

# Microbial Polysaccharides Extracted from Different Mature Muds of the Euganean Thermal District Show Similar Anti-Inflammatory Activity In Vivo

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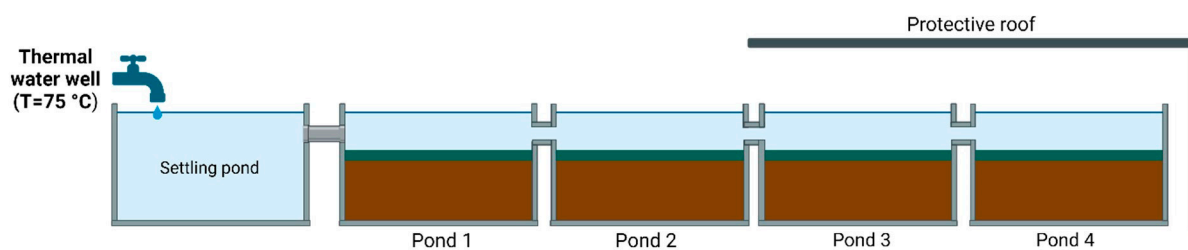
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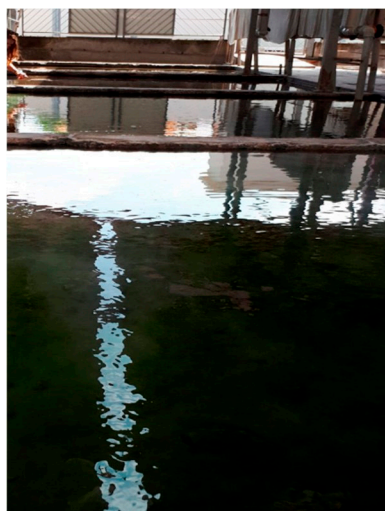
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**Figure S1:** Representation of a typical pond system. Image created with Biorender.com.



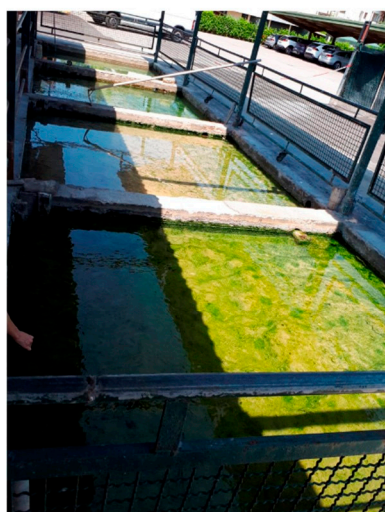
**P-1 = 36.6 °C**



**P-2 = 41.8 °C**



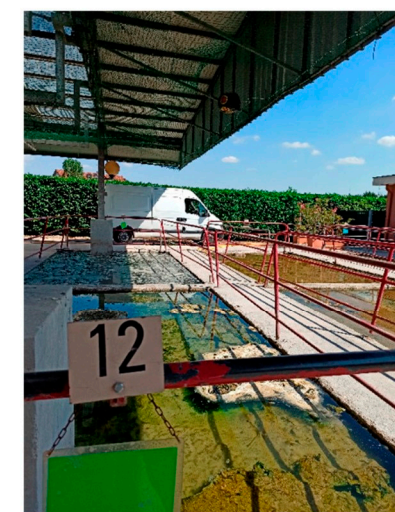
**P-3 = 46.5 °C**



**P-4 = 49.4 °C**

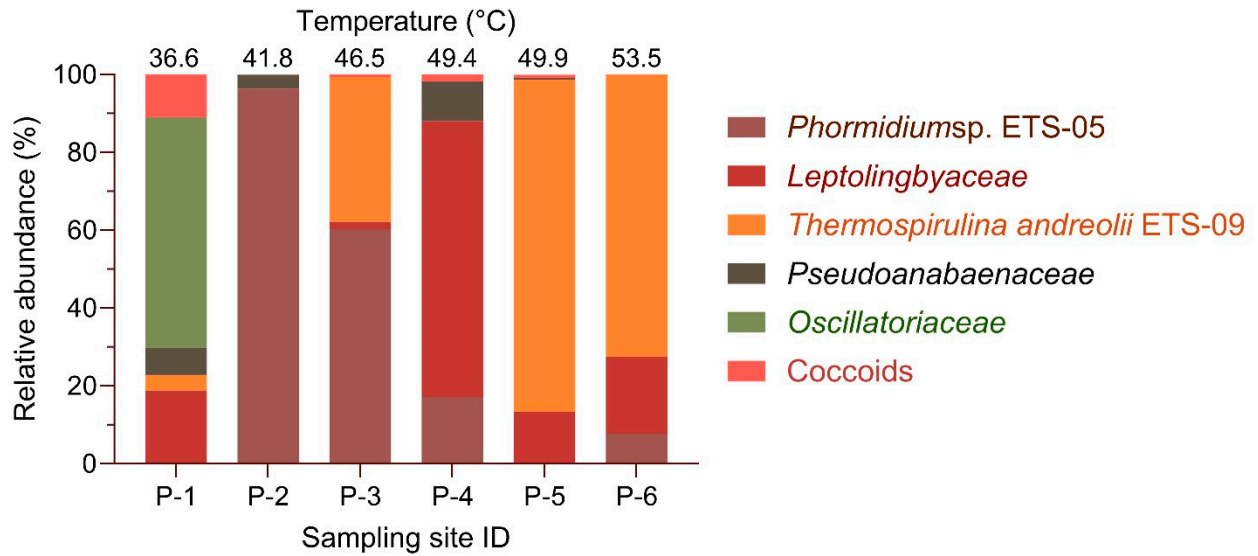


**P-5 = 49.9 °C**

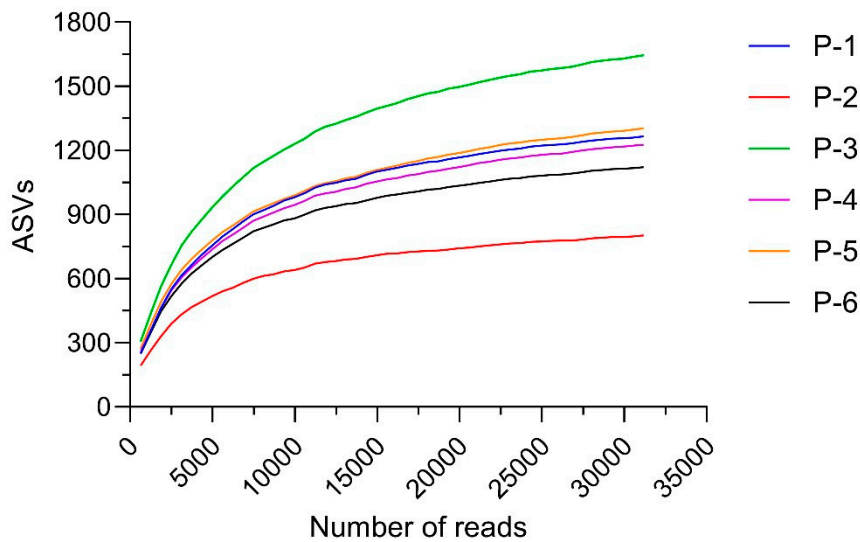


**P-6 = 53.5 °C**

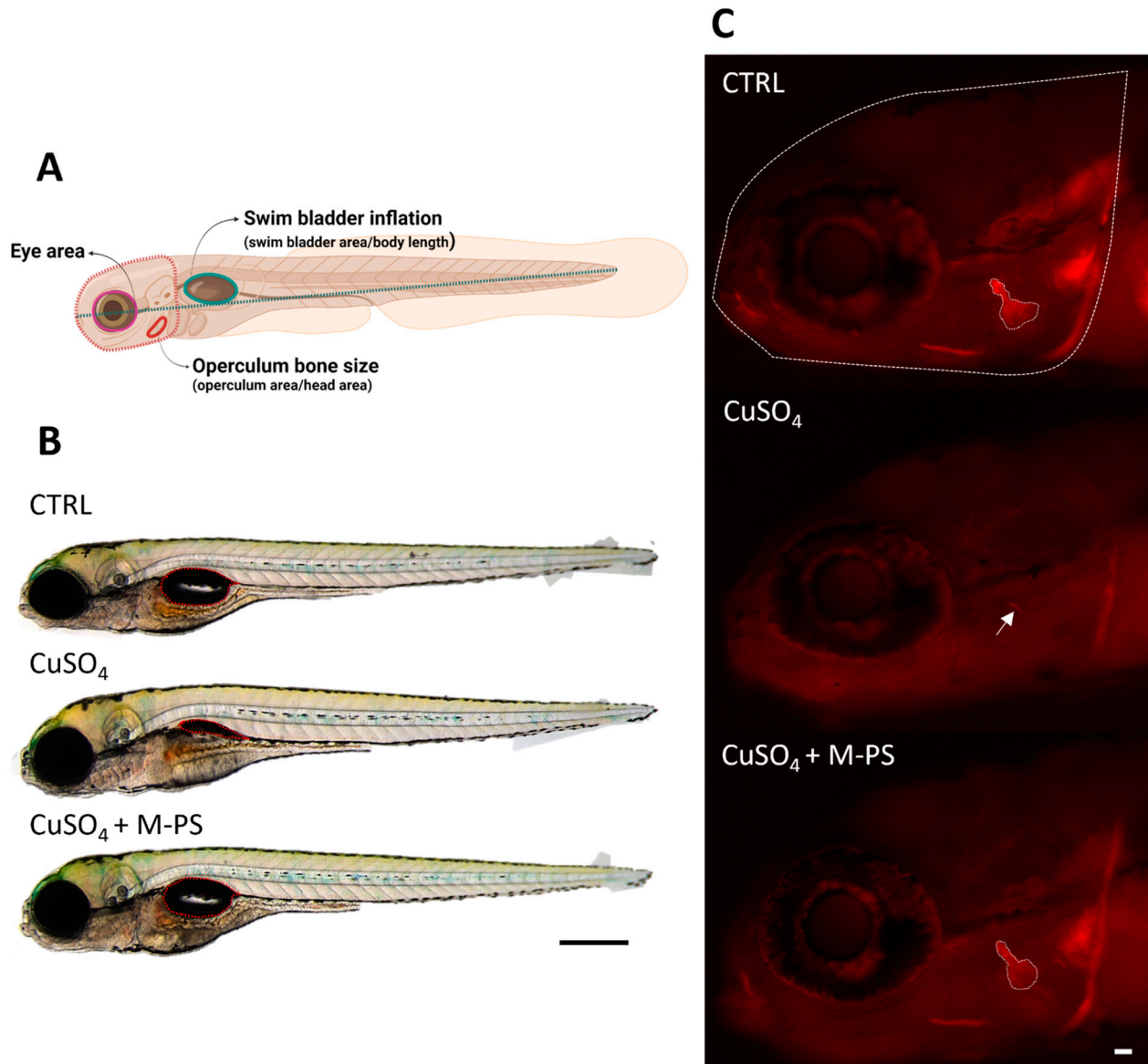
**Figure S2:** Pictures of the thermal environment of the sampled ponds.



**Figure S3:** Cyanobacteria population composition of the collected samples, obtained referring the calculated volumes of each morphogenera to the total Cyanobacteria volume, as described in Gris et al., 2020 (see main text). Briefly, two replicates of each collected biofilm sample were diluted with thermal water (1:20, *v/v*) and 40  $\mu$ l of formalin was added. Using a 40X objective, about 40 fields were analysed and images were acquired using both visible and fluorescent light (Leica DM6 B; Leica, Wetzlar, Germany). Images were then analysed using ImageJ software, considering filaments as cylinders and dividing coccoid species in groups based on cell size.

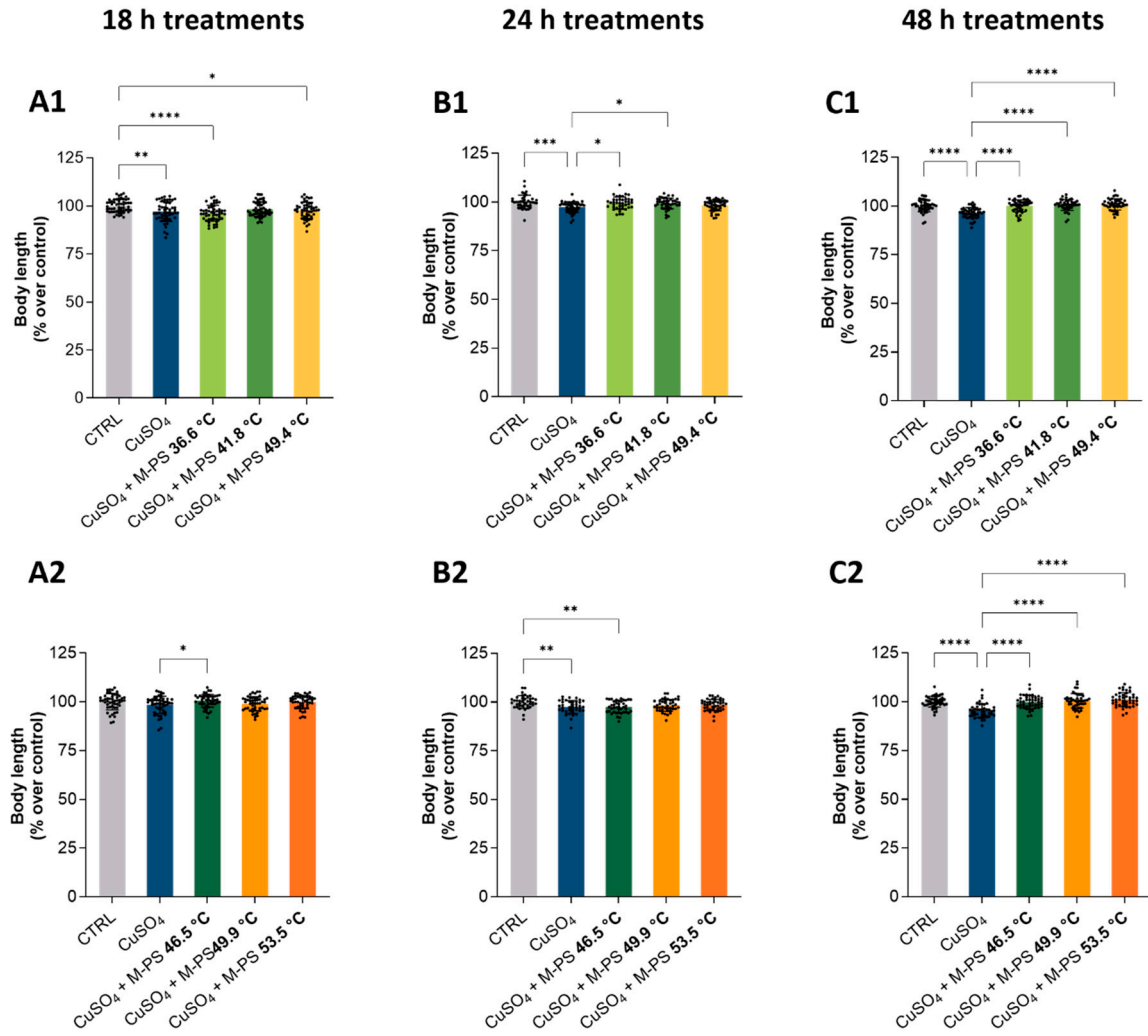


**Figure S4:** Average rarefaction curves ( $n=2$ ) of each sample.

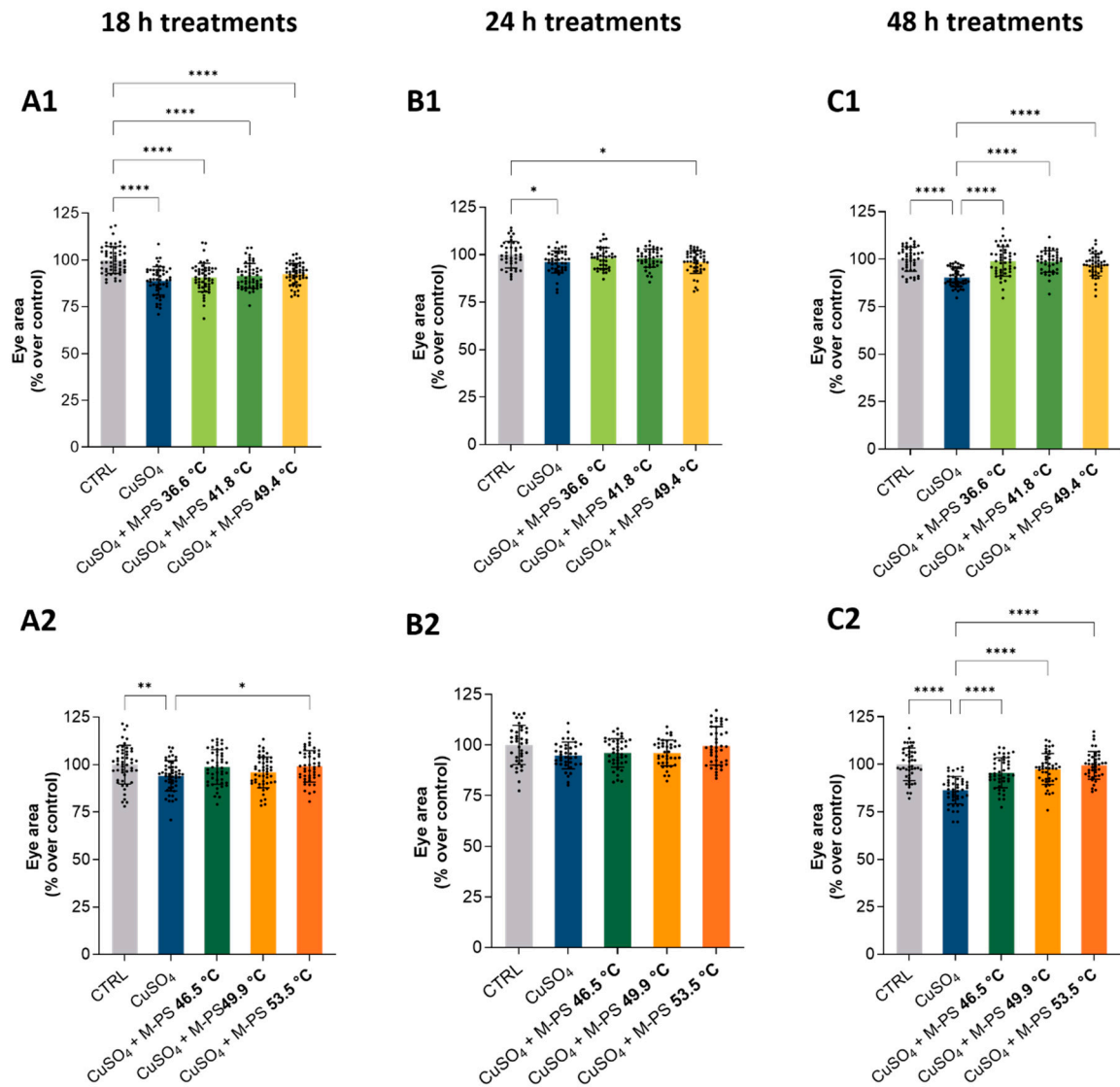


**Figure S5:** Representation of analysed morphological traits in zebrafish larvae. (A) Scheme representing the morphometric traits analysed on zebrafish larvae (Image created with Biorender.com). (B) Micrographs showing reduced inflation of swim bladder due to  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  exposure ( $\text{CuSO}_4$ ) and rescue of the inflation after 48 h M-PS treatment ( $\text{CuSO}_4 + \text{M-PS}$ ). Sibling larvae (CTRL) are shown for comparison. Swim bladder is highlighted by red dotted lines. Scale bar = 500  $\mu\text{m}$ . (C) Picture of 5-dpf larvae's head, stained with alizarin red S and showing the head and the operculum area evaluated through morphometric analyses. The operculum is highlighted by white dotted lines or white arrow. The micrographs show reduced operculum ossification after  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  exposure ( $\text{CuSO}_4$ ) and ossification rescue due to 48 h M-PS treatment ( $\text{CuSO}_4 + \text{M-PS}$ ). Sibling larvae (CTRL) are shown for comparison. Scale bar = 50  $\mu\text{m}$ .

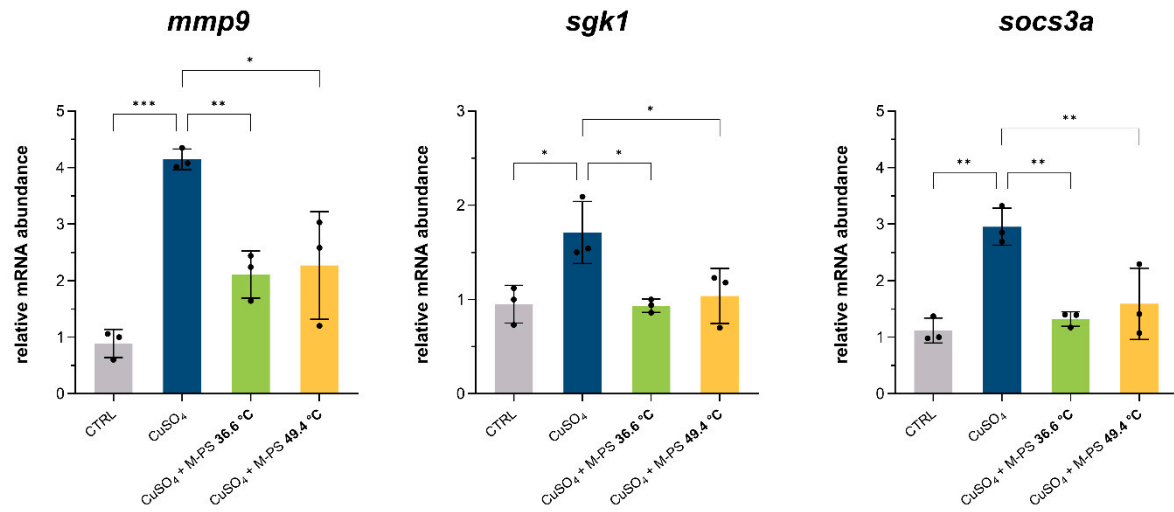




**Figure S6:** Analysis of body length on larvae treated with M-PS after CuSO<sub>4</sub> · 5H<sub>2</sub>O inflammation at 3 dpf. (**A1-A2**) Recovery from inflammation after 18 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (A1) and 46.5, 49.9, 53.5 °C (A2). (**B1-B2**) Recovery from inflammation after 24 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (B1) and 46.5, 49.9, 53.5 °C (B2). (**C1-C2**) Recovery from inflammation after 48 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (C1) and 46.5, 49.9, 53.5 °C (C2). Data are compared to control values and indicated as percentages over control. Black bars represent the mean  $\pm$  SD of three independent experiments conducted with 15-20 larvae per treatment. Statistical analysis was performed using GraphPad Prism 10 (ordinary oneway ANOVA followed by Tukey's multiple comparisons test with a single pooled variance in A2-B1-B2-C1-C2 and BrownForsythe and Welch ANOVA test followed by Dunnett's T3 multiple comparison test with individual variances computed for each comparison in A1). Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .



**Figure S7:** Analysis of eye area on larvae treated with M-PS after CuSO<sub>4</sub> · 5H<sub>2</sub>O inflammation at 3 dpf. (A1-A2) Recovery from inflammation after 18 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (A1) and 46.5, 49.9, 53.5 °C (A2). (B1-B2) Recovery from inflammation after 24 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (B1) and 46.5, 49.9, 53.5 °C (B2). (C1-C2) Recovery from inflammation after 48 hours of treatment with M-PS extract from mud matured at 36.6, 41.8, 49.4 °C (C1) and 46.5, 49.9, 53.5 °C (C2). The area was measured as the area of a polygon. Data are compared to control values and indicated as percentages over control. Black bars represent the mean ± SD of three independent experiments conducted with 15-20 larvae per treatment. Statistical analysis was performed using GraphPad Prism 10 (ordinary one-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance in A1-A2-B1-C2 and Brown-Forsythe and Welch ANOVA test followed by Dunnett's T3 multiple comparison test with individual variances computed for each comparison in C1). Statistical significance: \*p < 0.05, \*\*p < 0.01, \*\*\*p ≤ 0.001, \*\*\*\*p ≤ 0.0001.



**Figure S8:** Relative mRNA abundance of genes involved in inflammatory response, analysed in 3-dpf zebrafish larvae after CuSO<sub>4</sub> · 5H<sub>2</sub>O exposure, with and without M-PS treatment. M-PS were extracted from mud matured at 36.6 and 49.4 °C. The data represents the mean ± SD of three independent experiments conducted with 15-20 larvae. Statistical analysis was performed using GraphPad Prism 10 (ordinary one-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance). Statistical significance: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* ≤ 0.001.

**Table S1:** The Excel file contains a list of all the 16S rRNA sequences of Amplicon Sequence Variants (ASVs) obtained from the NGS analysis. Each mature mud sample was analysed in duplicate, so the file names are the following: P-1: A66-A67; P-4: A68-69; P-2: A70-A71; P-5: A72-A73; P-6: A74-75; P-3: A76-A77. Additionally, the spreadsheet includes ASVs corresponding to the most abundant taxa and Cyanobacteria (with abundances higher than 0.01%). Taxa are grouped based on the Phyla assigned by the pipeline.

**Table S2:** Average values of polysaccharides content  $\pm$  standard deviation of extracted M-PS samples and of lyophilized mature muds from thermal Spas in Abano and Montegrotto Terme in 2022. Sugar content was quantified using the Dubois method.

Sampling site ID	Temperature (°C)	Sugar content in extracted M-PS ( $\mu\text{g/g}_{\text{MUD}}$ )	Sugar content in mature mud ( $\text{mg/g}_{\text{MUD}}$ )
P-1	36.6	$54.02 \pm 5.63$	$2.80 \pm 0.27$
P-2	41.8	$43.24 \pm 4.70$	$2.52 \pm 0.20$
P-3	46.5	$72.02 \pm 29.27$	$3.35 \pm 0.84$
P-4	49.4	$76.10 \pm 19.26$	$6.31 \pm 1.04$
P-5	49.9	$58.06 \pm 3.88$	$3.17 \pm 0.87$
P-6	53.5	$75.27 \pm 29.93$	$2.74 \pm 0.25$

**Table S3:** Monosaccharide composition quantification, as molar %, of M-PS from the six mature muds. Abbreviations: fuc, fucose; rha, rhamnose; galN, galactosamine; ara, arabinose; glcN, glucosamine; gal, galactose; glc, glucose; man, mannose; xyl, xylose; galA, galacturonic acid; glcA, glucuronic acid.

Monosaccharides (mol %):	P-1	P-2	P-3	P-4	P-5	P-6
fuc	4.73	3.06	3.29	3.56	2.76	3.00
rha	5.43	4.84	4.66	6.25	5.14	6.58
galN	2.26	2.03	2.03	3.64	4.34	5.78
ara	2.42	2.23	1.68	3.39	2.47	3.06
glcN	3.94	3.97	3.15	6.07	5.09	5.01
gal	12.50	9.59	12.38	13.60	13.28	10.52
glc	17.91	27.02	19.80	16.10	16.77	14.07
man	17.71	17.27	19.68	18.18	17.06	15.82
xyl	22.52	20.10	18.34	19.56	18.55	19.80
galA	4.49	4.41	10.71	3.66	10.39	4.60
glcA	6.10	5.48	4.27	5.99	4.15	11.76



**Table S4:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs A1-B1-C1 of Figure 5 (Analysis of swim bladder area).

Dunnett's T3 multiple comparisons test	Adjusted P Value A1	Adjusted P Value B1	Adjusted P Value C1
CTRL vs. CuSO <sub>4</sub>	<0.0001	<0.0001	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 36.6 °C	<0.0001	0.1374	0.2772
CTRL vs. CuSO <sub>4</sub> + M-PS 41.8 °C	<0.0001	0.7660	0.0395
CTRL vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001	0.2017	0.3848
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 36.6 °C	0.0006	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 41.8 °C	0.0003	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001	<0.0001	<0.0001
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 41.8 °C	>0.9999	0.9011	0.9993
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.8954	>0.9999	>0.9999
CuSO <sub>4</sub> + M-PS 41.8 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.9260	0.9848	0.9967

**Table S5:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs A2-B2-C2 of Figure 5 (Analysis of swim bladder area).

Dunnett's T3 multiple comparisons test	Adjusted P Value A2	Adjusted P Value B2	Adjusted P Value C2
CTRL vs. CuSO <sub>4</sub>	<0.0001	<0.0001	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 46.5 °C	<0.0001	0.0184	0.9850
CTRL vs. CuSO <sub>4</sub> + M-PS 49.9 °C	<0.0001	0.8912	0.0038
CTRL vs. CuSO <sub>4</sub> + M-PS 53.5 °C	<0.0001	0.3992	0.0264
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 46.5 °C	0.0010	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.9 °C	<0.0001	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.0005	<0.0001	<0.0001
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.5167	0.8272	0.2286
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.9762	0.9909	0.6942
CuSO <sub>4</sub> + M-PS 49.9 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.9985	0.9999	0.9920

**Table S6:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs A1-B1 of Figure 6 (Analysis of operculum bone area).

Dunnett's T3 multiple comparisons test	Adjusted P Value A1	Adjusted P Value B1
CTRL vs. CuSO <sub>4</sub>	<0.0001	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 36.6 °C	<0.0001	>0.9999
CTRL vs. CuSO <sub>4</sub> + M-PS 41.8 °C	<0.0001	>0.9999
CTRL vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001	0.8922
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 36.6 °C	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 41.8 °C	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001	<0.0001
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 41.8 °C	0.9549	>0.9999
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.9998	0.9009
CuSO <sub>4</sub> + M-PS 41.8 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.5660	0.9833

**Table S7:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs A2-B2 of Figure 6 (Analysis of operculum bone area).

Dunnett's T3 multiple comparisons test	Adjusted P Value A2	Adjusted P Value B2
CTRL vs. CuSO <sub>4</sub>	<0.0001	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 46.5 °C	<0.0001	0.0558
CTRL vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.0004	0.1838
CTRL vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.0002	>0.9999
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 46.5 °C	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.9 °C	<0.0001	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 53.5 °C	<0.0001	<0.0001
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.4781	>0.9999
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.3663	0.0886
CuSO <sub>4</sub> + M-PS 49.9 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	>0.9999	0.2609

**Table S8:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs B1 of Figure 7 (Analysis of locomotor activity, 24 hours).

Dunnett's T3 multiple comparisons test	Adjusted P Value B1
CTRL vs. CuSO <sub>4</sub>	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 36.6 °C	>0.9999
CTRL vs. CuSO <sub>4</sub> + M-PS 41.8 °C	0.9795
CTRL vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.6498
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 36.6 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 41.8 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 41.8 °C	0.9992
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.9266
CuSO <sub>4</sub> + M-PS 41.8 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	>0.9999

**Table S9:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs B2 of Figure 7 (Analysis of locomotor activity, 24 hours).

Dunnett's T3 multiple comparisons test	Adjusted P Value B2
CTRL vs. CuSO <sub>4</sub>	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 46.5 °C	0.9847
CTRL vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.9868
CTRL vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.9942
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 46.5 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.9 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 53.5 °C	<0.0001
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 49.9 °C	>0.9999
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	>0.9999
CuSO <sub>4</sub> + M-PS 49.9 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	>0.9999

**Table S10:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs B1 of Figure 8 (Analysis of locomotor activity, 48 hours).

Dunnett's T3 multiple comparisons test	Adjusted P Value B1
CTRL vs. CuSO <sub>4</sub>	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 36.6 °C	0.9998
CTRL vs. CuSO <sub>4</sub> + M-PS 41.8 °C	0.9987
CTRL vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.1923
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 36.6 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 41.8 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.4 °C	<0.0001
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 41.8 °C	>0.9999
CuSO <sub>4</sub> + M-PS 36.6 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.8054
CuSO <sub>4</sub> + M-PS 41.8 °C vs. CuSO <sub>4</sub> + M-PS 49.4 °C	0.7526

**Table S11:** Exact adjusted P value resulted from Brown-Forsythe and Welch ANOVA test followed by post hoc Dunnett's T3 multiple comparison test of graphs B2 of Figure 8 (Analysis of locomotor activity, 48 hours).

Dunnett's T3 multiple comparisons test	Adjusted P Value B2
CTRL vs. CuSO <sub>4</sub>	<0.0001
CTRL vs. CuSO <sub>4</sub> + M-PS 46.5 °C	0.0124
CTRL vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.1503
CTRL vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.6910
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 46.5 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 49.9 °C	<0.0001
CuSO <sub>4</sub> vs. CuSO <sub>4</sub> + M-PS 53.5 °C	<0.0001
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 49.9 °C	0.9864
CuSO <sub>4</sub> + M-PS 46.5 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.5308
CuSO <sub>4</sub> + M-PS 49.9 °C vs. CuSO <sub>4</sub> + M-PS 53.5 °C	0.9907

**Table S12:** Primer pairs used for RT-qPCR.

Gene name	GeneBank accession No	Primer sequences (5' - 3')
<i>gapdh</i>	NM_001115114	<i>Fw</i> : GTGGAGTCTACTGGTGTCTTC <i>Rv</i> : GTGCAGGAGGCATTGCTTAC
<i>mmp9</i>	NM_213123	<i>Fw</i> : CATTAAAGATGCCCTGATGTATCCC <i>Rv</i> : AGTGGTGGTCCGTGGTTGAG
<i>sgk1</i>	NM_199212	<i>Fw</i> : GGTAGCTTCGGCAAGGTTCT <i>Rv</i> : CAGTAACACGTTGCGCTCTGA
<i>socs3a</i>	DQ333315.1	<i>Fw</i> : GGAAGACAAGAGCCGAGACT <i>Rv</i> : GCGATACACACCAAACCCTG