoxification even in the absence of environmental H_2S in the laboratory, which suggests evolutionary divergence in gene regulation between sulphidic and non-sulphidic ecotypes [130].

Other traits likely diverged through indirect effects of H_2S [131]. For example, the presence of H_2S affects energy budgets of sulphide spring residents, both because sulphide detoxification is energetically costly and energy acquisition is constrained by aquatic surface respiration [125]. As a consequence, some sulphide spring *Poecilia* are in worse nutritional condition [132] (see [71]), exhibit changes in energy metabolism [133], and diverged in life history strategies, producing fewer but substantially larger offspring [71,74,134]. In addition, colonizing sulphide springs was accompanied by changes in trophic resource use, where *Poecilia* switched from a typically algivorous diet in non-sulphidic habitats to a diet consisting of sulphur-metabolizing bacteria and invertebrates in sulphidic habitats [94,131]. This shift in resource use was paralleled by changes in both viscerocranial and gastrointestinal morphology [131].

3.2.3. Ecological Speciation in Sulphide Spring Environments?

The relatively high levels of endemism and the presence of divergent ecotypes in sulphide springs indicate that adaptation to H_2S-rich environments may be linked to speciation processes. Indeed, ecological speciation theory posits that reproductive isolation between populations can emerge incidentally as a by-product of adaptive trait divergence [135,136], and multifarious selection generated by the complex environmental differences between adjacent sulphidic and non-sulphidic environments may further promote the speciation process [137]. The majority of sulphide spring *Poecilia* and *Gambusia* are genetically differentiated from adjacent, ancestral populations in non-sulphidic environments, despite small geographic distances (in some instances <100 meters) and a lack of physical barriers that could prevent fish migration [70,77,138]. In the genus *Poecilia*, reproductive isolation is at least in part mediated by selection against immigrants. Natural selection mediated through H_2S toxicity and the correlated hypoxia drastically reduces survival particularly of migrants from non-sulphidic to sulphidic habitats, and sexual selection mediated through ecotype assortative mating and male-male aggression reduces fitness of migrants from sulphidic to non-sulphidic habitats [138–141]. Since selection against immigrants by itself cannot explain the degree of genetic differentiation between pairs of

sulphidic and non-sulphidic populations [138], empirical studies on other isolating mechanisms are highly warranted. Considering that adaptive changes have been documented in genes encoded by both the mitochondrial and nuclear genomes [127], there is a distinct possibility for the presence of postzygotic reproductive isolation through cyto-nuclear incompatibilities [142]. Whether and how ecological speciation processes are consistently associated with adaptation to sulphide spring environments remains to be tested in a broader group of taxa, as detailed investigations on invertebrates, pupfishes, and even the majority of poeciliid lineages are still missing.

4. A Case Study: Comparative Analyses of Sulphide Springs and Adjacent Non-Sulphidic Habitats in Mexico

The published literature about the global biodiversity in sulphide springs provides a general overview about taxa that have successfully invaded these extreme environments; however, the lack of systematic surveys precludes any quantitative assessments of biodiversity patterns at that scale. In an attempt to elucidate some quantitative trends, the following sections provide a yet unpublished case study of biodiversity patterns of macroinvertebrates and fishes in sulphidic springs and adjacent non-sulphidic habitats of southern Mexico.

In Southern Mexico, sulphide springs occur in a replicated fashion in the states of Tabasco and Chiapas, particularly in the region around the city of Teapa [76,77]. Here, the mountains of the Sierra Madre de Chiapas meet the wide floodplains of northern Tabasco, and sulphide springs can be found in four major tributaries of the Rio Grijalva: the Rios Tacotalpa, Puyacatengo, Ixtapangajoya, and Pichucalco (from east to west; Figure 3). In the upper reaches where the sulphidic springs are located, these tributaries are separated by mountains, while they all eventually join the Rio Grijalva and are widely interconnected in the lowlands, at least during the wet season [143]. Since all sulphide springs eventually discharge into non-sulphidic streams and rivers, there are no physical barriers that prevent migration of aquatic organisms between adjacent sulphidic and non-sulphidic environments.

Figure 3. Map of the locations of southern Mexican sulphide springs (yellow arrows) and non-sulphidic reference habitats (blue arrows) used in the case study on patterns of biodiversity in sulphidic habitats. Numbers correspond to IDs in Table 2.
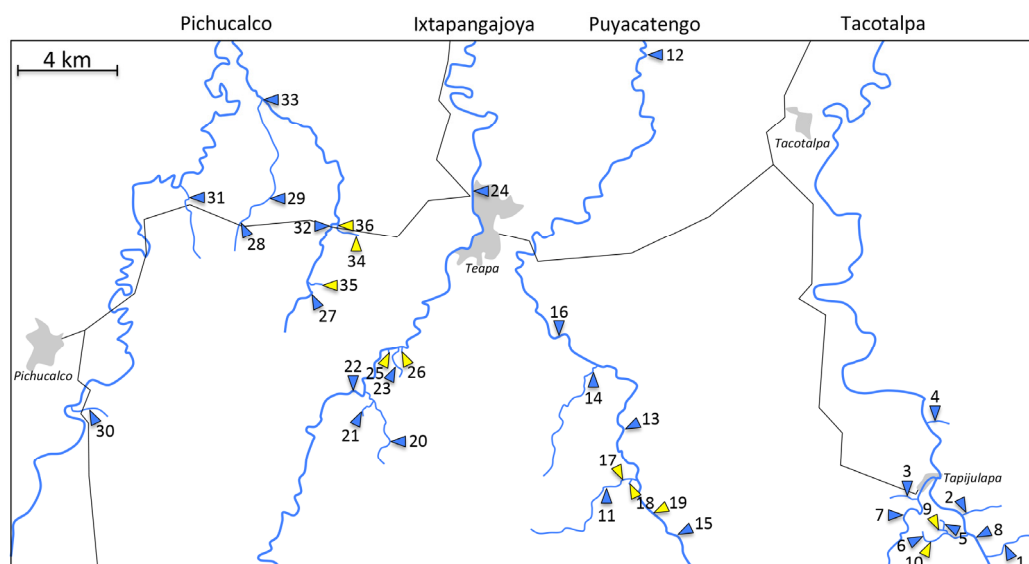
Table 2. Sites investigated for the case study comparing diversity patterns between sulphidic and adjacent, non-sulphidic habitats in southern Mexico. Provided are locality information, summary statistics of fish communities (number of times sampled for qualitative surveys and cumulative number of species recorded) and invertebrate communities (number of times sampled, average number of genera per m^2, and density per m^2).

ID	Location	H₂S	Latitude	Longitude	Times Sampled (fish)	Number of Species (fish)	Times Sampled (Invertebrates)	Number of Genera (Invertebrates)	Density (Invertebrates)
	Tacotalpa drainage								
1	Arroyo Bonita *	-	17.427	−92.752	11	16	1	8.6	3.54×10^5
2	Arroyo Cristal	-	17.451	−92.764	9	15	1	8.3	4.11×10^4
3	Arroyo Tacubaya	-	17.454	−92.785	10	15	2	8.9	1.06×10^5
4	Arroyo Tres	-	17.484	−92.776	9	11	1	9.0	1.59×10^5
5	El Azufre, tributary	-	17.442	−92.775	4	4			
6	El Azufre, upstream	-	17.436	−92.77431	5	6	2	4.1	3.77×10^5
7	Rio Amatán	-	17.449	−92.787	1	12			
8	Rio Oxolotán	-	17.444	−92.763	3	19			
9	El Azufre I*	+	17.442	−92.775	13	1	2	0.4	9.01×10^5
10	El Azufre II	+	17.438	−92.775	7	2	2	1.0	1.01×10^6
	Puyacatengo drainage								
11	Arroyo La Lluvia, upstream *	-	17.461	−92.897	5	8	1	5.2	8.56×10^4
12	Rio Puyacatengo, Miguel Hidalgo	-	17.668	−92.900	1	10			
13	Rio Puyacatengo, road crossing	-	17.470	−92.896	8	12	1	5.2	1.77×10^5
14	Rio Puyacatengo, tributary	-	17.505	−92.908	5	8			
15	Rio Puyacatengo, upstream	-	17.456	−92.888	3	10	1	7.5	1.60×10^5
16	Rio Puyacatengo, Vicente Guerrero	-	17.510	−92.915	8	11			
17	La Lluvia, big spring	+	17.462	−92.896	7	1			
18	La Lluvia, small spring *	+	17.464	−92.895	10	2	1	0.3	4.49×10^5
19	Puyacatengo springs	+	17.458	−92.889	5	1	1	0.8	3.11×10^3
	Ixtapangajoya drainage								
20	Arroyo Chiflon	-	17.476	−92.986	1	3			
21	Arroyo La Joya	-	17.499	−92.993	1	5			

Table 2. *Cont*.

ID	Location	H$_2$S	Latitude	Longitude	Times Sampled (fish)	Number of Species (fish)	Times Sampled (Invertebrates)	Number of Genera (Invertebrates)	Density (Invertebrates)
	Ixtapangajoya drainage								
22	Rio Ixtapangajoya	-	17.495	−92.998	9	7			
23	Rio Ixtapangajoya, tributary	-	17.510	−92.980	3	5			
24	Rio Teapao	-	17.495	−92.997	1	15			
25	La Esperanza, big spring	+	17.511	−92.983	3	1			
26	La Esperanza, small spring	+	17.511	−92.980	2	1			
	Pichucalco drainage								
27	Arroyo Caracol	-	17.534	−93.016	3	7			
28	Arroyo Rafael I	-	17.558	−93.043	6	11	1	9.6	4.4×10^5
29	Arroyo Rafael II	-	17.564	−93.039	1	11			
30	Arroyo Rosita	-	17.485	−93.104	11	15	1	6.6	1.45×10^5
31	Arroyo Santa Ana	-	17.566	−93.064	4	7			
32	Rio El Azufre, west branch *	-	17.556	−93.008	11	13	2	8.0	6.96×10^4
33	Rio Pichucalco	-	17.605	−93.036	9	22			
34	Baños del Azufre *	+	17.552	−92.999	13	2			
35	La Gloria springs	+	17.532	−93.015	10	4			
36	Rio El Azufre, east branch	+	17.557	−93.006	9	2	2	0.4	4.06×10^4

* Sites included in the quantitative comparison of fish assemblages.

H$_2$S in springs of this region stems from bacterial sulphate reduction in aquifers fed by meteoric water [39]. Sulphur and carbon sources to fuel bacterial metabolism are associated with hydrocarbon deposits [39] and potentially volcanic activity [34]. This leads to biologically significant and sustained concentrations of H$_2$S. Depending on the spring, average sulphide concentrations range between 23 and 190 μM, with peak concentrations exceeding 1000 μM [69,76,106]. Spatial variation in sulphide concentrations is relatively pronounced in all springs; concentrations decrease with increasing distance from the springheads due to volatilization [144] and oxidation of sulphide molecules [83,84]. In contrast, temporal variation in both sulphidic and non-sulphidic habitats is minimal by comparison. In addition to the presence of H$_2$S, sulphidic habitats also differ from non-sulphidic habitats in exhibiting higher average temperatures, higher specific conductivities, lower pH, and lower dissolved oxygen concentrations [76].

4.1. Aquatic Macroinvertebrate Communities

To compare patterns of benthic macroinvertebrate diversity between sulphidic and non-sulphidic environments, we sampled sites with ($N = 5$) and without H$_2$S ($N = 11$; Table 2) in the Tacotalpa, Puyacatengo, and Pichucalco drainages. Macroinvertebrates were collected using a Surber sampler (900 cm^2 sampling area, 500 μm mesh size) at three to six locations within each site to represent different microhabitats. Samples were preserved in 80% ethanol, sorted in the laboratory, and identified to the lowest practical taxon (typically genus level) using keys provided by Merritt and Cummins [145] and other taxonomic references for particular groups [146,147]. All sites were sampled in March of 2009 (dry season), and a subset of sites was also sampled in August of 2008 (wet season). If sites were sampled both during the wet and the dry season, all values were averaged across visits. For data analysis, we calculated the number of genera, the Shannon-Wiener diversity index, as well as the density of individuals for each site. We compared these metrics using analysis of covariance (ANCOVA) with total area sampled as the covariate to account for potential effects of sampling effort. Furthermore, we compared Bray-Curtis dissimilarity among site-pairs to estimate the compositional similarity among habitat types.

Overall, we identified 74 different genera of aquatic invertebrates. In addition, we found 14 groups, particularly dipterans, which were not identified to genus level (Table 3; note that these taxa were treated as genera for data analysis). Sulphidic habitats had significantly fewer genera per unit area sampled than non-sulphidic habitats (Figure 4a; $F_{1,16} = 50.765$, $p < 0.001$; estimated marginal means ± s.e.m. from ANCOVA, sulphidic sites: 3.29 ± 3.31; non-sulphidic sites: 31.91 ± 2.22; actual mean ± s.e.m., sulphidic sites: 2.30 ± 0.60; non-sulphidic sites: 32.36 ± 2.86), and there was a non-significant trend of higher richness for sites where larger areas were sampled ($F_{1,16} = 3.917$, $p = 0.069$). The same pattern was evident when comparing Shannon-Wiener diversity indices among sites: sulphidic sites had significantly lower diversity than non-sulphidic ones (Figure 4b; $F_{1,16} = 85.112$, $p < 0.001$). There was no effect of sampling effort on Shannon-Wiener indices ($F_{1,16} = 0.156$, $p = 0.699$). Finally, differentiation of aquatic invertebrate communities between sulphidic and non-sulphidic habitats was evident by comparisons of Bray-Curtis dissimilarities. Dissimilarities were highest when comparing sites from sulphidic and non-sulphidic habitats (mean ± SD: 0.981 ± 0.010), indicating little faunal overlap between these distinct habitat types. In contrast, dissimilarities among sites from sulphidic habitats were relatively low (0.307 ± 0.108). Dissimilarities among non-sulphidic sites were intermediate (0.753 ± 0.166), reflecting pronounced heterogeneity of communities in non-sulphidic streams of the region.

Figure 4. Patterns of macroinvertebrate diversity and density in sulphidic springs and adjacent non-sulphidic reference habitats of Southern Mexico. Bar graphs depict the number of aquatic arthropod genera (**A**); Shannon-Wiener Diversity Index (**B**); and aquatic arthropod densities (**C**) recorded for each site investigated. Non-sulphidic sites are in blue, sulphidic sites are in yellow. Site numbers correspond to IDs in Table 2.
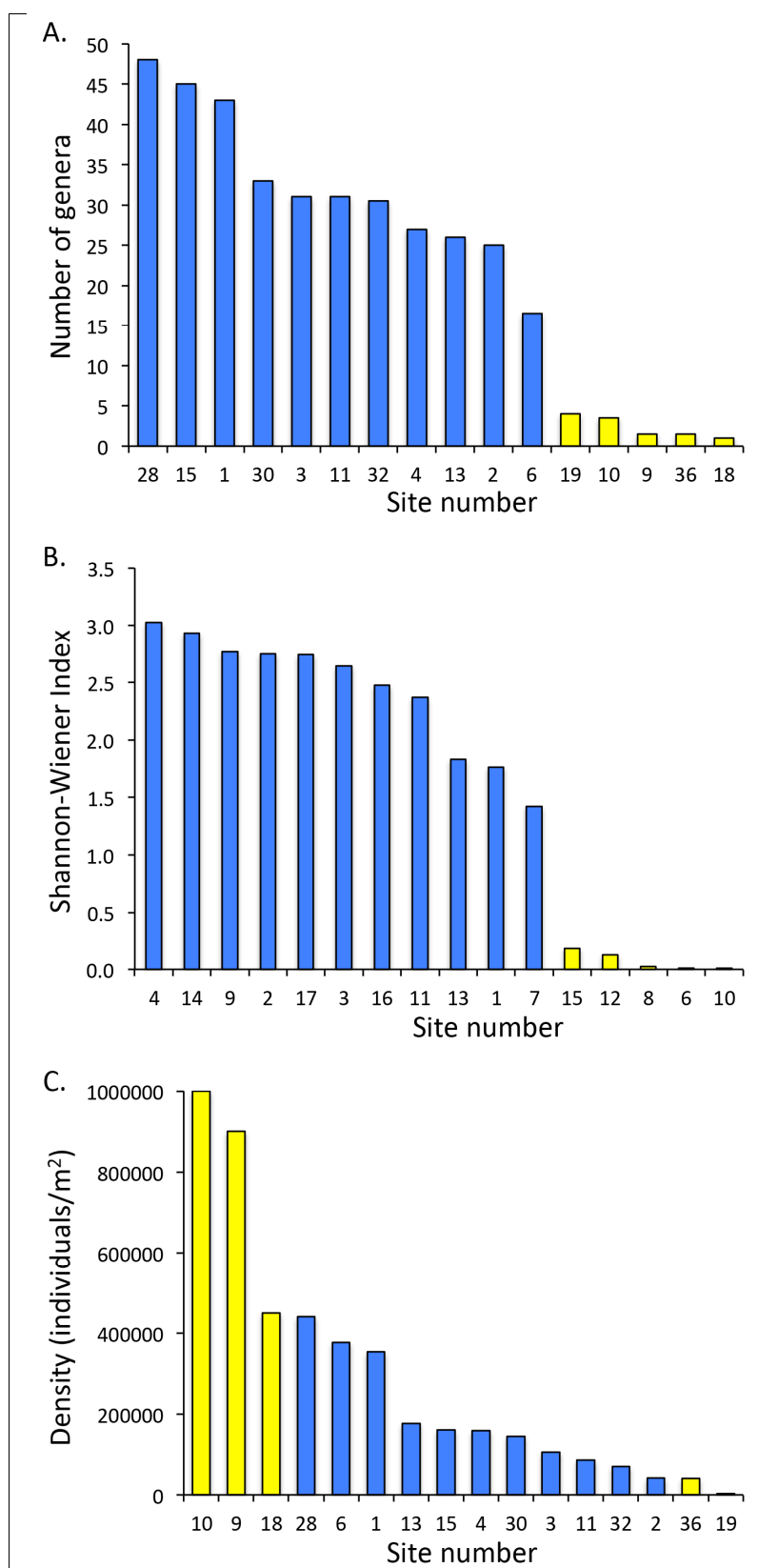
Table 3. Summary of arthropod genera identified in samples from sulphidic and non-sulphidic habitats in three river drainages of Southern Mexico.

	Pichucalco Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic
Ephemeroptera: Baetidae						
Americabaetis	+	-	+	-	+	-
Baetodes	+	-	+	-	+	-
Camelobaetidius	+	-	+	+	+	-
Cloeodes	+	+	+	-	+	-
Fallceon	+	-	+	-	+	+
Paracloeodes	+	-	+	-	-	-
Ephemeroptera: Caenidae						
Caenis	+	-	+	-	+	-
Ephemeroptera: Heptageniidae						
Mccaffertium	+	-	-	-	+	-
Ephemeroptera: Leptohyphidae						
Allenhyphes	+	-	-	-	+	-
Asioplax	+	-	-	-	-	-
Leptohyes	+	-	+	-	+	-
Tricorythodes	+	-	+	-	+	-
Vacupernius	+	-	+	-	+	-
Ephemeroptera: Leptophlebiidae						
Farrodes	+	-	+	-	+	-
Leptophlebia	-	-	+	-	+	-
Thraulodes	+	-	+	-	+	-
Ulmeritus	-	-	-	-	+	-
Odonata: Calopterygidae						
Hetaerina	+	-	-	-	-	-
Odonata: Coenagrionidae						
Argia	+	-	+	-	+	-
Odonata: Gomphidae						
Erpetogomphus	-	-	+	-	+	-
Odonata: Libellulidae						
Brechmorhoga	+	-	+	-	+	-
Macrothemis	-	-	-	-	+	-
Odonata: Platystictidae						
Palaemnema	+	-	+	-	+	-
Plecoptera: Perlidae						
Anacroneuria	+	-	+	-	+	-
Hemiptera: Belostomatidae						
Belostoma	-	-	-	-	+	+
Hemiptera: Gerridae						
Trepobates	-	-	+	-	-	-
Hemiptera: Naucoridae						
Ambrysus	+	-	+	-	-	-
Cryphocricos	+	-	+	-	+	-
Limnocoris	+	-	+	-	+	-

Table 3. *Cont.*

	Pichucalco Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	**Sulphidic**	**Non-Sulphidic**	**Sulphidic**	**Non-Sulphidic**	**Sulphidic**
Hemiptera: Veliidae						
Rhagovelia	+	-	+	-	+	-
Neuroptera: Corydalidae						
Corydalus	+	-	+	-	+	-
Coleoptera: Curculionidae						
Unidentified genus	-	-	+	-	-	-
Coleoptera: Elmidae						
Austrolimnius	+	-	-	-	+	-
Heterelmis	+	-	+	-	+	-
Hexacylloepus	+	-	+	-	+	-
Macrelmis	+	-	+	-	+	-
Microcylloepus	+	-	+	-	+	-
Neocylloepus	+	-	-	-	-	-
Neoelmis	+	-	+	-	+	-
Phanocerus	-	-	-	-	+	-
Stenelmis	+	-	-	-	+	-
Coleoptera: Hydrophilidae						
Tropisternus	-	-	-	+	-	-
Coleoptera: Lutrochidae						
Lutrochus	-	-	+	-	-	-
Coleoptera: Psephenidae						
Psephenus	+	-	+	-	+	-
Coleoptera: Ptilodactylidae						
Anchytarsus	+	-	-	-	-	-
Trichoptera: Calamoceratidae						
Phylloicus	-	-	+	-	-	-
Trichoptera: Glossomatidae						
Protoptila	-	-	+	-	+	-
Trichoptera: Heliocopsychidae						
Heliocopsyche	-	-	+	-	+	-
Trichoptera: Hydropsychidae						
Cheumatopsyche	+	-	-	-	-	-
Rhyacophylax	+	-	+	-	-	-
Smicridea	+	-	+	-	+	+
Trichoptera: Hydroptilidae						
Hydroptila	+	-	+	-	+	-
Leucotrichia	+	-	+	-	+	-
Mayatrichia	-	-	-	-	+	-
Neotrichia	+	-	+	-	+	-
Oxytheria	+	-	-	-	-	-
Trichoptera: Leptoceridae						
Nectopsyche	+	-	+	-	-	-
Oecetis	+	-	+	-	+	-

Table 3. *Cont.*

	Pichucalco Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic
Trichoptera: Philopotamidae						
Chimarra	-	-	-	-	+	-
Wormaldia	+	-	-	-	+	-
Unidentified genus	-	-	-	-	+	-
Trichoptera: Polycentropodidae						
Cernotina	+	-	-	-	-	-
Cyrnellus	-	-	-	-	+	-
Polyplectropus	+	-	+	-	+	-
Trichoptera: Xiphocentronidae						
Xiphocentron	+	-	+	-	+	-
Lepidoptera: Unidentified family						
Unidentified genus	+	-	+	-	+	-
Diptera: Athericidae						
Atherix	-	-	-	-	+	-
Unidentified genus	+	-	-	-	+	-
Diptera: Ceratopogonidae						
Atrichopogon	-	-	-	-	+	+
Ceratopogon	+	-	-	-	+	-
Culicoides	+	-	-	+	+	-
Forcipomyia	-	-	-	-	+	+
Probezzia	+	-	+	-	+	-
Diptera: Chironomidae						
Unidentified genus (Chiromini)	+	+	+	+	+	+
Unidentified genus (Orthocladinae)	+	-	+	-	+	-
Unidentified genus (Pseudochironomini)	+	-	+	-	-	-
Unidentified genus (Tanypodinae)	+	-	+	-	+	-
Unidentified genus (Tanytarsini)	+	-	+	-	+	-
Diptera: Empididae						
Hemerodromia	+	-	+	-	+	-
Diptera: Ephyridae						
Unidentified genus	-	-	-	+	-	-
Diptera: Psychodidae						
Maruina	+	-	-	-	-	-
Diptera: Simulidae						
Simulium	+	-	-	-	+	-
Diptera: Stratiomyiidae						
Nemotelus	-	-	-	-	+	-
Diptera: Tabanidae						
Unidentified genus	-	-	-	-	+	-
Diptera: Tipulidae						
Unidentified genus (Limoniinae)	+	-	+	-	+	-
Unidentified genus (Tipulinae)	+	-	-	-	+	-

Table 3. *Cont.*

	Pichucalco Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic
Crustacea: Ostracoda						
Unidentified genus	+	-	-	-	-	-
Crustacea: Palaemonidae						
Macrobrachium	-	-	-	-	+	-

Similar to benthic invertebrate communities in other areas of Central America [148–150], communities in non-sulphidic habitats were dominated by Ephemeroptera, Coleoptera (particularly the family Elmidae), Trichoptera, and Diptera (particularly the family Chironomidae). In contrast to other studies [151], however, shrimps and crabs were relatively scarce in our samples. This is likely an artefact of our sampling technique, as benthic sampling generally underestimates the abundance of decapods [152], and shrimp of the genus *Macrobrachium* (particularly *M. carcinus*), *Atya scabra*, and crabs of the genus *Avotrichodactylus* can regularly be collected in non-sulphidic habitats using seines and baited traps (Tobler, personal observation).

Macroinvertebrate communities are substantially less diverse in sulphidic springs than in other habitats. The impoverished invertebrate communities in sulphidic habitats were dominated by dipterans (especially chironomids; >98% of specimens collected in all sulphidic habitats). Dipterans also represented the only group that was consistently present in all sulphidic habitats examined, which likely explains the low Bray-Curtis dissimilarities among sites from sulphidic habitats. Chironomids are known for their hypoxia tolerance [153,154], and while there is much variation among different taxa, particularly species of the tribe Chironomini are able to withstand adverse environmental conditions, including anthropogenically altered habitats [155–157]. It is currently unclear whether chironomids generally have an increased tolerance to the presence of H_2S. Some studies found survival and high recovery rates of chironomids after temporal exposure to sulphide [158], while others documented avoidance behaviour and high sulphide-induced mortality [159]. Detailed studies, including species level identification of specimens from different habitats and sulphide tolerance tests, will need to corroborate whether chironomids in sulphidic springs represent locally adapted populations or species, or whether the group generally has high tolerance levels that allow them to thrive in the toxic and hypoxic sulphidic springs.

Among the few other groups that were occasionally recorded in sulphidic habitats were the genera *Cloeodes* and *Fallceon* (Baetidae, Ephemeroptera) in the Tacotalpa and the Pichucalco drainages, respectively, the genera *Smicridea* (Hydropsychidae, Trichoptera) and *Tropisternus* (Hydrophilidae, Coleoptera) in the Puyacatengo drainage, as well as the genus *Belostoma* (Belostomatidae, Hemiptera) in the Tacotalpa drainage. Although not represented in our quantitative sampling, crabs of the genus *Avotrichodactylus* have also been reported from sulphidic springs in the Pichucalco drainage (Tobler, personal observation) and from a sulphidic cave in the Tacotalpa drainage (*A. bidens* [160]). For the most part, there is little known about potential adaptations to H_2S-rich environments of these groups. Ephemeropterans and trichopterans occur in wide variety of freshwater environments but are most diverse in lotic habitats [112,161]. They acquire dissolved oxygen through abdominal gills and are generally considered indicators for good water quality because of their low to moderate tolerance to

disturbance and pollution [162,163]. The few species investigated to date show imminently low tolerance to H$_2$S exposure [164,165]. Nonetheless, tolerance to physico-chemical stressors can vary widely among genera and species of these families [166–168], and it remains to be tested whether members of the genera *Cloeodes*, *Fallceon*, and *Smicridea* generally have an increased tolerance to environmental stressors or whether they exhibit specific adaptations to sulphidic habitats. Hydrophilid scavenger beetles are mostly found in stagnant and slow moving waters [112,145]. Accordingly, they are adapted to low dissolved oxygen concentrations, and *Tropisternus*—like most other hydrophilids—have the ability to breathe air. Larvae use paired spiracles at the end of abdomen to acquire atmospheric oxygen, while adults collect air with modified antennae and extract oxygen from an air bubble stored at the underside of the body (plastron respiration) [169–171]. Tolerance to environmental stressors varies not only within the family, but also within the genus *Tropisternus* [172]. To our knowledge nothing is known about H$_2$S tolerance in these beetles. Finally, most information is available for giant water bugs of the genus *Belostoma* that occur in sulphidic surface and cave habitats of the Tacotalpa drainage. Waterbugs have been documented to be able to complete their entire life cycle in sulphidic waters [173], where they act as apex predators foraging on fishes [102,174,175]. Their ability to breathe air through respiratory siphons—rather than acquiring dissolved oxygen through gills—may reduce their exposure to toxic H$_2$S [111].

While patterns of macroinvertebrate diversity were highly consistent across sulphide springs from different drainages, their density varied tremendously among sulphide spring sites. Although densities were overall slightly lower in non-sulphidic habitats, there was no significant difference among habitat types ($F_{1,16} = 3.331$, $p = 0.091$; note there was no effect of total area sampled: $F_{1,16} = 0.008$, $p = 0.931$). In fact, sulphidic habitats included sites with the highest and the lowest invertebrate densities (Figure 4c). High densities were particularly present in springs of the Tacotalpa drainage and the small La Lluvia spring in the Puyacatengo. These sites share low to moderate H$_2$S concentrations (multiple year averages between 23 and 129 μM H$_2$S [76]) and continuously flowing water that potentially mitigates the reduction in oxygen concentration through sulphide oxidation. Extremely low densities were recorded particularly in the large Puyacatengo springs, which are characterised by high sulphide concentrations (peak concentrations >500 μM), stagnant water, and higher temperatures (>33 °C [76]). More thorough spatial sampling will be required to elucidate factors driving chironomid densities, but the current results may indicate that these dipterans thrive in sites with moderate sulphide concentrations, where competition with other invertebrates and predation by fish is likely reduced compared to non-sulphidic environments, but avoid more harsh sites potentially because of limited capabilities of sulphide detoxification.

4.2. Fish Communities

To compare patterns of fish diversity between sulphidic and non-sulphidic environments, we took a two pronged approach: (1) In a qualitative approach, we used presence and absence data of fish species across a broad sample of sulphidic and non-sulphidic sites (most of which were visited repeatedly over the course of multiple years) to compare species richness between sulphidic and non-sulphidic sites in the Tacotalpa, Puyacatengo, Ixtapangajoya, and Pichucalco drainages; (2) For a subset of sites, we also

used standardised sampling techniques to compare fish species richness and density patterns between sulphidic and non-sulphidic habitats.

For the qualitative analysis, we compared the total number of fish species collected at sites with ($N = 10$) and without H$_2$S ($N = 26$; see Table 2). Sites were visited between 1 and 13 times between October 2004 and June 2013. At each visit, fish were caught by seine, identified on site using keys provided by Miller *et al.* [176], and subsequently released at the collection site. Since sampling efforts were not consistent among sites and visits and sampling occurred in different seasons, we counted the number of fish species that were recorded over the years at each site and compared this number across sulphidic and non-sulphidic sites using ANCOVA. Since the number of species recorded should be increasing with each sampling visit, we included the number of visits as a covariate. A subset of sites (Table 2) was also sampled quantitatively in June of 2013 by seining riffle-pool sequences along three 20-meter transects. The area sampled was measured for each seine haul. The number of species present and fish density (individuals/m^2) were used as dependent variables in ANCOVA; sample area (m^2), presence of H$_2$S, river drainage, and the H$_2$S by drainage interaction served as independent variables.

Overall, we identified 39 different fish species during our qualitative sampling across years (Table 4). When comparing the species richness recorded for each site, the number of visits had a significant effect ($F_{1,36} = 6.644$, $p = 0.015$). Nonetheless, even when controlling for the number of visits, sulphidic habitats harboured significantly fewer species than non-sulphidic ones (Figure 5a; *ew* corroborated these results. ANCOVA results indicated a significant H$_2$S by drainage interaction ($F_{2,10} = 21.145$, $p < 0.001$); sulphidic sites consistently had a lower number of species present, but the magnitude of difference between sulphidic and non-sulphidic habitats varied between drainages (Figure 5b). The area sampled did not have a significant effect on the number of species present ($F_{1,10} = 3.309$, $p = 0.099$).

Non-sulphidic habitats in Southern Mexico were generally characterised by harbouring diverse communities composed of species from different orders and families (Table 4). Fish communities in non-sulphidic waters were dominated by cichlid and poeciliid species, as typical for the wider geographic region [143,176]. Comparatively low species diversity in a non-sulphidic habitat was only documented in Arroyo Chiflon, a small, high gradient stream with fast currents, harbouring only three species (*Astyanax aeneus*, *Pseudoxiphophorus bimaculatus*, and *Vieja intermedia*) typical for low order streams. In contrast to the majority of non-sulphidic habitats, sulphidic springs were characterised by reduced species richness (between one and four species depending on the spring complex). It seems noteworthy that the changes in communities are very abrupt at ecotones between sulphidic and non-sulphidic habitats even in the absence of physical barriers for fish dispersion. This is highlighted in the different number of species occurring at adjacent sites that differ in the presence or absence of H$_2$S, but are separated by <100 m (e.g., the different branches of the Rio El Azufre in the Pichucalco drainage, and La Lluvia big spring and the upstream site of Arroyo La Lluvia in the Puyacatengo drainage).

Table 4. Summary of fish species identified in qualitative samples from sulphidic and non-sulphidic habitats of four river drainages between 2004 and 2013. Differences in species composition among drainages primarily reflect patterns of endemism (e.g., different *Priapella* species with allopatric distributions occur in the Tacotalpa and the Pichucalco drainages) and differences in habitat characteristics. For example, non-sulphidic habitats in the Tacotalpa and Puyacatengo drainages primarily included small streams and swiftly flowing piedmont rivers that are characterised by species like *Heterophallus milleri*, *Priapella chamulae*, *Paraneetroplus gibbiceps*, and *Theraps lentiginosus*. In contrast, habitats in the Pichucalco drainage also included sluggish lowland rivers, where species like *Hyphessobrycon compressa*, *Belonesox belizanus*, *Phallichthys fairweatheri*, *Astatheros robertsoni*, and *Petenia splendida* occur.

	Pichucalco Drainage		Ixtapangajoya Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic
Characidae								
Astyanax aeneus	+	-	+	-	+	-	+	-
Brycon guatemalensis	-	-	-	-	+	-	-	-
Hyphessobrycon compressa	+	-	-	-	-	-	-	-
Ariidae								
Potamarius nelsoni	-	-	-	-	-	-	+	-
Pimelodidae								
Rhamdia guatemalensis	+	-	+	-	+	-	+	-
Rhamdia laticauda	-	-	+	-	+	-	-	-
Loricariidae								
Pterygoplichthys pardalis*	+	-	+	-	+	-	+	-
Batrachoidae								
Batrachoides goldmani	-	-	-	-	+	-	+	-
Atherinopsidae								
Atherinella alvarezi	+	-	+	-	+	-	+	-
Belonidae								
Strongylura hubbsi	+	-	-	-	+	-	+	-
Poeciliidae								
Belonesox belizanus	+	-	-	-	-	-	-	-
Carlhubbsia kidderi	+	-	-	-	-	-	-	-
Gambusia eurystoma	-	+	-	-	-	-	-	-
Gambusia sexradiata	+	-	-	-	+	-	-	-
Heterandria bimaculata	+	+	+	-	+	-	+	-
Heterophallus milleri	+	-	+	-	-	-	+	-
Phallichthys fairweatheri	+	-	-	-	-	-	-	-
Poecilia mexicana	+	-	+	-	+	+	+	+
Poecilia petenense	+	-	-	-	-	-	-	-
Poecilia sulphuraria	-	+	-	-	-	-	-	-
Poecilia thermalis	-	-	-	+	-	-	-	-
Priapella chamulae	-	-	-	-	-	-	+	-
Priapella compressa	+	-	-	-	-	-	-	-
Xiphophorus hellerii	+	+	+	-	+	-	+	-

* Introduced species.

Table 4. *Cont.*

	Pichucalco Drainage		Ixtapangajoya Drainage		Puyacatengo Drainage		Tacotalpa Drainage	
	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic	Non-Sulphidic	Sulphidic
Synbranchidae								
Ophisternon aenigmaticum	-	+	+	-	-	-	+	+
Cichlidae								
Astatheros robertsoni	+	-	-	-	-	-	-	-
"Cichlasoma" urophthalmus	+	-	-	-	-	-	-	-
"Cichlasoma" salvini	+	-	+	-	+	-	+	-
Oreochromis cf. aureus *	-	-	+	-	+	-	+	-
Parachromis managuensis *	+	-	-	-	-	-	-	-
Paraneetroplus gibbiceps	-	-	-	-	+	-	+	-
Petenia splendida	+	-	-	-	-	-	-	-
Rocio octofasciata	+	-	-	-	-	-	-	-
Theraps lentiginosus	+	-	+	-	+	-	+	-
Thorichthys helleri	+	-	+	-	+	-	+	-
Thorichthys meeki	+	-	-	-	+	-	-	-
Vieja bifasciata	+	-	+	-	+	-	+	-
Vieja intermedia	-	-	+	-	+	-	+	-
Eleotridae								
Gobiomorus dormitor	-	-	-	-	-	-	+	-

* Introduced species.

Figure 5. Patterns of fish diversity and density in sulphidic springs and adjacent non-sulphidic reference habitats of Southern Mexico. (**A**) Bar graph depicting the number of species collected at each site during the multiple-year, qualitative surveys; (**B**) Estimated marginal means (± s.e.m) for the number of species per site from quantitative surveys; (**C**) Estimated marginal means (± s.e.m) for the density of individuals per site from quantitative surveys. Estimated marginal means were derived from ANCOVA models as described in the main text. Non-sulphidic sites are in blue, sulphidic sites are in yellow. Site numbers correspond to IDs in Table 2.
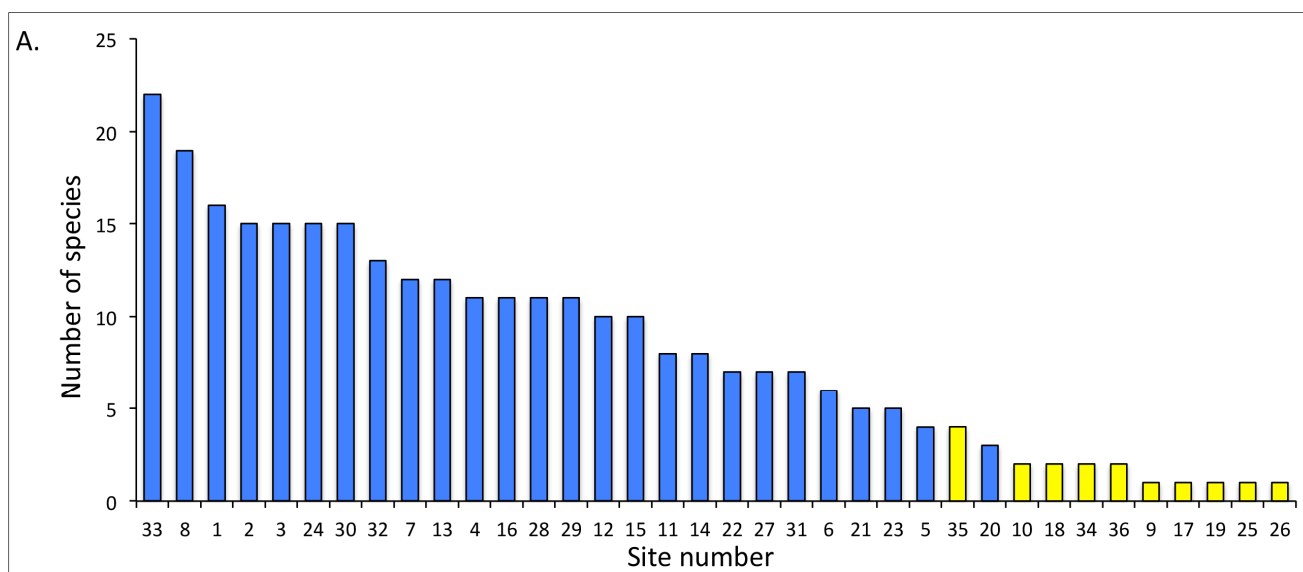
Figure 5. *Cont.*
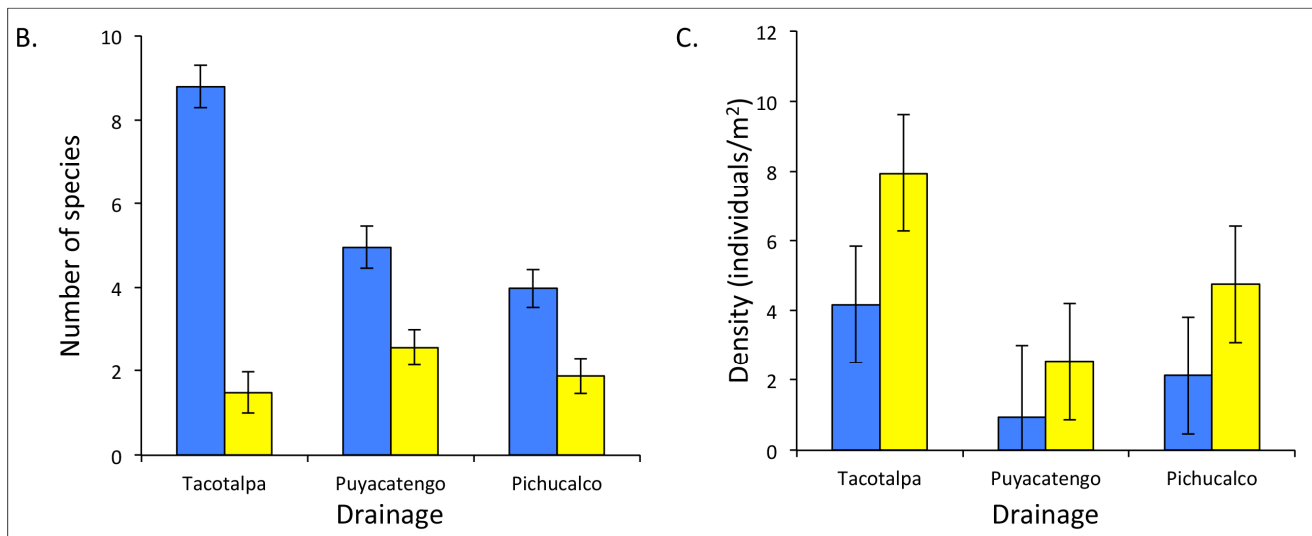
Species of the genus *Poecilia* were collected in all sulphide springs investigated; nominally, this included sulphidic ecotypes of *Poecilia mexicana* in the springs of the Tacotalpa and Puyacatengo drainages, *P. thermalis* in the Ixtapangajoya (endemic to the two La Esperanza springs), and *P. sulphuraria* in the Pichucalco (endemic to the Baños del Azufre and its outflows, as well as the La Gloria springs; see [77] for a discussion of the convoluted taxonomy of this group). Sulphidic ecotypes in all drainages are reproductively isolated, genetically differentiated, and differ from conspecifics from adjacent non-sulphidic habitats in a series of phenotypic traits, including physiological, morphological, behavioural, and life history characteristics (see Sections 3.2.2. and 3.2.3.).

In conjunction with resident *Poecilia*, some sulphidic habitats were inhabited by additional poeciliid species. The sulphide spring endemic *Gambusia eurystoma* was present in the Baños del Azufre and its outflows (Pichucalco), and it represents a sulphide-adapted form closely related to *G. sexradiata* [67–69,71]. Furthermore, *Xiphophorus hellerii* occurred in the small La Lluvia (Puyacatengo) and the La Gloria springs (Pichucalco), and *Pseudoxiphophorus bimaculatus* in the La Gloria springs. Whether these species occur predominantly in microhabitats with low concentrations of H_2S or whether they actually exhibit similar adaptations as the *Poecilia* inhabiting sulphide springs remains to be investigated in detail; although preliminary data indicates that at least *X. hellerii* from the La Gloria springs show similar trait divergence from non-sulphidic ancestors as observed in *Poecilia* [177]. Finally, synbranchid eels were occasionally collected in the El Azufre II (Tacotalpa) and La Gloria springs. In contrast to variation in species richness, fish densities did not change starkly among drainages and habitat types, although there was a non-significant trend towards higher densities in sulphidic habitats ($F_{1,11} = 3.521$, $p = 0.087$; Figure 5c).

5. Synthesis and Open Questions

Freshwater springs with high concentrations of hydrogen sulphide occur around the globe; yet, little remains known about the faunas inhabiting these extreme environments. Based upon–mostly qualitative and often observational–data available to date, two critical patterns emerge. Firstly, sulphide spring habitats are characterised by low levels of diversity compared to adjacent non-sulphidic habitats,

which was particularly evident in the case study focusing on macroinvertebrate and fish diversity patterns in Southern Mexico. The reduction of species richness in sulphidic habitats is likely directly related to the toxic properties of H_2S [16,69], although correlated environmental factors (such as the rampant hypoxia, high ionic strength, and sometimes high temperatures) may complement and exacerbate the adverse physiological effects of sulphide [178,179]. Dipterans (among aquatic macroinvertebrates) and cyprinodontiforms (among fishes) appear to be the dominant groups in the sulphide spring faunas investigated to date. Interestingly, the majority of invertebrate and fish taxa present in sulphide springs environments are able rely on alternative respiratory strategies (other than acquisition of dissolved oxygen through gills), which accordingly could represent a critical exaptation for the colonization of these extreme environments.

Secondly, at least in fishes, sulphide spring habitats are characterised by high levels of endemism, harbouring species or populations with specific adaptations to cope with the extreme environmental conditions. It remains unclear whether similar patterns of endemism are present in invertebrates documented from sulphide springs, either because comparisons between sulphidic and adjacent non-sulphidic habitats do not have adequate taxonomic resolution (most taxa were identified to genus level only), or because there have been no thorough comparative analyses between populations in sulphide springs with closely related forms in non-sulphidic environments.

Overall, biodiversity patterns in sulphide springs appear to be similar to other marginal freshwater habitats [7–12]. But first and foremost, this review has highlighted the dearth of our current knowledge about freshwater sulphide spring faunas, and there are some obvious questions that need to be addressed in the future: (1) Considering the abundance of sulphide springs worldwide and the attention many springs have received from microbiologists, quantitative studies on metazoan biodiversity are almost entirely lacking. Consequently, rigorous surveys in a variety of springs worldwide are needed to corroborate the trends of reduced species diversity and the presence of highly endemic species and populations at larger spatial scales; (2) The inhospitable conditions in sulphide springs warrant studies investigating what traits predispose certain taxonomic groups (such as dipterans and cyprinodontiforms) to colonize sulphide springs, and how colonizers subsequently adapt over evolutionary times to cope with sulphide toxicity. Such studies are requisite to test for potential convergent evolution particularly of physiological coping mechanisms that may have evolved in similar fashions across broad taxonomic groups. Due to the increasing interest in the role of sulphur metabolism and processing in disease formation, sulphide spring inhabitants with modifications in relevant physiological pathways may also provide critical insights for biomedical applications [180,181]; (3) The presence of endemic species and divergent populations exclusively inhabiting sulphide springs raises questions about whether and how speciation processes are linked to the continuous presence of environmental H_2S. While there is increasing evidence for ecological speciation in sulphidic spring fishes, comparable processes remain to be investigated in other taxonomic groups; (4) The uniqueness of sulphide spring faunas raise potential concerns for conservation, as many populations clearly represent unique evolutionary lineages with highly restricted distributions meriting separate management and a high priority for protection [181]. Among all the sulphide endemics documented to date, only *Gambusia eurystoma* [182] and *Poecilia sulphuraria* [184] have been assessed; both species are listed as "critically endangered" by the IUCN [185] and are federally protected in Mexico [186]. Our own fieldwork in North America, the Caribbean, and the Neotropics, however, has indicated that many–if not most–sulphide springs are

disturbed or developed through a variety of mechanisms, including deforestation, farming and agriculture, as well recreational activities (*i.e.*, the conversion of spring heads into swimming pools). Ultimately, the unique ecological setting of sulphide springs and the evolutionary uniqueness of their inhabitants make them valuable study systems in basic and applied biological sciences. These springs provide readily accessible models to understand how some organisms challenge the boundaries of what most others can tolerate and to investigate life's capacities and limitations to deal with far from average conditions [13].

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Author Contributions

Ryan Greenway, Lenin Arias-Rodriguez, and MT Michael Tobler conducted the fieldwork, analysed the data, and wrote the paper. Pete Diaz identified invertebrate samples.

Conflicts of Interest

The authors declare no conflict of interest.

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