Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supplementary Information

Toxicity of nanoplastics to zooplankton is influenced by temperature, salinity, and natural particulate matter

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Characterization of nanoplastics

We verified the size distribution of nanoplastics considered in this study in deionized water under dynamic light scattering after dialysis (Figure S1). The detected radius size of 40-50 nm agreed with the reported size by the manufacturer.

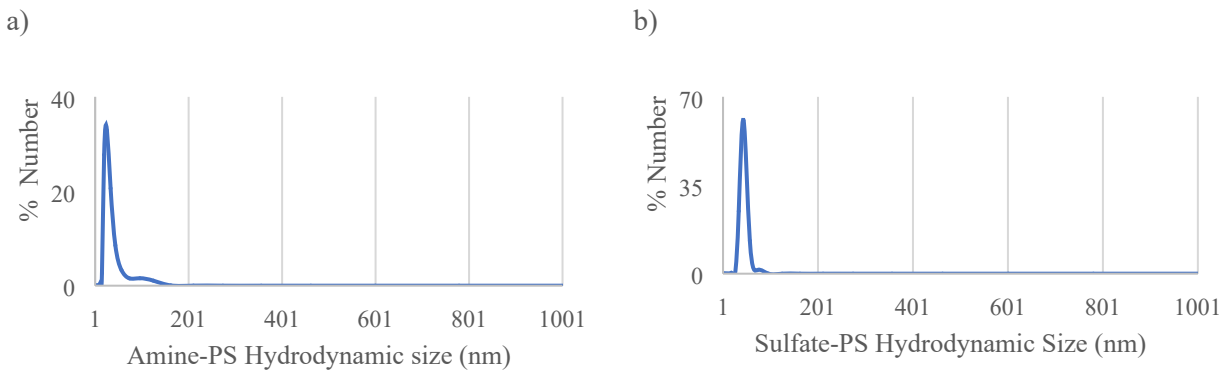


Figure S1: Size distribution of PS NP with (a) amine-modification, (b) sulfate-modification.

We verified the sizes of both PS nanoparticles in media, with the addition of HA and NC (see Figure S2). Overall, amine-modified NPs remained stable, except for very high salinities where we also observed a substantial polydispersity.

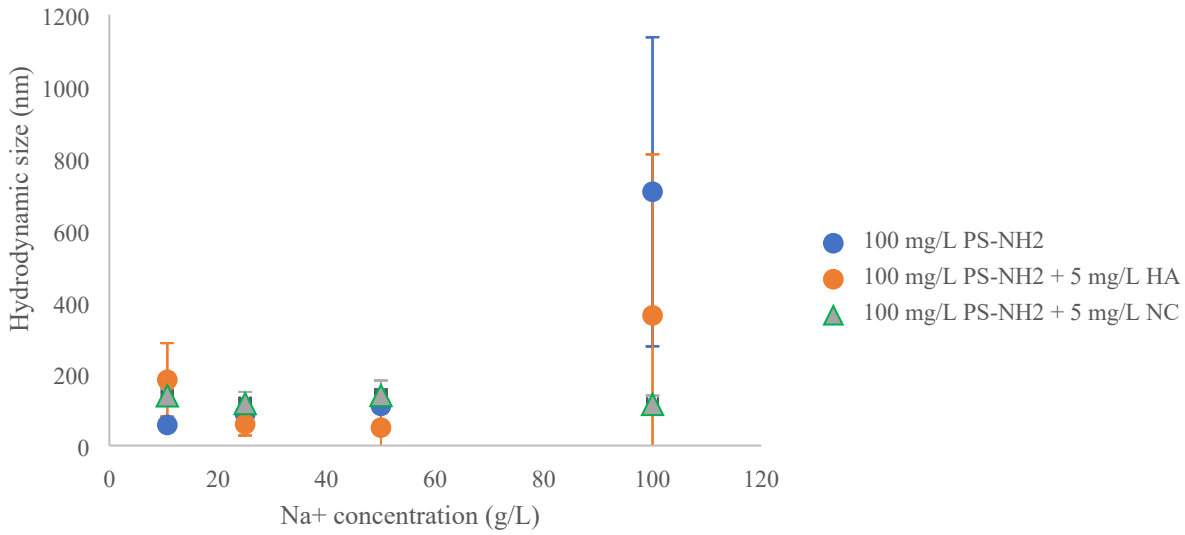


Figure S2: Stability of amine-modified PS NP (PS-NH2) under a range of salinities (x-axis). Points show mean hydrodynamic size at each salinity level, with 5 mg/L HA (orange circles), 5 mg/L NC (green triangles), or neither (blue circles). Bars represent +/- one standard error of the mean.

We also verified the behavior of sulfate-modified NP under a number of scenarios, as shown in Figure S3. These nanoparticles exhibit greater aggregation when in the presence of increasing salinities, with humic acid having a stabilizing role.

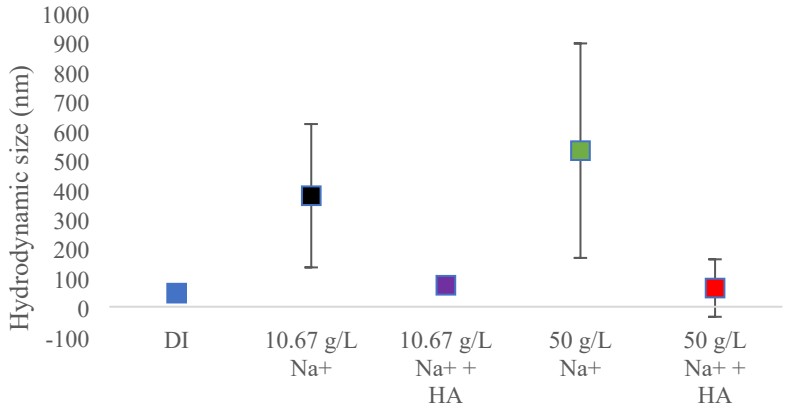


Figure S3: Stability of 100 mg/L sulfate-modified PS under different salinities and with the addition of 5 mg/L HA. Points show mean hydrodynamic size, with bars showing +/- one standard error of the mean.

Culturing and Setup details

Artemia franciscana cysts (BrineShrimp) were maintained at -20^{o}C. Eggs were transferred into 0^{o}C prior to hatching. We hatched cysts by immersing them in 50% ASW in DI water solution in plastic petri dishes with minimal volume to enhance oxygen diffusion. Hatching was conducted in an enclosed plastic box to avoid contamination and a gentle humidified air stream was provided. Most eggs were observed to hatch within 30 hours. After 48 hours to allow full hatching, live brine shrimp were pipetted out of the medium. The aid of LED lights enabled separation from dead eggs and empty shells. Hatched brine shrimp were transferred into a 30 mL flask, and four samples of 100 µL of the live brine shrimp solution were pipetted onto a kimwipe paper. Brine shrimps in each samples were counted with a handheld microscope to estimate the number concentration of individuals per mL in the flask. With this approach, we calculated the volume of hatched shrimp solution expected to contain 15 individuals, and pipetted this volume into each well of a 48-wellplate. We then added the volume of media, salt and nanoplastics solutions to achieve treatment concentrations in a total of 200 µL per well. The 200 µL volume allows for easy tracking and counting of organisms in each well by maintaining a small depth of solution, while allowing for adequate survival as similarly implemented elsewhere.[35]

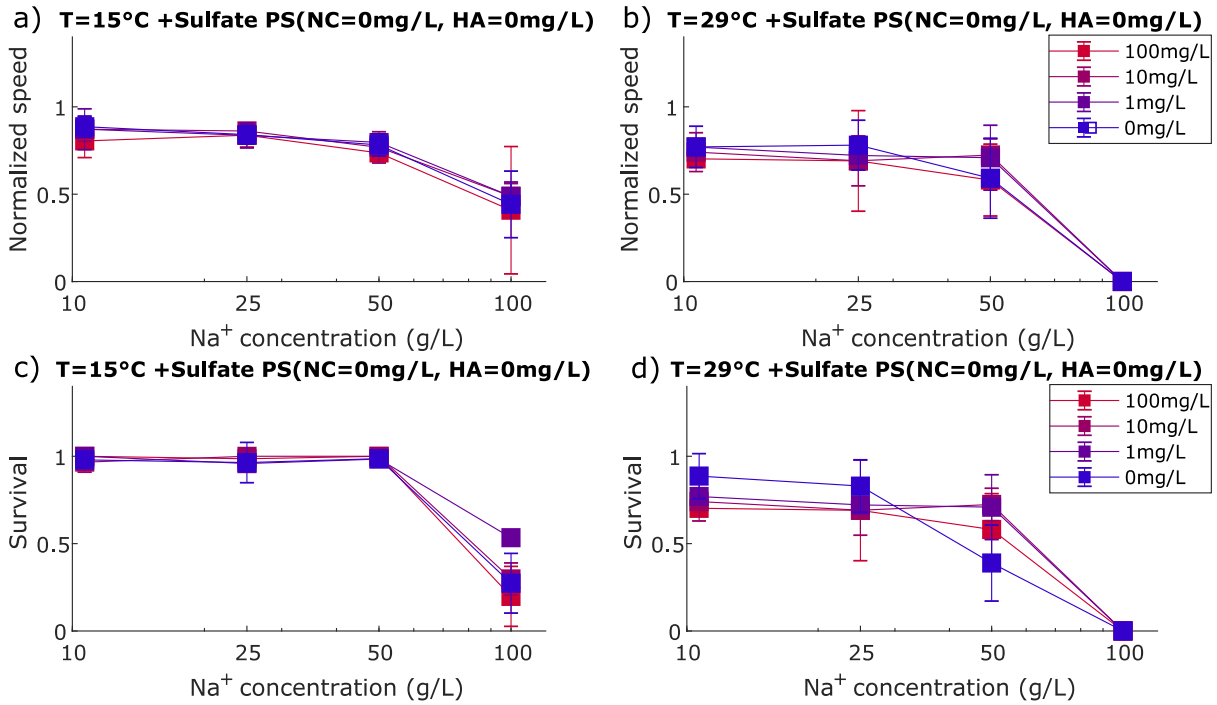


Figure S4: **Impact of multiple stressors on the normalized speed and survival of *A. franciscana*.** Combination of sulfate-modified nanoplastic (negatively charged, NP) and salinity levels on normalized speed under 15 °C (a) and 29 °C (b), and on survival under 15 °C (c) and 29 °C (d), with levels of salinity (Na^+ concentration) separated along the x-axis. Points are treatment means, and bars show ± 1 standard error of the mean. Treatments that have the same concentration of nanoplastics are connected by lines and distinguished by color.

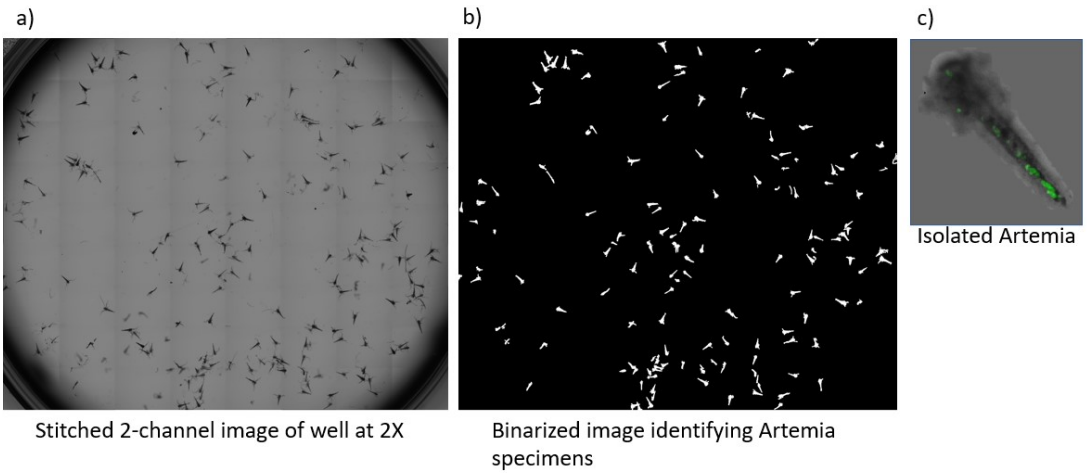


Figure S5: Imaging of dead *A. franciscana* to quantify nanoplastic uptake. Filtered specimens are placed in a 6-wellplate, and are imaged with both optical and GFP-fluorescence microscopy (a), followed by binarization of image to identify where *A. franciscana* are located in the image (b-c).

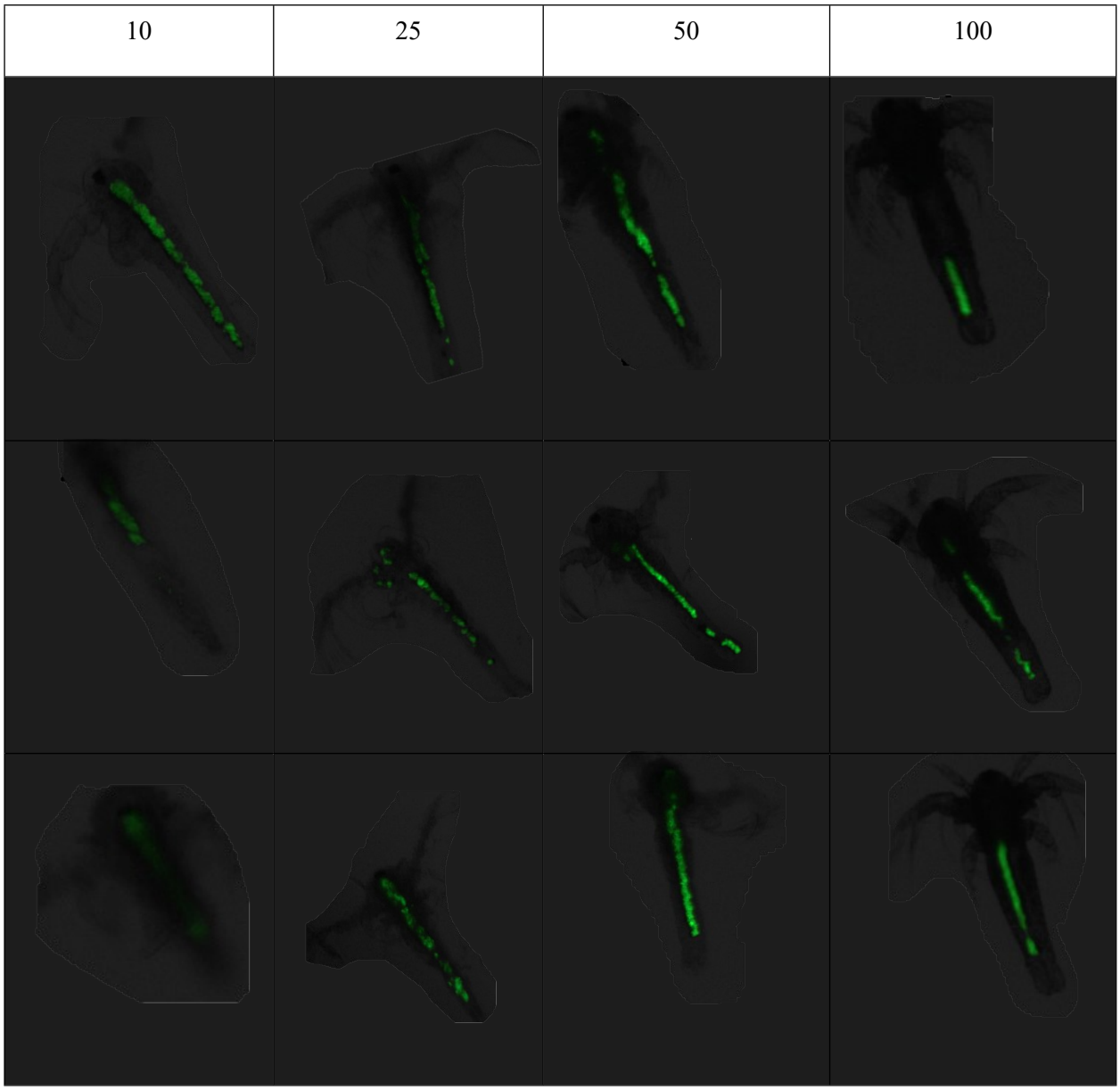


Figure S6: Sample of fluorescence images at different salt levels (g Na+ /L on top) showing the uptake of nanoplastics by *Artemia franciscana*

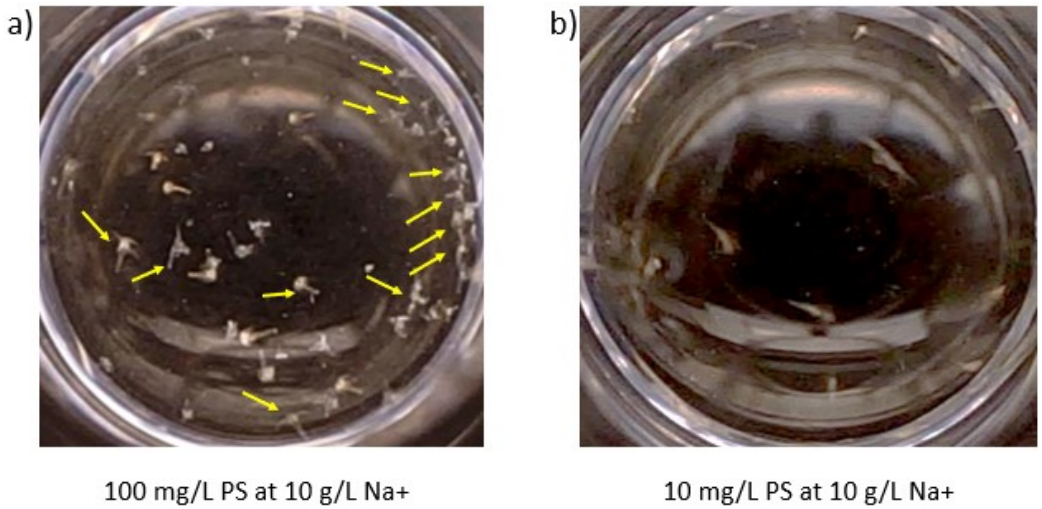


Figure S7: Several molting events observed at high Amine-modified nanoplastic concentration (a), but none were observed at the low concentrations (b). Yellow arrows indicated molted exoskeletons.

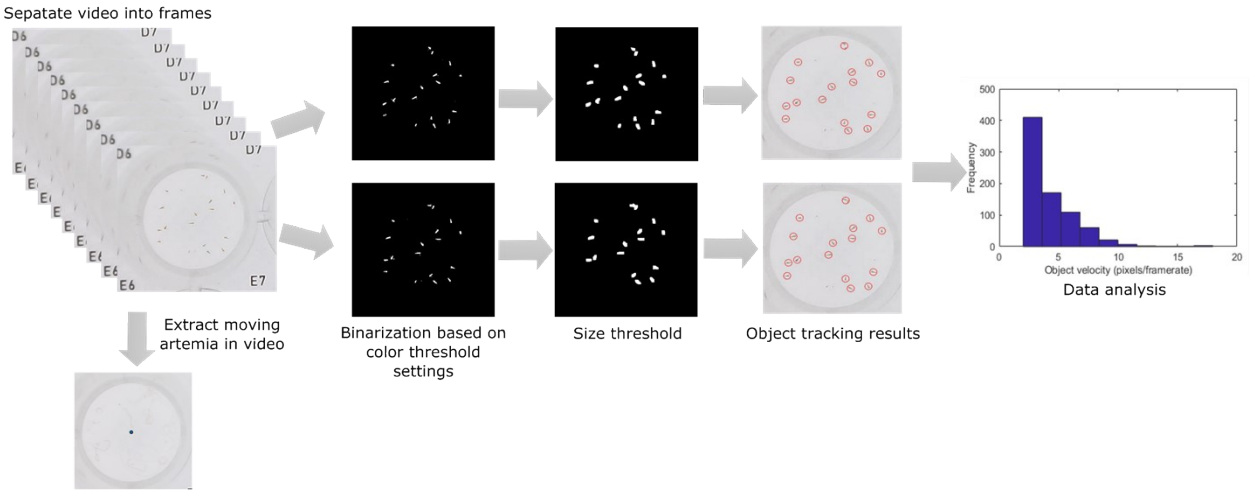
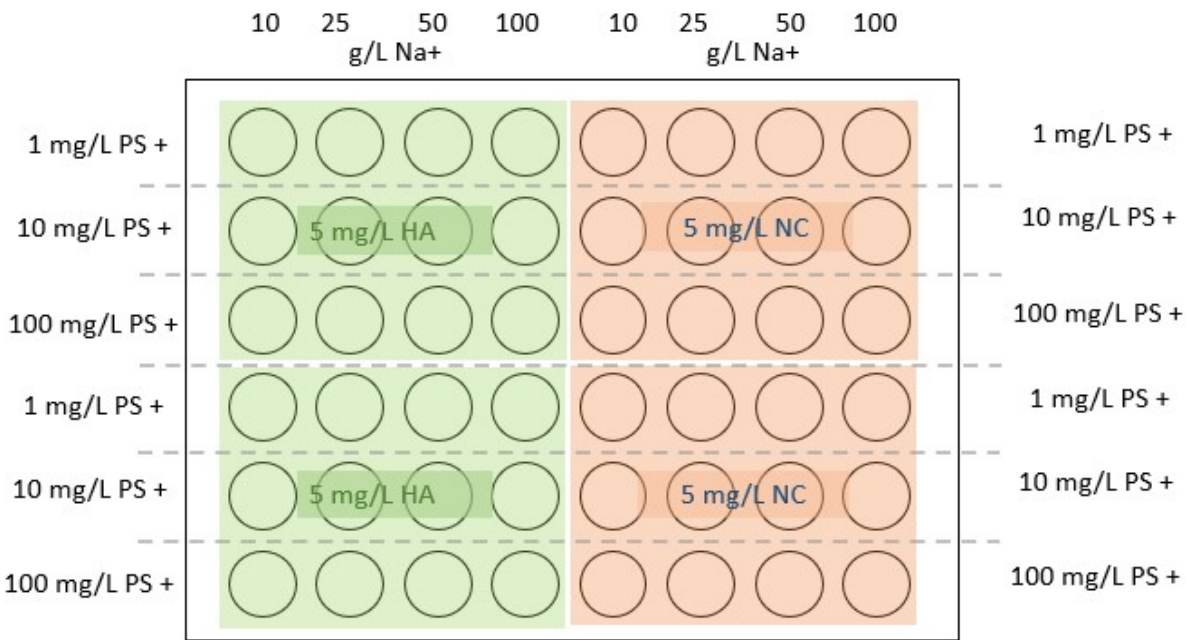


Figure S8: Image segmentation for object detection and tracking using *A. franciscana*. We conducted object tracking by first detecting well and defining its area for each separate frame of the recording, followed by color-threshold to identify *A. franciscana* in each frame, in which we impose a binary mask. We threshold the size to remove noise from the color threshold. This allows us to define where the *A. franciscana* are located, while also quantifying its motion per frame. Note that we prevent code from predicting *A. franciscana* to be located at the edge or outside of the well by restricting the radii of all wells.

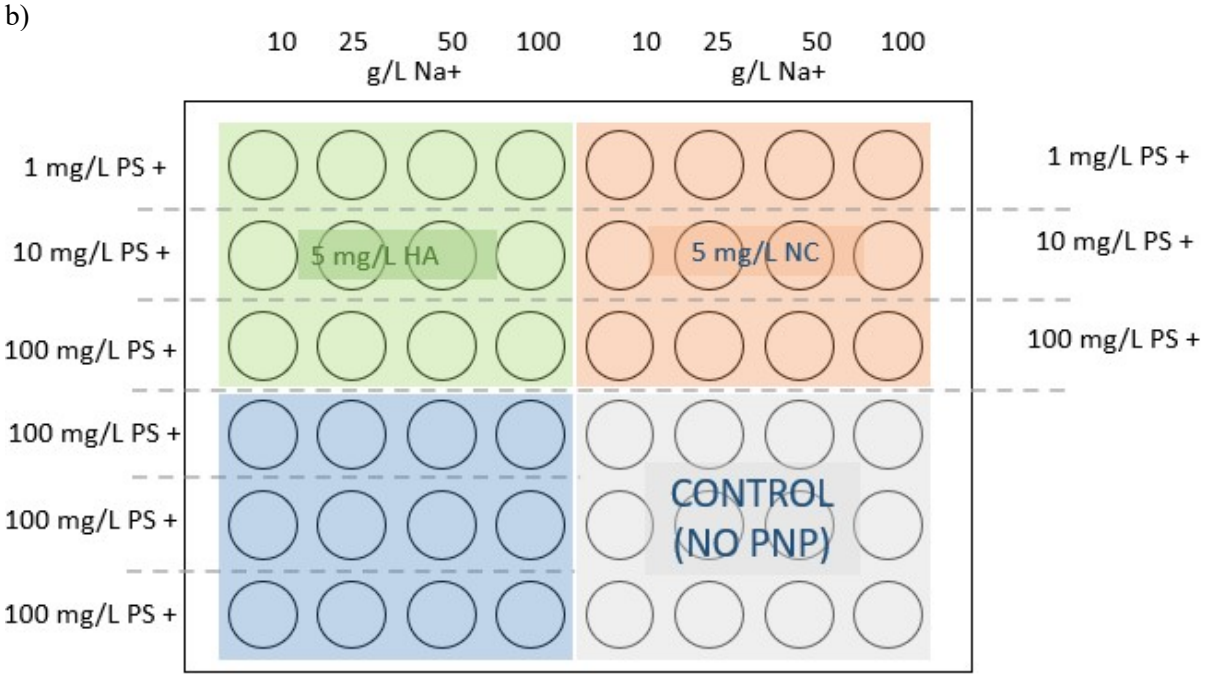
Table S1: Selected ranges of parameters

NP (+ or -) concentration (mg/L)	Na+ concentration (g/L)	Temperature (°C)	NC concentration (mg/L)	HA concentration (mg/L)
0	10.67	15	0	0
1	25	22	5	5
10	50	29		
100	100			

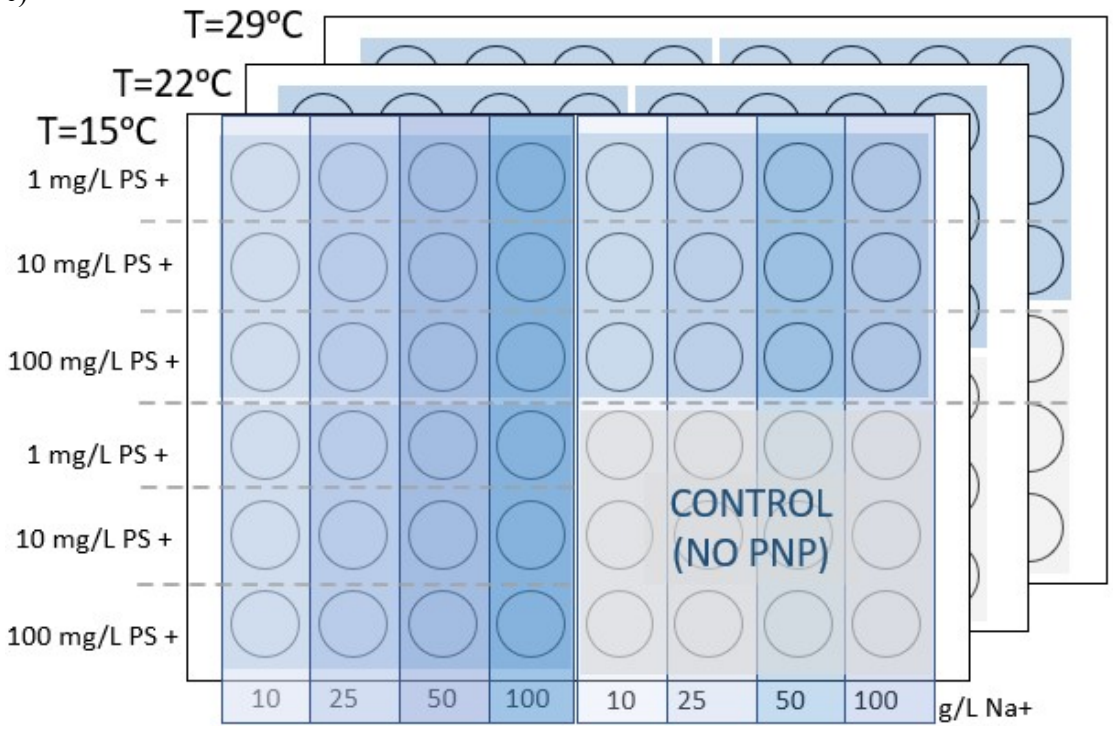
a)



b)


10 25 50 100
g/L Na+
10 25 50 100
g/L Na+
1 mg/L PS +
10 mg/L PS +
100 mg/L PS +
100 mg/L PS +
100 mg/L PS +
100 mg/L PS +
5 mg/L HA
5 mg/L NC
CONTROL
(NO PNP)
1 mg/L PS +
10 mg/L PS +
100 mg/L PS +

c)


T=29°C
T=22°C
T=15°C
1 mg/L PS +
10 mg/L PS +
100 mg/L PS +
1 mg/L PS +
10 mg/L PS +
100 mg/L PS +
CONTROL
(NO PNP)
10 25 50 100 10 25 50 100
g/L Na+

d)

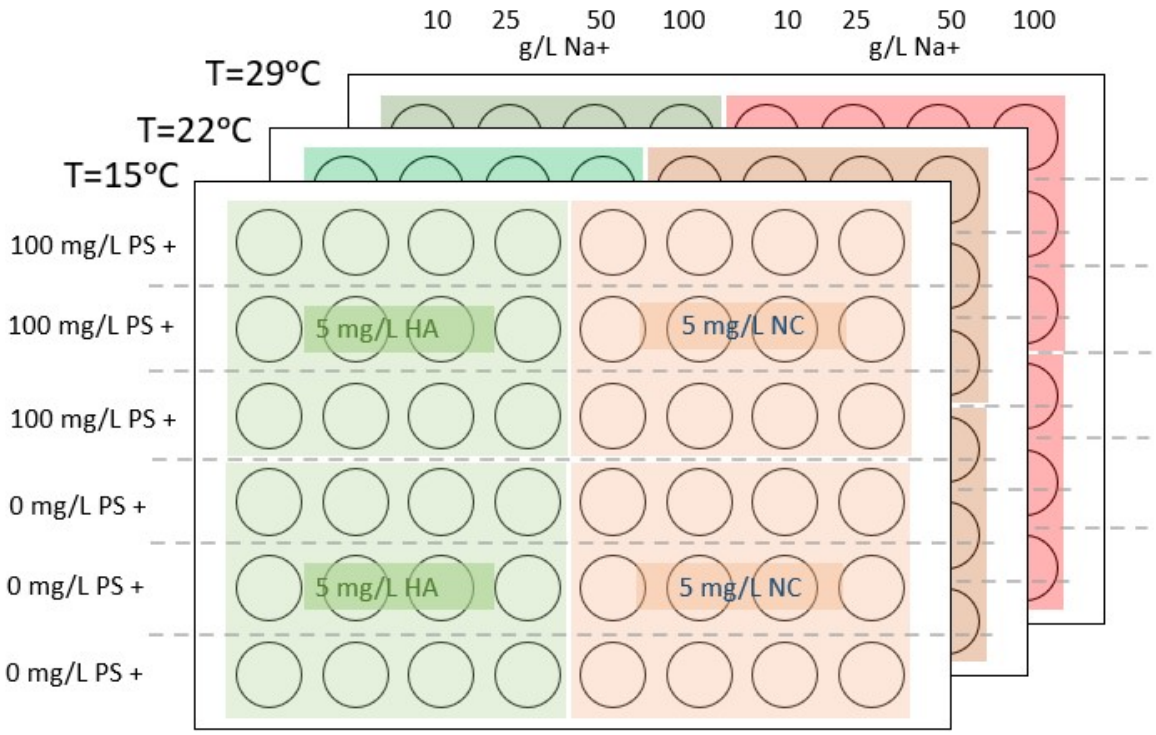


Figure S9: Experiment design in 48 well plates to evaluate the combined effects for nanoplastics with salinity, NC, HA and temperature. a-b) Experiment with a paired-plate design combining three levels of nanoplastics with the four levels of sodium concentration, with three replicates for each of these treatments in a background of HA or NC. This plate design also included a control with no nanoplastics present (grey), and a control with 100 mg/L nanoplastics (blue) in a background of 5mg/L of either HA or NC. All plates of this design were incubated at 22°C. c) Additionally, three well plates with the same combinations of NP levels and salinity levels in identical design were set up at different temperatures (15°C, 22°C and 29°C), and in combination with HA or NC for control and 100 mg/L Amine-modified NP. Designs in panels a/b and c were repeated twice. Identical experiments were conducted for the negatively charged sulfate-modified particles as shown in part d), with only two temperature levels (15°C and 29°C).

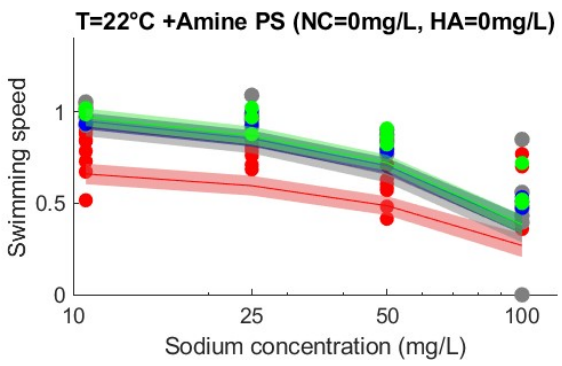
Model fitting

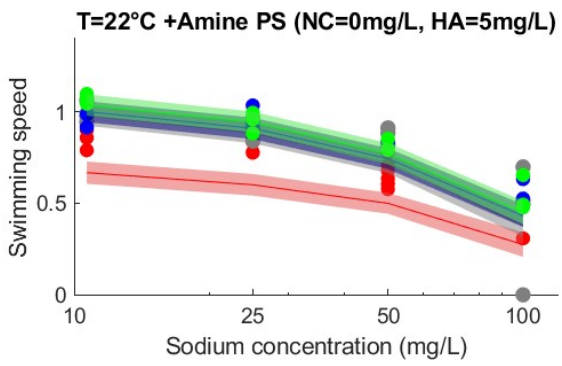
The following equation is used to fit the whole dataset:

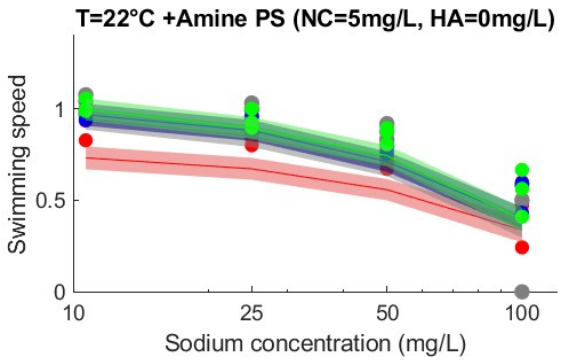
$$Y_{pred} = \beta_0 + \sum_{i=1}^{6} \beta_i X_i + \sum_{i=1}^{6} \sum_{j=1, j \neq i}^{6} \beta_{ij} X_i X_j + \sum_{i=1}^{6} \sum_{j=1, j \neq i}^{6} \sum_{k=1, k \neq j, k \neq i}^{6} \beta_{ijk} X_i X_j X_k \quad \text{(S1)}$$

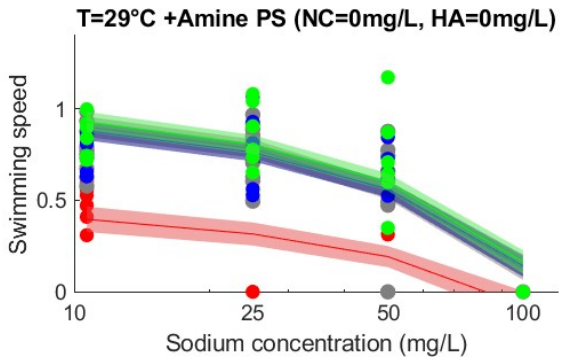
Where Y_{pred} corresponds to the predicted value of either the survival or the normalized swimming speed of organisms under each respective treatment. The terms X_i, X_j, X_k correspond to the parameter, which are standard normalized (with values ranging from -1 to 1) prior to fitting to ensure proper comparison between coefficients. The first term β_0 consists of the bias/intercept term, the indexes represent the categorical or numerical variables (1=amine-PS concentration, 2=sulfate-PS concentration, 3=sodium concentration, 4= temperature, 5=NC concentration, 6=HA concentration), the first summation corresponds to the individual effects of each parameter with, and the second summation represents the interactive effects incorporated by the linear model, with β_i and β_{ij} as the fitting coefficients. Experiment date and number of individuals in each replicate are included as random effects. We implemented this method in MATLAB 2020b.

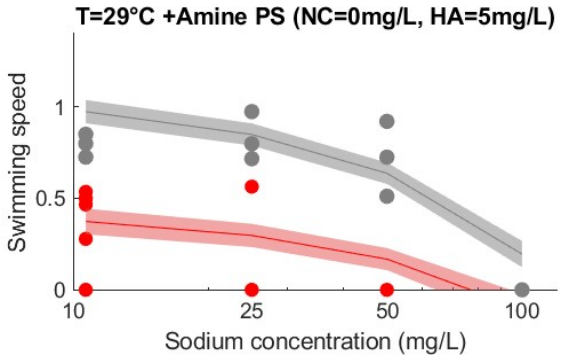
Resulting fits are shown in Figure S10, S11 and S12.

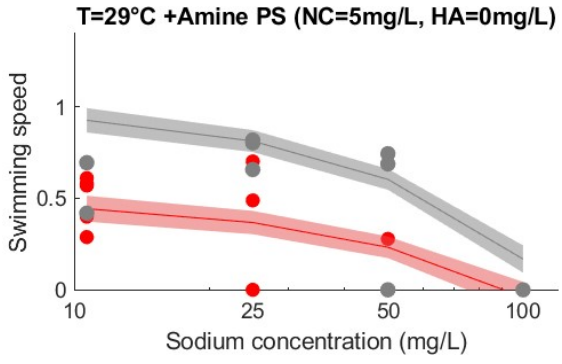

T=22°C +Amine PS (NC=0mg/L, HA=0mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)

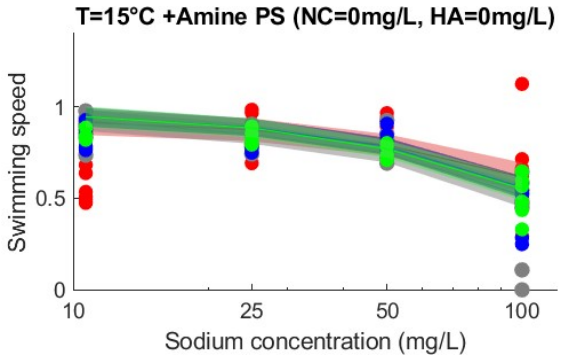

T=22°C +Amine PS (NC=0mg/L, HA=5mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)

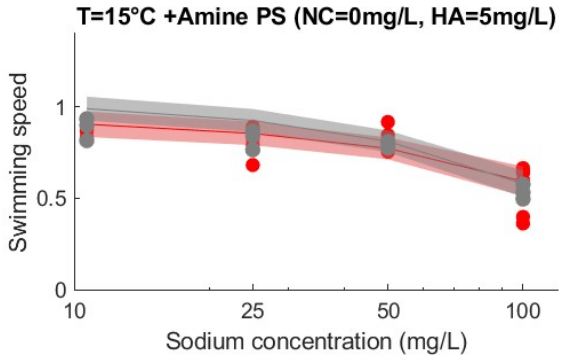

T=22°C +Amine PS (NC=5mg/L, HA=0mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)


T=29°C +Amine PS (NC=0mg/L, HA=0mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)


T=29°C +Amine PS (NC=0mg/L, HA=5mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)


T=29°C +Amine PS (NC=5mg/L, HA=0mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)


T=15°C +Amine PS (NC=0mg/L, HA=0mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)


T=15°C +Amine PS (NC=0mg/L, HA=5mg/L)
Swimming speed
0
0.5
1
10
25
50
100
Sodium concentration (mg/L)

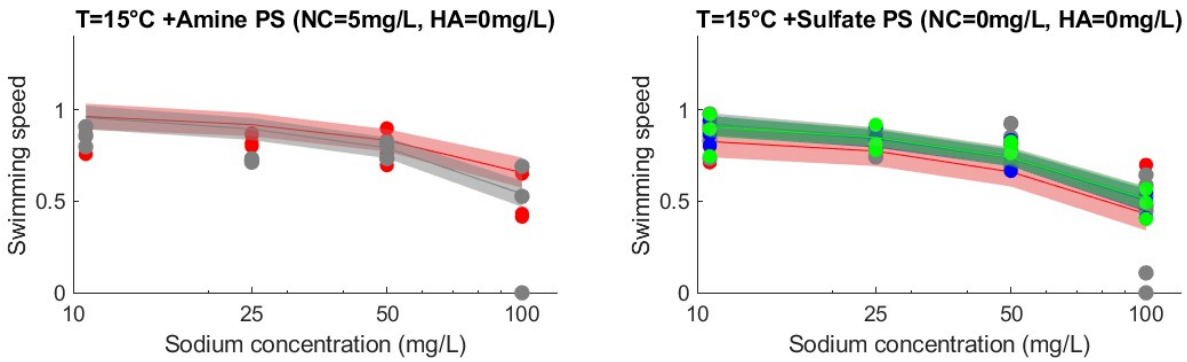
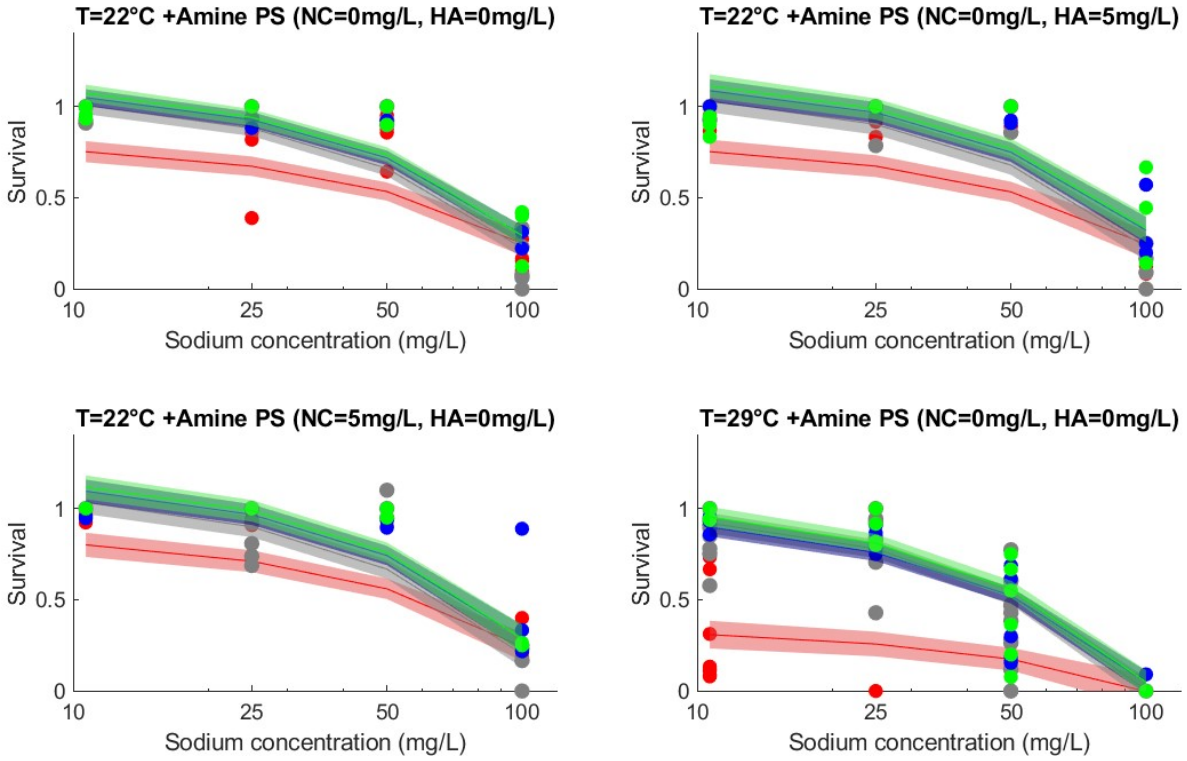


Figure S10: Sample of results showing linear fit of swimming speed data (concentrations of nanoplastics represented by different colors, red=100mg/L, blue=10mg/L, green=1mg/L, gray=0mg/L). Points represent the collected data, including all replicates. Lines correspond to the fitted linear model with shaded regions accounting for the confidence interval.



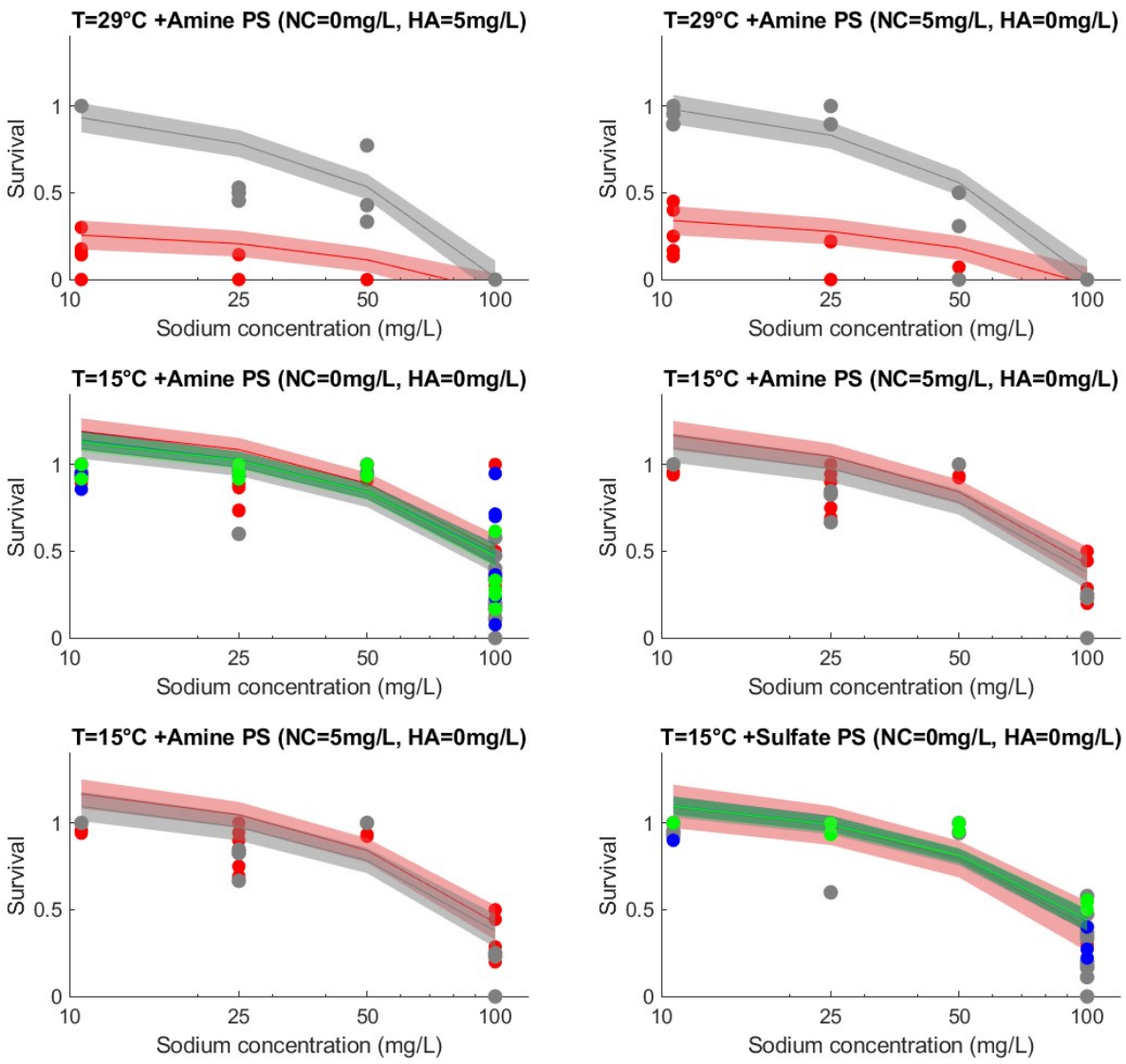


Figure S11: Sample of results showing linear fit of survival after 48 hours (concentrations of nanoplastics represented by different colors, red=100mg/L, blue=10mg/L, green=1mg/L, gray=0mg/L). Points represent the collected data, including all replicates. Lines correspond to the fitted linear model with shaded regions accounting for the confidence interval.

Parameter Name	Estimate	p-value
(Intercept)	2.64E+03	4.76E-79
Temperature	-1.50E+03	8.51E-28
Salinity	-7.84E+02	1.19E-08
NC	-3.29E+03	3.49E-90
HA	-3.44E+03	3.18E-98
Temperature:Salinity	5.80E+02	2.43E-05
Temperature:NC	1.89E+03	4.72E-31
Salinity:NC	1.04E+03	7.43E-11
Temperature:HA	2.07E+03	3.40E-38
Salinity:HA	1.14E+03	1.72E-12
Temperature:Salinity:NC	-6.34E+02	8.47E-05
Temperature:Salinity:HA	-6.21E+02	8.37E-05

Table S2: Generalized linear model fitting for green fluorescence intensity (adjusted R^2=0.2707).

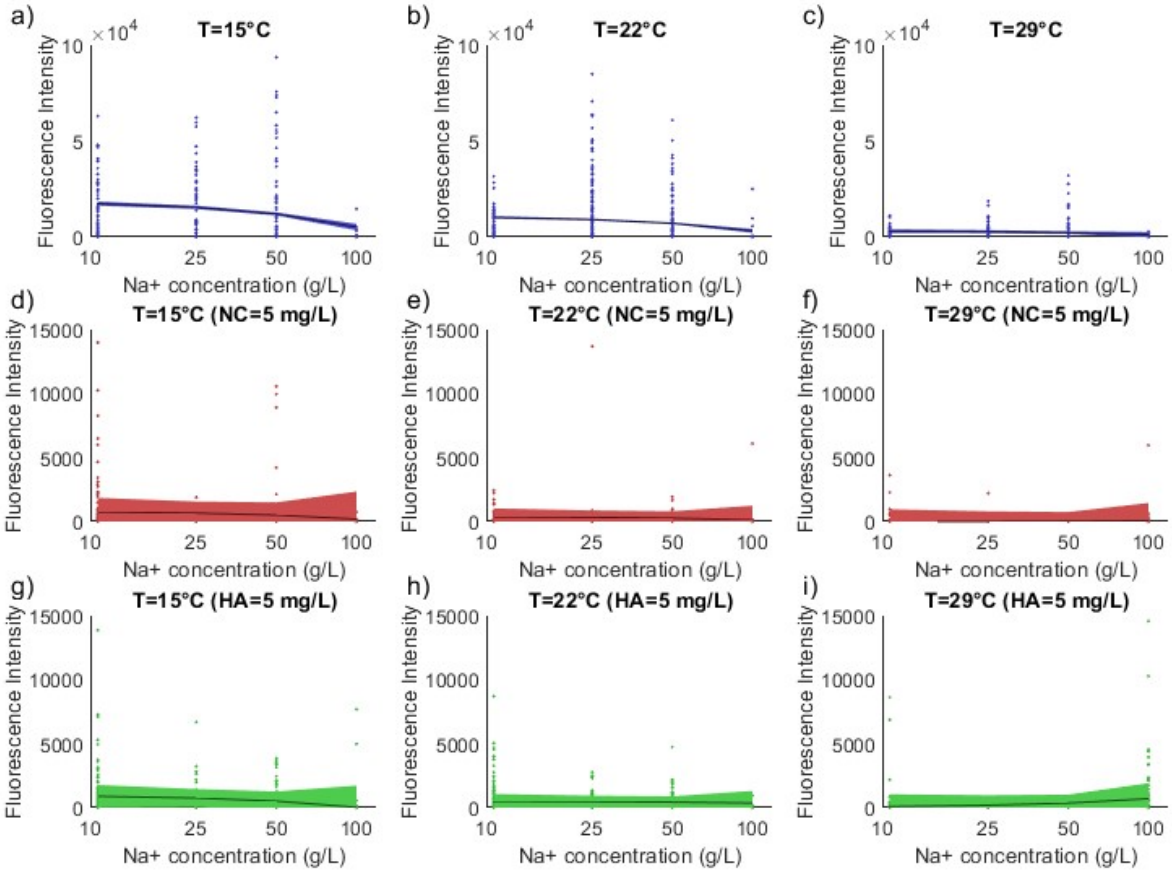


Figure S12: Data and model fit of NP uptake based on fluorescence intensity. Combination of nanoplastics and salinity levels under 15 °C (a), 22 °C (b), and 29 °C (c), with levels of salinity separated along the x-axis. (d-f) show addition of NC under same temperature and salinity

conditions as (a-c). (g-i) show addition of HA under same conditions as (a-c). Points are the total intensity per imaged organisms in each well, and lines correspond to the fitted linear model with shaded regions accounting for the confidence interval.