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Anomalies of Sponge Spicules: Exploring Links to Environmental Pollution

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Abstract: The aim of this study was to assess the frequency of spicule malformations in freshwater sponges in relation to selected environmental parameters of the streams and the presence of river pollutants. A total of 50 sponge samples were collected from ten rivers in Serbia. Selected parameters of the water varied considerably at every site where sponges were found. After spicule preparation, the samples were subjected to morphological analysis by light and scanning electron microscopy, and the number of anomalies were recorded (spicules with bulbous enlargements, sharply bent, bifurcated, scissor- and cross-like, and t-shaped). The frequencies and types of malformations within the analyzed specimens varied from 1 to 100 per 1000 spicules, with an average number of 12 per 1000. The main types of anomalies were single- and double-bent spicules. The highest number of anomalies was found in a specimen of *Eunapius fragilis* collected at Markovac (Velika Morava River), and the lowest number was found in a specimen of *Ephydatia fluviatilis* from Kanjiža (Tisa River). The sites with the lowest and the highest numbers of anomalies showed statistically significant differences in concentrations of ammonia, orthophosphates, sodium, chloride, manganese, and lead. This study indicates that several pollutants potentially affect the occurrence of spicule anomalies.

Keywords: Spongillidae; sponges; freshwater; spicule; anomalies; malformations; pollution; heavy metals; environmental parameters

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1. Introduction

Freshwater sponges are a relatively neglected group of macroinvertebrates, and yet, they are omnipresent. Sponges are predominantly marine, with about 240 species, grouped in 48 genera and 6 families, and inhabit freshwater ecosystems all over the world [1]. Freshwater sponges (Phylum Porifera, family Spongillidae) are sessile organisms attached to hard substrates of all kinds, and similarly to their marine relatives, they produce many bioactive compounds. Freshwater sponges are widespread and often a common component of many ecosystems. They can be found in rivers, streams, springs, rapids, estuaries, lakes, caves, ponds, anthropogenic reservoirs, pools, etc. [1]. They are mainly attached to a solid substrate, which includes stones, rocks, submerged branches, roots, and vegetative organs of various aquatic plants. They can often be found on mollusk shells, as well as on live shellfish or snails. They also show great affinity for various types of anthropogenic substrates submerged in water, such as glass, cement, plastic, and metal objects [1]. Artificial bank fortifications are especially suitable for the colonization of sponges. Even so, their distribution is fairly irregular. In some habitats, they are sporadic, with very little share in the total biomass, while in others, they are very abundant [2–4].

Sponges spend their full vegetative life cycle in one place, exposed to the conditions of the water body they inhabit. Moreover, they are filter feeders with substantial filtering potential (often amounting to hundreds of liters per hour) and consequently, with a role in processes of water purification. This makes them a vital element of aquatic ecosystems and a potentially suitable water quality bioindicator. Freshwater sponges show a propensity towards calmer and cleaner waters, in which there is less possibility of silting up of the pores and obstruction of breathing and nutrition. However, they are often found in polluted waters as well, for example near industrial plant discharges or waste water collectors, where there is a higher level of organic input [5].

Freshwater sponges are frequently found in symbiosis with green algae, which affect host gemmule germination rates, enhance its growth rate due to net gain from photosynthesis, and provide fixed carbon for metabolism [6]. Due to their low nutritional value and high mineral content, sponges do not have many predators. However, some organisms do feed on them. Rare sponge predators include ducks [7], crayfish [8], some aquatic insects, and snails [9,10].

Aquatic ecosystems of southeast Europe have been poorly investigated in terms of freshwater sponge presence and their precise distribution. Though they represent an important component in many aquatic ecosystems, they are often overlooked, as they are rarely collected during standard sampling procedures. Previous investigations of Serbian waters revealed mainly *Spongilla lacustris* (Linnaeus, 1759) species, sporadically found in the benthic macroinvertebrate communities of large rivers (Globaqua, JDS3 report). Recent studies on freshwater sponges of the western Balkans and some parts of the Pannonian Plain by Andjus et al. [11,12] confirmed the presence of five species in different water bodies.

In recent years, data regarding Spongillidae morphology, distribution, phylogeny, etc. are increasing. Spicules, as structural elements of the skeleton of most sponges, have been the subject of numerous research efforts. Interestingly, one aspect of freshwater sponge biology remains rather uninvestigated. Namely, different studies have mentioned the existence of spicule malformations, yet very few have elaborated on this topic. Earlier studies have shown that environmental factors have a fundamental impact on Spongillidae spicule size and shape [13,14]. Among the most important factors in the synthesis of these structural elements are silica concentration, water temperature, and depth [15–19]. The importance of these parameters has been assessed both in field and in laboratory experiments. Lowering concentrations of silica in experimental conditions inhibits spicule synthesis. Also, most continental freshwater sponges prefer summer temperatures when they grow, while they are receding, and in dormant gemmule form during cold periods [20]. The toxicity of copper and zinc rises with water "softness" (lower mineral content), contributing to altered spiculogenesis as well [21]. For other benthic organisms, particularly diatoms, numerous studies have shown a connection between morphological changes and environmental stressors, mostly trace metals and multiple stressors [22,23]. It was noted that some of the genera from this algae group tend to develop deviations from the normal shape or ornamentations of the cell wall, which reflect sub-lethal responses to contaminants [24].

Although molecular genetics offers great possibilities in the sponge identification process, morphological analysis of the sponge mineral skeleton remains an unavoidable tool in Porifera taxonomy. However, it is not rare that ecomorphs are mistakenly registered as a new species due to aberrant spicules, and thus, additional information on sponge skeletal malformations is needed. The aims of the present study were to describe and quantify the most frequently encountered spicule anomalies in different sponge species collected in Serbian rivers and, if possible, to relate their presence to the streams' physico-chemical characteristics and the pollutants' presence.

2. Materials and Methods

2.1. Study Area

For the present study, ten rivers of the Danube River Basin in Serbia were investigated as major representatives of the basin: Velika Morava, Zapadna Morava, Južna Morava, Tisa, Kolubara, Porečka River, Mlava, Beli Timok, Crni Timok, and Nišava. On average, three to five localities in the upper, middle, and lower sections of the rivers were inspected in the period from August–November 2017 (Figure 1).

Figure 1. Map of localities searched during the study.

2.2. Sponge Sampling

Wadeable rivers were searched in their entire width, and the larger ones were in the coastal zone. They were examined on selected localities within 100 m stretches of wadeable areas and at depths usually between 0 to 1.5 m. The main sponge substrates were medium-sized rocks, wood debris, and submerged tree roots, but some specimens were also found attached to barrels and concrete underwater constructions, like piers. A total of 50 sponge specimens were collected and kept in 96% ethanol until spicule preparation.

2.3. Analysis of Selected Environmental Parameters of the Rivers

Physico-chemical parameters considered relevant for sponge ecology based on literature data [25] were included in the statistical analysis: water temperature, pH, electrical conductivity, suspended solids, dissolved oxygen, oxygen saturation, total water hardness, dissolved carbon dioxide, bicarbonates, total dissolved salts, silicates, and calcium. Temperature, pH, conductivity, and dissolved oxygen were measured in situ, using the WTW probe model Multi 3630 IDS SET G.

For other selected parameters, such as heavy metal concentrations, nitrates, etc., data were obtained from the Agency for Environmental Protection of Serbia [26]. The Environmental Protection Agency measures a vast number of physical and chemical parameters on a monthly basis, and average values for the period of sponge collection were calculated from the available data.

2.4. Spicule Preparation and Light Microscopy

The nitric acid technique, as described by Manconi and Pronzato [27], was used to dissolve sponge tissue and prepare spicules for light microscope analysis. Sponge fragments of approximately 2–5 mm were washed with ethanol, dried, and put into glass tubes. Then, they were covered with 2–5 mL of concentrated nitric acid and left to decompose for 24 h. The acid was removed with a pipette, and the spicule pellet was washed repeatedly with distilled water. Finally, the spicules were rinsed with and re-suspended in 96% ethanol. A drop of suspension was placed on a cover slip, and after the alcohol dried, the cover slip was placed over the microscope slides with a drop of Canada balsam. Slides were analyzed under magnifications of 20× and 40× on a light microscope (model ZEISS AXIO Lab.A1), and spicule malformations were scored. Spicule sizes were measured as well.

2.5. Quantification and Classification of Spicule Malformations

For the quantification of anomalies, light microscopy was used, and all spicules without apparent malformations and within normal size ranges [28] in five randomly chosen fields of view (FOV) were counted. Then, the average number of spicules per FOV was multiplied by the total number of FOV on the microscopic slide. The anomalies were counted on the entire slide, and the results were expressed in the number of anomalies per thousand spicules (1×10^3). All detected anomalies were sorted into main groups and named. The classification of known types of anomalies was proposed (Section 3.4).

2.6. Scanning Electron Microscopy

To analyze the sponge spicules more closely, specimens were prepared for SEM analysis. Drops of spicule suspension in ethanol were placed on specimen holders and coated with gold in a gold sputter at 18 mA for 1 min. Elements of the mineral skeleton were analyzed and photographed in a VEGA TS 5133MM Scanning Electron Microscope (SEM) in high-vacuum mode using the SE detector with accelerating voltage. Measurements of spicules (widths and lengths) were taken, and a more accurate observation of anomalies was performed.

2.7. Statistical Analysis

Differences in selected parameters between the rivers containing sponges were analyzed. A comparison was made between the measured values of parameters at sites with the highest number of sponge spicule anomalies and values at sites with the lowest number of anomalies. Statistical analyses were performed using IBM SPSS Statistics for Windows Software (Version 22.0; IBM Corp, Armonk, NY, USA). The statistical significance between numerical data was determined by Student's t-test. Assumptions of normality were verified using Kolmogorov–Smirnov and Shapiro–Wilks tests. All p-values less than 0.05 were considered significant.

3. Results

3.1. Sponge Distribution

In the ten investigated rivers, 51 localities were selected and analyzed. Three to five locations in the upper, middle, and lower sections of each river were chosen as sampling sites. Sponges were found at 18 out of the 51 investigated localities (Figure 1), and 50 samples were collected in total. Among all examined samples, five species of sponges were recorded.

The detected species, from the most to the least frequent in the samples, were the following: *Ephydatia fluviatilis* (Linnaeus, 1759) (24 samples), *Ephydatia muelleri* (Lieberkühn, 1856) (8 samples), *Spongilla lacustris* (Linnaeus, 1759) (9 samples), *Trochospongilla horrida* Weltner, 1893 (5 samples), and *Eunapius fragilis* (Leidy, 1851) (4 samples). It must be emphasized that not every species was detected at each of the 18 locations, indicating diverse community compositions. Interestingly, the presence of one of the species, characteristic of this part of Europe, *Heteromeyenia stepanowii* (Dybowski, 1884), was not confirmed.

3.2. Sponge Characteristics

Briefly, the appearance of *E. fluviatilis* was highly variable, from a relatively thin lichen-like layer covering the substrate to massive capped or ridged forms. The color of the body also varied greatly from whitish to brown, and due to symbiosis with algae, green specimens could be found as well (Figure 2).

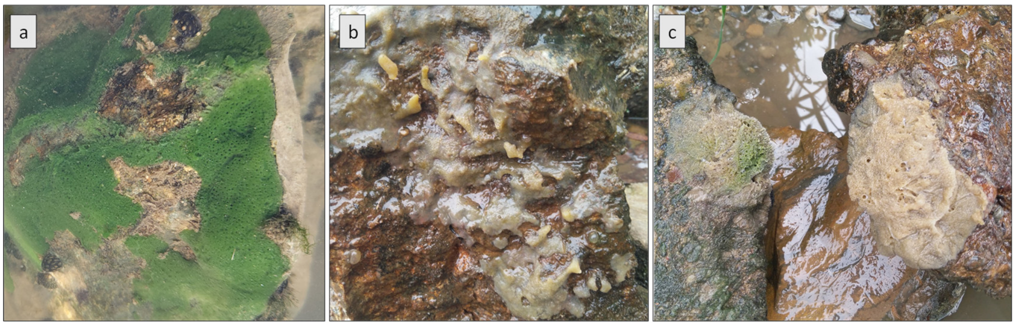

Figure 2. In situ appearances of three of the five sponges encountered on typical rocky substrates: *S. lacustris* (green color indicating symbiosis with algae)—(**a**); *E. fragilis*—(**b**); *E. fluviatilis*—(**c**).

E. muelleri was phenotypically very similar to *E. fluviatilis*. *E. muelleri* was found in various shades of brown, gray, and yellow, but as a result of symbiosis with algae, some green specimens were also seen. The shape of the body was irregular, and the surface was often papillar.

The vegetative body of *S. lacustris* was characterized by cylindrical, finger-like outgrowths that extended and branched from the axis of the body attached to the substrate. Thanks to these outgrowths, *S. lacustris* could be fairly accurately distinguished from other species in situ. Due to the presence of symbiotic algae, the body color was usually green.

E. fragilis was also often in symbiosis with algae, which turned its color green. Otherwise, it was grayish to brown. After growing a couple of centimeters wide, it had a cushion-like form, with visible ostia and sometimes bud-like protuberances.

The body of *T. horrida* was mostly flat and covered the substrate in the form of a thin layer with an irregular border. The color ranged from yellowish to dark brown.

3.3. Spicules Characteristics

Before analyzing spicule anomalies, normal spicules of the five sponge species were examined, and their morphology and size were recorded. The appearance of the spicules was generally in agreement with previous reports. The mineral skeleton of *E. fluviatilis* is characterized by two types of spicules: megascleres and gemmuloscleres. There are no microscleres in this species. The sizes of the megascleres (oxa) are in the 250–380 µm range in length and the 12–19 µm range in width. They are needle-shaped, slightly curved, and rarely straight. The surface of the megascleres may be smooth or covered with micro-thorns (Figure 3B). Gemmuloscleres are in the form of birotules, with a smooth or thorny axis, flat rotules of the same diameter, and micro-thorns and irregularly incised edges, with 15–20 teeth. The dimensions of the gemmuloscleres range from 21 to 25 µm in length, while the shafts are 2–4 µm wide. The diameters of the rotules are 18–23 µm (Figure 3B). *E. muelleri* is characterized by the presence of two variants of megascleres. They are usually densely covered with micro-thorns (except at the ends), but they can be completely smooth. The megascleres measure 170–320 µm in length. This species does not have microscleres. The microskeleton of *S. lacustris* differs from the previously mentioned species in that it has another class of spicules—microscleres. These spicules are located between megascleres and give the sponge extra support. Megascleres are in the shape of smooth amphioxea, 160–350 µm long, and 5–17 µm wide. The microscleres are also of the amphioxea type, slightly to strongly curved, and densely covered with minuscule thorns. The microscleres measure 35–95 µm in length. Gemmuloscleres are slightly to strongly curved and covered with microspines. The gemmuloscleres range from 18 to 70 µm in length and 3 to 10 µm in width. *E. fragilis* is characterized by amphioxa-type megascleres that are smooth, 165–261 µm long, and 10–14 µm wide. This type of sponge does not have microscleres. Gemmuloscleres belong to the amphistrongile type. They have blunt ends and are covered with thorns, mostly concentrated toward the tips (Figure 3A). The length ranges from 50 to 110 µm, and the width ranges from 4 to 9 µm.

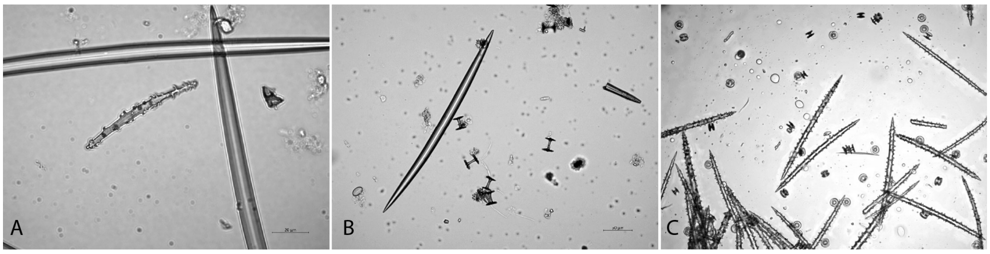

Figure 3. Representative spicules of *E. fragilis* (megascleres detail and gemmulosclere)—(**A**); *E. fluviatilis* (megasclere and gemmuloscleres–birotules)—(**B**); and *T. horrida* (numerous megascleres and birotules)—(**C**).

Megascleres of *T. horrida* species are of the amphioxea type and densely covered with blunt, thorny outgrowths. Dimensions range from 165 to 245 µm. Microscleres do not exist. Gemmuloscleres are in the form of birotules, with a short, smooth axis (the length of the axis is shorter than the diameter of the rotule) and rotules with smooth edges (Figure 3C). One is usually slightly larger in diameter than the other. The ranges of different spicule dimensions are shown in Table 1, while several representative skeletal elements of different Spongillida species are displayed in Figure 3.

Table 1. Spicule types and size ranges for species collected during the study.

Species/Spicule Sizes (µm)	Megasclere		Gemulosclere			Microsclere	
	Length	Width	Length	Width	Rotule Diameter	Length	Width
E. fluviatilis	250–380	12–19	21–25	2–4	18–23	/	/
E. muelleri	170–320	12–21	8–24	2–4	9–25	/	/
S. lacustris	160–350	5–17	18–70	3–10	/	35–95	4–7
E. fragilis	165–261	10–14	50–110	5–9	/	/	/
T. horrida	165–245	8–12	8–10	2–5	13–15	/	/

3.4. Spicule Anomalies

The types and incidences of spicule malformations varied considerably within the analyzed specimens. The frequency of anomalies ranged from 1×10^{-3} to 97×10^{-3}, with an average number of 12×10^{-3}. Among different spicule types, megascleres were the most affected by malformations, followed by microscleres. Gemmulosclere anomalies were seldom detected. The most frequent anomalies were spicules sharply bent at different angles near one end, both ends, or medially (Figure 4A–C). Spicules with split ends (bifurcated) were also common in different species (Figure 4D–F). Scissor-like or cross-like spicules (Figure 4G–I) and T-shaped spicules were recurrent as well (Figure 4J–L).

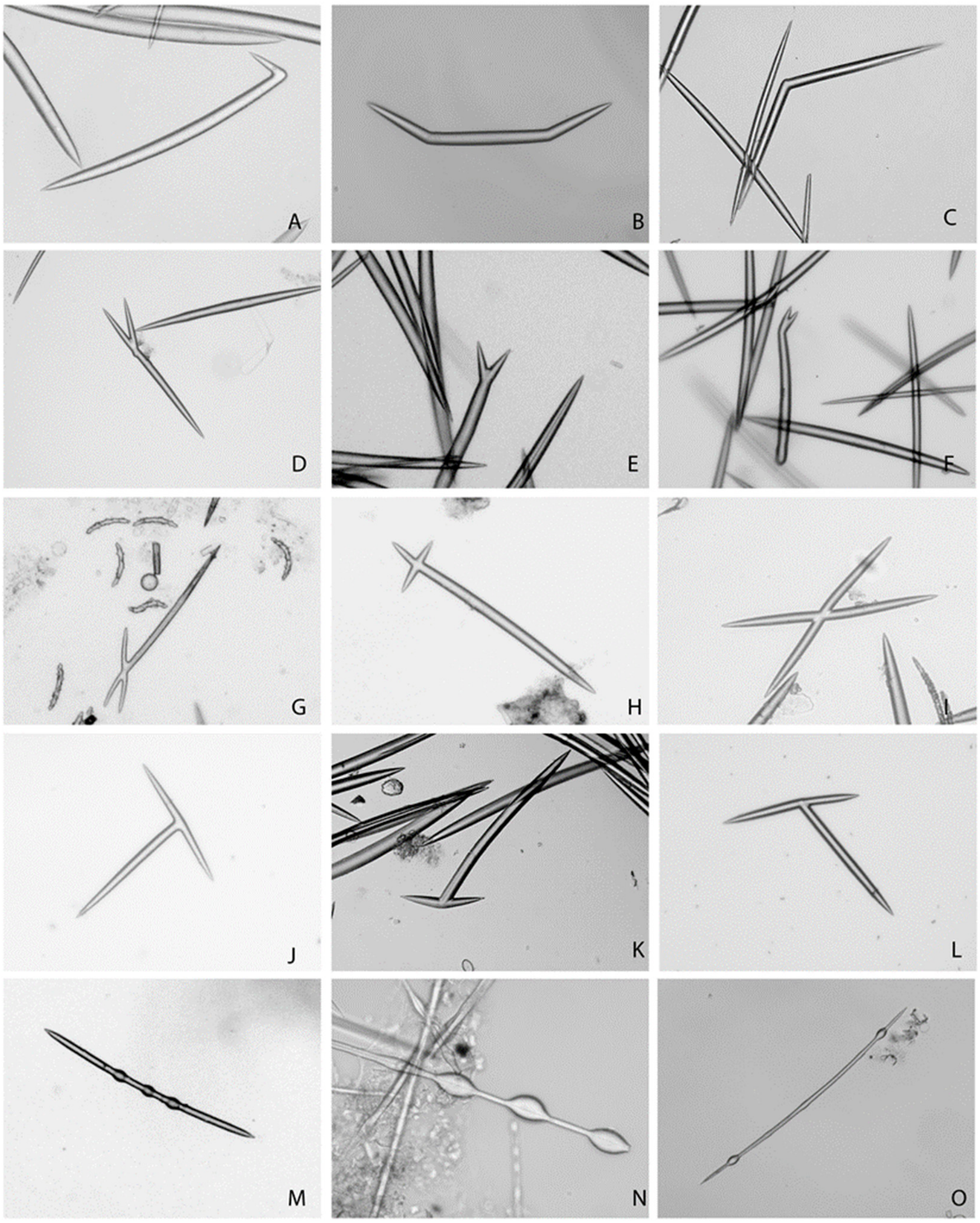

Figure 4. Compilation of encountered forms of spicule anomalies seen in specimens of freshwater sponges (light microscopy): megasclere bent near one end, both ends, and medially (**A**–**C**); bifurcated (split-end) spicules (**D**–**F**); scissor-like or cross-like spicule formations (**G**–**I**); T-shaped spicules (**J**–**L**); and spicules with bulbous, spherical enlargements (**M**–**O**).

As an example of a peculiar anomaly—spicules with bulbous, spherical enlargements along the axis were found at high frequency in specimens of *E. fragilis* but could be seen in *E. fluviatilis* and *E. muelleri* as well. Single or multiple bulbous enlargements were recorded on both megascleres and microscleres (Figure 4M–O). These were the most often encountered anomalies. A few anomalies appeared extremely rarely in our samples, such as gemmuloscleres forming loops, polyaxial spicules resembling skeletal structures in marine sponges, and spicules with complex malformation (Figure 5A–C).

Some of the most frequent malformations were observed with SEM as well (Figure 6).

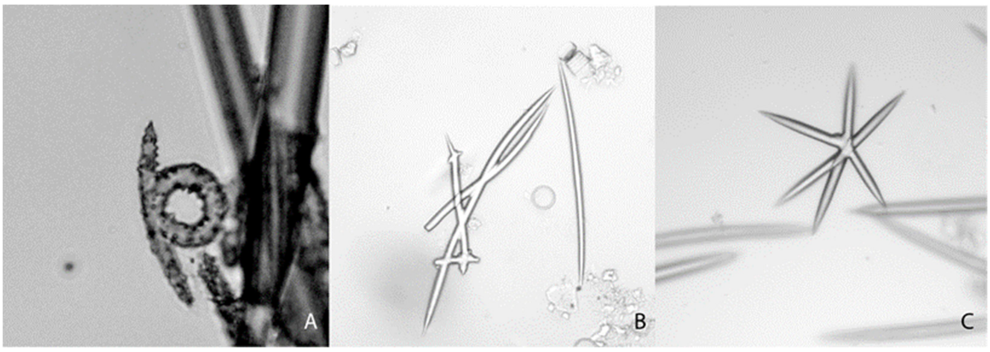

Figure 5. Rare types of spicule anomalies: gemmulosclere of *S. lacustris,* usually straight or slightly curved, forming a circle here (**A**); spicule with multiple anomalies (**B**); polyaxial spicule aberration, resembling spicules from marine species (**C**).

3.5. Spicule Anomalies in Relation to Environmental Parameters and Pollutants

Considerable variations among the ten surveyed rivers were noted regarding the main parameters (oxygen saturation, pH, temperature, and conductivity), levels of metals (iron, copper, led, etc.), and other pollutants, such as nitrates, orthophosphates, sodium, chlorides, etc. However, those levels did not exceed limits defined by legislation [29]. In order to uncover potential factors conditioning the appearance of spicule anomalies, we compared the rivers that harbored sponges with extreme values of anomaly frequency (lowest and highest). The two rivers, Tisa and Velika Morava, showed statistically significant differences in the following factors: pH, ammonia, orthophosphates, sodium, chloride, manganese, and lead (Table 2).

Table 2. Physico-chemical parameters and pollutants on sites with the least detected anomalies (Tisa, Martonoš) versus the most detected spicule anomalies (Velika Morava, Bagrdan); ns—non significant.

Parameter	Tisa, Martonoš	V. Morava Bagrdan	*t*-Test	
	Mean ± SD	**Mean ± SD**	**df**	**Sign.**
Watertemperature (°C)	13.6 ± 9.5	14.6 ± 9.1	20	ns
Turbidity (NTU)	23.4 ± 17.6	58.3 ± 10.8	20	ns
pH	8.00 ± 0.13	8.21 ± 0.16	20	0.010
Oxygensaturation (%)	88.6 ± 4.7	95.9 ± 10.0	20	ns
Conductivity (μS/cm)	427.4 ± 71.9	402.9 ± 41.7	20	ns
Ammonia(NH_4-N)	0.067 ± 0.069	0.207 ± 0.228	20	0.048
Nitrates(N-NO_3) mg/L	0.675 ± 0.314	1.200 ± 0.455	20	ns
Orthophosphate(O-PO_4) mg/L	0.045 ± 0.012	0.083 ± 0.031	20	0.006
Sodium(Na) mg/L	32.5 ± 8.4	13.1 ± 4.5	20	0.000
Chloride(Cl) mg/L	38.3 ± 14.5	12.9 ± 3.6	20	0.000
Manganese(Mn) μg/L	61.6 ± 35.9	139.2 ± 73.8	20	0.028
Zink(Zn) μg/L	32.6 ± 25.2	13.6 ± 7.9	20	ns
Copper(Cu) μg/L	5.8 ± 1.0	7.0 ± 3.6	20	ns
Led(Pb) μg/L	2.2 ± 1.1	9.5 ± 8.7	20	0.046
Aluminum(Al) μg/L	599.3 ± 452.6	1086.8 ± 1049.8	20	ns

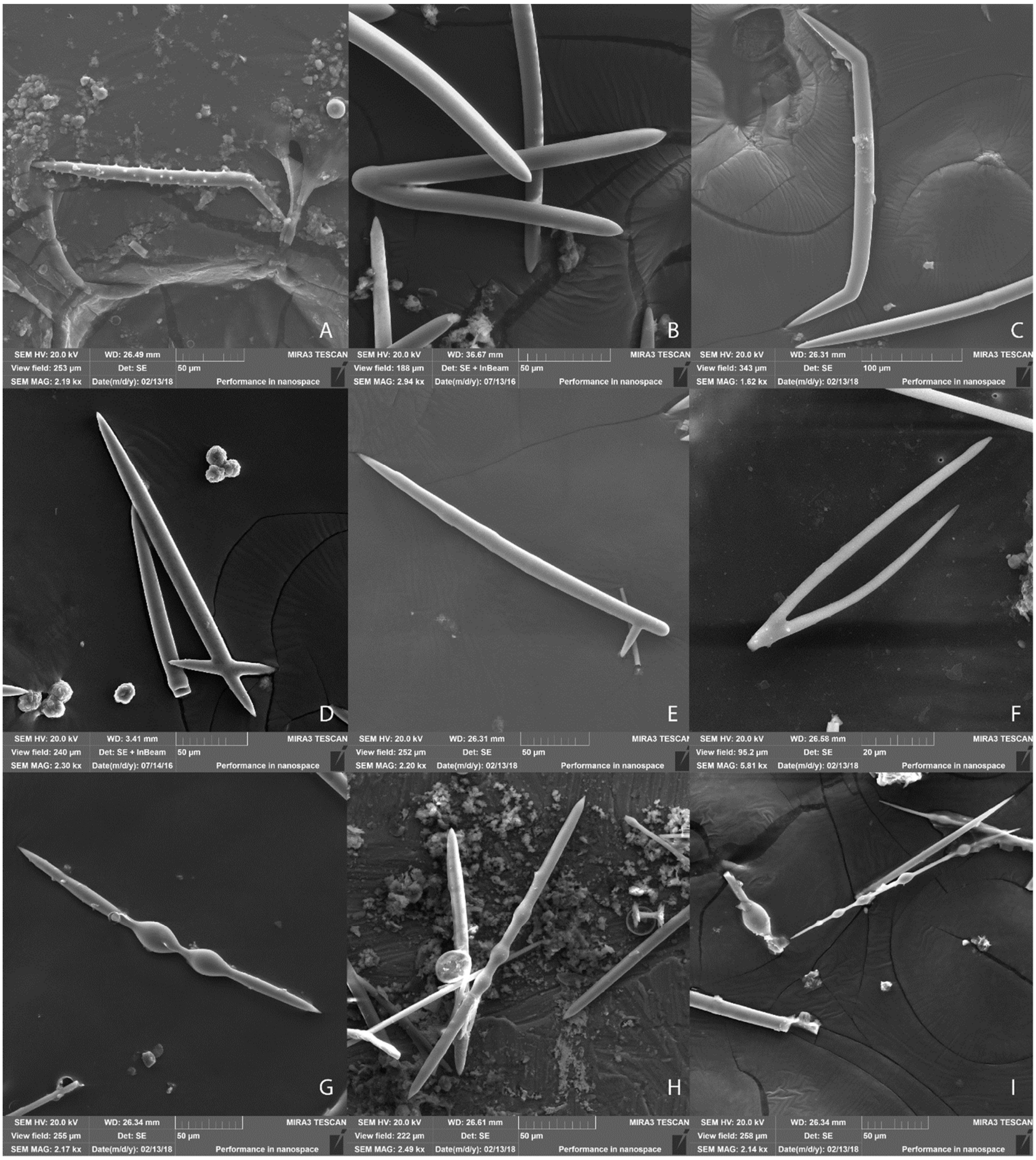

Figure 6. Collection of spicule anomalies analyzed with SEM: a thorny spicule of *T. horrida* sharply bent near one end (**A**); spicule sharply bent medially (**B**); spicule symmetrically bent on both ends (**C**); cross-like spicule (**D**); spicule with a thorn (**E**); bifurcating spicule (**F**); spicule anomaly in the shape of bulbous enlargements on megascleres (**G**,**H**); and microscleres (**I**).

The lowest number of anomalies was found in a specimen of *E. fluviatilis* (1×10^{-3}) from Kanjiža near Martonoš (Tisa River), and the main type of anomaly was the bent megasclere (single distal bend). The highest number of anomalies was found in a specimen of *E. fragilis* (97×10^{-3}) collected at Markovac near Bagrdan (Velika Morava River), and the predominant anomalies were bulbous megascleres. The orthophosphates concentration was lower in Tisa than in V. Morava, while sodium and chloride were both present at higher concentrations on the site that harbored sponges with less anomalies.

4. Discussion

According to current as well as our previous fieldwork, freshwater sponges were not frequent organisms in the studied water bodies. The number of sites where sponges were recorded represents a smaller part of the total number of searched sites. Their abundance, with a few exceptions, was low as well, since only a small number of specimens were collected from the sites where their presence was established. They were observed to display a tendency towards coastal zones and areas with slower water currents. However, in shallow rivers, they were discovered in locations further away from the banks, even in the midst of the stream. Sponges were mostly detected and collected at depths ranging from 0.5 to 1.5 m, and can be found on both natural and artificial substrates. Furthermore, they exhibited a distinct affinity for slightly alkaline and well-oxygenated water, as well as higher temperatures and conductivity.

In the present study, we tried to make a catalogue of the most frequent spicule anomalies encountered in Porifera from Serbian rivers and look for a relationship, if any, between the occurrence of anomalies and selected environmental parameters. Quite remarkably, a number of studies have dealt with the process of spiculogenesis and its regulation in freshwater and marine sponges, both in vitro and in vivo [30–34], and yet, information regarding spicule anomalies is very limited. Holwoet and Van der Vyer, for instance, demonstrated the substantial impact of silicate concentration on spicule formation in *Ephydatia fluviatilis* in terms of their quantity. The number of spicules was inversely related to silicate concentrations, both when sponges were grown under standard conditions in mineral medium, as well as when the differentiation of the aquiferous system was inhibited (hydroxyurea added to the medium), or the integration of spicules into a tridimensional network was disrupted (puromycine added to the medium). Yet, there was no comment about the possible impact on spicule morphology [35]. In the study by Nakayama et al., the complex steps of spicule formation and positioning, which include the activities of different classes of cells, were described in great detail in *E. fluviatilis*, but again, only normal morphogenesis was considered [34].

Interestingly, novel data on the specific topic of spicule malformations are missing. Several important historical papers have raised the question of spicule anomalies and their relation to water pollution. For instance, malformations were observed in spicules of *Trochospongilla leidyi* growing in iron water pipes [36] and megascleres of *E. fluviatilis* growing in water polluted by industrial wastes [37]. Some field observations have suggested that sponges are sensitive to chemical pollution [5], while other studies have been unable to find a clear relationship between spicule malformations and specific causal agents [3].

Sponge skeletal elements in the present study were observed using both light microscopy and scanning electron microscopy, and the morphologies of normal spicules belonging to the five recorded species, as well as their dimensions, corresponded with literature data [38,39]. On the other hand, the encountered anomalies varied greatly in terms of both morphology and frequency; the frequencies of spicules with structural anomalies varied practically two orders of magnitude between the localities and between the species.

The highest percentage of anomalies was found in *E. fragilis* (almost 10% of spicules were aberrant), while the lowest was in *E. fluviatilis* (only 1‰). The two "antipode" rivers, i.e., the rivers with sponges that had the biggest (Velika Morava) and smallest (Tisa) numbers of anomalies, showed statistically significant differences in a number of pollutants. Hence, we can speculate that they might have impacted anomaly occurrence. For instance, the concentrations of ammonia, manganese, and lead were 3, 2.3, and 4.3 times, higher in Velika Morava, respectively, compared to Tisa and could potentially affect spiculogenesis. This finding is in line with the study of Richelle et al., who demonstrated that *E. fluviatilis* was affected by Cd and Hg and that relatively low metal concentrations could cause spicule malformations [13]. However, in the mentioned study, the sponges were artificially grown in selected portions of rivers characterized by different types of pollution. On the other hand, it seems that the scarcity of some elements, such as sodium and chloride (2.5 and

3 times, respectively, lower concentrations in Velika Morava than in Tisa) might play a role in the development of malformations, given the statistically highly significant difference.

Interestingly some authors have pointed to the influence of copper and zinc on spiculogenesis [21]. In the present study, these metals did not seem to influence the appearance of anomalies.

The fact that *E. fragilis* had 10 times more aberrations than recorded on average, in the absence of extreme environmental conditions (none of the pollutant's concentrations exceeded limits defined by legislation), should be emphasized. It suggests that *E. fragilis* might be more sensitive to different pollutants than other species. This finding is in agreement with a report on sponges from Lake Ilopango, El Salvador [14]. Namely, a very high frequency of anomalies (bulbous structures) was registered in *Spongilla alba*, and their occurrence was related to elevated concentrations of arsenic, while at the same time, spicules of *E. fluviatilis* from this lake were without malformations, pointing again to differences in sensitivity, depending on the species.

Freshwater sponges were poorly represented in the surveyed localities in Serbia; a small number of sponges was collected, i.e., their abundance was low, which represents a limitation in the present study. In light of the scarcity of sponge samples, our focus in the analysis was on exploring potential trends and possibilities rather than establishing definitive correlations. We believe that even with a limited sample size, our study provides valuable insights into the interplay between physical and chemical parameters and sponge anomalies. The second limitation of sponge paucity was, consequently, the fact that not all sponge species were present at the same localities, preventing the comparison of species–specific propensities to aberrant spiculogenesis in a given environment.

5. Conclusions

The present study describes the pattern of sponge spicule malformations in different rivers from inland Serbia. Several factors can explain the observed pattern, ranging from environmental parameters to aberrant spiculogenesis, the origin of which is still unclear. The use of this invertebrate taxon as a bioindicator could be significantly increased if studies confirm a relationship between the changes in the chemical and physical parameters of streams and the frequency of anomalies found in spicules. Future investigations on larger samples of Porifera are needed.

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