Electronic Supplementary Information

Reverse Trojan-horse effect decreased wastewater toxicity in the presence of inorganic nanoparticles

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Fig. S1. Amount adsorbed (size of bubble proportional to the amount adsorbed in μg/m) as a function of Dow and Kow (Table S3). The results are shown for compounds significantly adsorbed with respect to the experimental error. (Compounds numbered as in Table S3.)

Fig. S2. Percent recovery of wastewater pollutants after washing the filter two times with methanol as indicated in the text. (The results are showed only for compounds significantly adsorbed in the nanoparticles.)

Table S4. Dose-effect relationship parameters obtained using the computer software CompuSyn for wastewater (WW), individual nanoparticles and binary mixtures.

Table S5. Toxicity reported in the literature for the wastewater pollutants identified in this work.

Fig. S3. Staining and visualization of lipid droplets. Representative confocal images of Anabaena sp. PCC 7120 CPB4337 cells (a) non exposed and cells exposed to (b) 451.5 mg/L of SiO2, and (c) 442.6 mg/L of SiO2-NH2 nanoparticles. Images are (left to right) bright field, chlorophyll fluorescence (red), and Bodipy 505/515 fluorescence (green). Arrows indicate single cells detached from filaments.

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Experimental Section

Combination index (CI) for determining combined toxicities. The response to combined toxicities exposure in *Anabaena* CPB4337 test was estimating using the median-effect equation (Chou and Talalay, 1984) based on the mass-action law:

\[
\frac{fa}{fu} = \left( \frac{D}{D_m} \right)^m
\]

where \(fa\) is the fraction affected by a certain dose, \(D\), expressed as concentration of toxicant, \(fu\) is the unaffected fraction \((fa = 1-fu)\), \(D_m\) represent the dose for 50% effect (median effect-dose, EC\(_{50}\)), and \(m\) is the coefficient of the sigmoidicity of the dose-effect curve: \(m = 1, m > 1,\) and \(m < 1\) indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively. Therefore, the method takes into account both the potency \((D_m)\) and shape \((m)\) parameters. Eq.1 may be arranged as follows:

\[
D = D_m \left( \frac{fa}{1-fa} \right)^{1/m}
\]

The \(D_m\) and \(m\) values for each individual nanoparticle and wastewater, or mixture were determined by the median-effect plot: \(x = \log(D)\) versus \(y = \log(\frac{fa}{fu})\), which is based on the logarithmic form of Eq. (1). In the median effect plot, \(m\) is the slope and \(\log(D_m)\) is the \(x\)-intercept. The conformity of the data to the median-effect principle can be ready assessed by the linear correlation coefficient \((r)\) of the data to the logarithmic form of Eq.2. These parameters were then used to calculate doses of individual compounds and their combinations required to produce various effect levels according to Eq. (1).

Combination index values (CI) for each effect level were calculated according to the general CI equation (Chou, 2006):

\[
n_{(CI)_x} = \frac{n}{\sum_{j=1}^{n} (D_{m_j})} = \frac{n}{\sum_{j=1}^{n} [D_j]} \left( \frac{\sum_{j=1}^{n} [D_j]}{[D]} \right)
\]

where \(n\) \((CI)_x\) is the combination index for \(n\) chemicals at \(x\)% inhibition; \((D_x)_{j-n}\) is the sum of the dose of \(n\) chemicals that exerts \(x\)% inhibition in combination, \((\frac{[D_j]}{\sum_{i=1}^{n} [D_i]})\) is the proportionality of the dose of each of \(n\) chemicals that exerts \(x\)% inhibition in combination; and \((D_{m_j}) (\frac{fa_{x_j}}{[1-(fa_{x_j})]^{1/m_j}}\) is the dose of each drug alone that exerts \(x\)% inhibition. From Eq. (3), \(CI < 1\), \(CI = 1\) and \(CI > 1\) indicates synergism, additive effect and antagonism, respectively (Chou 2006).

Apparent octanol-water distribution coefficient. The pH-dependent or apparent octanol-water distribution coefficient, \(D_{ow}\), which considers the dissociation constant of acidic or basic solutes, \(pK_a\), and the current pH of wastewater, can be derived from the Herderson-Hasselbalch equations (Scheytt et al., 2005). For acidic and basic compounds, the equations are as follows:
In the case of compounds with two pKₐ, both acidic or both basic, the apparent partition coefficient can be calculated from the following equations:

For two acidic groups:

\[
D_{ow} = \frac{K_{ow}}{1 + 10^{pH - pK_{a1}}} + \frac{K_{ow}}{\left(10^{pH - pK_{a1}}\right)\left(10^{pH - pK_{a2}}\right)}
\]

For two basic groups:

\[
D_{ow} = \frac{K_{ow}}{1 + 10^{pK_{a1} - pH}} + \frac{K_{ow}}{\left(10^{pK_{a1} - pH}\right)\left(10^{pK_{a2} - pH}\right)}
\]

For compounds in which acidic and basic groups coexist, the following equation stands if pKₐ(base) > pKₐ(acid):

\[
D_{ow} = \frac{K_{ow}}{1 + 10^{pH - pK_{a1}}} + 10^{pK_{a1} - pH}
\]

In this case, the compound can be neutral for a certain pH interval. Conversely, if pKₐ(acid) > pKₐ(base) the compound is always charged, with a pH zone in zwitterionic form and Dₐₐ doesn't apply.

For neutral substances, \(D_{ow} = K_{ow}\).

**Transmission electron micrographs of cyanobacterial cells.** High-resolution transmission electron microscopy (TEM) images were taken on a JEOL JEM 1400 microscope operating at 100 kV in combination with energy dispersive X-ray spectroscopy (EDS). TEM samples were prepared as follows. *Anabaena* CPB4337 cells were exposed to the EC₅₀ of each nanoparticle or wastewater dilution, and to binary combinations with a fixed ratio for 24 h. Non-exposed cyanobacterial cells (control) and exposed cells were collected by centrifugation, washed three times in phosphate buffer 0.1 M, pH 7.2 for 10 min, and fixed using 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer for 4 h at 4 °C. The samples were subsequently rinsed three times with phosphate buffer and stored at 4 °C overnight. Postfixation was performed on 1 mm 2% agar blocks using 1% osmium tetroxide in distilled water for 1 h at room temperature. The samples were rinsed with three more times and dehydrated through a graded acetone series of 30-50-70-80-90-95-100% for 15 min. Infiltration and embedding of Spurr-resin was conducted by increasing resin concentrations in acetone (25%, 50%, 75% and 100%) for 15 min. Infiltration and embedding of Spurr-resin was conducted by increasing resin concentrations in acetone (25%, 50%, 75% and 100%). The samples were subsequently embedded in pure resin at room temperature overnight. Finally, polymerization resin polymerization tool place at 60 °C for 48 h. The sectioned samples in semi-thin (0.5 µm) and ultra-thin sections (60 nm) were stained with uranyl acetate and lead citrate.

**Staining and observation of lipid droplets.** The staining of lipid droplets was performed using borondipyrromethene difluoride (Bodipy) 505/515 as described Cooper et al. (2010). *Anabaena* CPB4337 cells were stained in vivo with a 50 µM aqueous solution of Bodipy 505/515 dissolved in DMSO (1%) to achieve a final concentration of 1.5 µM (0.03
% DMSO). Upon addition of the fluorochrome, *Anabaena* cells were incubated in the dark for 20 min at room temperature prior to visualization. Bodipy 505/515 fluorescence was visualized using confocal fluorescence microscope (Espirital Leica TCS SP5) with excitation at 488 nm. The emission filter was settled at 665 nm for chlorophyll fluorescence and at a window of 510-550 nm for Bodipy 505/515 fluorescence. Images were acquired with a Leica Confocal Software (LCS Lite). All comparative images were obtained under identical microscope and camera settings.

**References**


Table S1. Properties of studied nanomaterials. Average particle diameter (DLS) and ζ-potential of suspensions in water, AA/8+N and wastewater at pH 8 after 24 in contact.

<table>
<thead>
<tr>
<th>Particle</th>
<th>Concentration (mg/L)</th>
<th>Water*</th>
<th>AA/8+N</th>
<th>Wastewater</th>
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<tr>
<td></td>
<td>d (nm)</td>
<td>ζ-potential (mV)</td>
<td>d (nm)</td>
<td>ζ-potential (mV)</td>
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<td>-</td>
</tr>
<tr>
<td>AA/8+N</td>
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<td>180 ± 35</td>
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<td>443 ± 78</td>
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<td>100</td>
<td>692 ± 28</td>
<td>-26.5 ± 0.3</td>
<td>742 ± 51</td>
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<td>SiO₂-NH₂</td>
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<td>486 ± 30</td>
<td>-28.9 ± 1.3</td>
<td>190 ± 38</td>
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<td>100</td>
<td>650 ± 50</td>
<td>+3.7 ± 0.6</td>
<td>449 ± 50</td>
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<td>TiO₂</td>
<td>10</td>
<td>132 ± 7</td>
<td>+5.7 ± 0.6</td>
<td>158 ± 59</td>
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<td>100</td>
<td>445 ± 96</td>
<td>+42.5 ± 2.1</td>
<td>591 ± 85</td>
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<td>Fe₃O₄</td>
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<td>+32.4 ± 0.3</td>
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<td>38.6 ± 4.5</td>
<td>+42.6 ± 0.8</td>
<td>2380 ± 196</td>
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</table>

* Minor peaks occasionally detected in the tens of nanometer range.

Table S2. Wastewater characterization parameters (0.45 μm filtered samples).

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<th>value</th>
<th>Anions and cations</th>
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<td>Turbidity (NTU)</td>
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<td>Chloride</td>
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<td>Conductivity (mS/cm)</td>
<td>1.23 ± 0.03</td>
<td>Sulphate</td>
<td>143.7</td>
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<tr>
<td>COD (mg/L)</td>
<td>49.2 ± 1.2</td>
<td>Fluoride</td>
<td>&lt;0.80</td>
</tr>
<tr>
<td>NPOC (mg/L)</td>
<td>17.2 ± 0.5</td>
<td>Nitrite</td>
<td>&lt;0.10</td>
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<td>Alkalinity (mg CaCO₃/L)</td>
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<td>Bicarbonate</td>
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<td></td>
<td>Magnesium</td>
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<td>Calcium</td>
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<td>Ammonium</td>
<td>68.4</td>
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Table S3. Concentrations of pollutants in wastewater and physicochemical properties of pollutants contained in wastewater.

<table>
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<tr>
<th>No.</th>
<th>Compound</th>
<th>Concentration (ng/L)</th>
<th>CAS Number</th>
<th>Molecular formula</th>
<th>log K&lt;sub&gt;ow&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;a(1)&lt;/sub&gt;</th>
<th>pK&lt;sub&gt;a(2)&lt;/sub&gt;</th>
<th>Acid/Base</th>
<th>log D&lt;sub&gt;ow&lt;/sub&gt; *</th>
<th>Main use</th>
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<td>1</td>
<td>4-Aminoantipyrine (4-AA)</td>
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<td>Weakly basic</td>
<td>-0.07</td>
<td>Metabolite of amipyrine</td>
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<td>N-acetyl-4-aminoantipyrine (4-AAA)</td>
<td>1050</td>
<td>83-15-8</td>
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<td>-0.13</td>
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<td>-0.13</td>
<td>Metabolite of metamizole</td>
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<tr>
<td>3</td>
<td>N-formyl-4-aminoantipyrine (4-FAA)</td>
<td>904</td>
<td>1672-58-8</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;H&lt;sub&gt;13&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-0.41</td>
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<td>-0.41</td>
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<td>4</td>
<td>Antipyrine</td>
<td>49</td>
<td>60-80-0</td>
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<td>Weakly basic</td>
<td>0.38</td>
<td>Analgesic</td>
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<td>9.6</td>
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<td>Basic</td>
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<td>β-blocker</td>
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<td>8.8</td>
<td>Basic</td>
<td>-1.07 Bronchodilator/Vasodilator</td>
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<td>1.59 Antidepressant</td>
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</table>

* wastewater at pH 7.8
Fig. S1. Amount adsorbed (size of bubble proportional to the amount adsorbed in μg/g) as a function of $D_{ow}$, and $K_{ow}$ (Table S3). The results are shown for compounds significantly adsorbed with respect to the experimental error. Compounds numbered as in Table S3. a) SiO$_2$, b) SiO$_2$-NH$_2$, c) TiO$_2$, d) Fe$_3$O$_4$. 
Fig. S2. Percent recovery of wastewater pollutants after washing the filter two times with methanol as indicated in the text. (The results are showed only for compounds significantly adsorbed in the nanoparticles.)
Table S4. Dose-effect relationship parameters obtained using the computer software CompuSyn for wastewater (WW), individual nanoparticles and binary mixtures.

<table>
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<tr>
<th>Drug combo</th>
<th>Dose-effect parameters</th>
<th>(D_m)</th>
<th>(m)</th>
<th>(r)</th>
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<td>WW</td>
<td>(D_m)</td>
<td>1.08</td>
<td>0.98</td>
<td>0.97</td>
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<td>SiO(_2)-wastewater</td>
<td>SiO(_2)</td>
<td>402</td>
<td>0.41</td>
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<td>WW-SiO(_2)</td>
<td>628</td>
<td>0.49</td>
<td>0.92</td>
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<tr>
<td>SiO(_2)-NH(_2)-wastewater</td>
<td>WW</td>
<td>1.08</td>
<td>0.92</td>
<td>0.97</td>
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<td>SiO(_2)-NH(_2)</td>
<td>440</td>
<td>0.33</td>
<td>0.93</td>
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<td>SiO(_2)-NH(_2)-WW</td>
<td>244</td>
<td>0.58</td>
<td>0.94</td>
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<td>TiO(_2)-wastewater</td>
<td>TiO(_2)</td>
<td>17.8</td>
<td>0.66</td>
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<td>TiO(_2)-WW</td>
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<td>1.08</td>
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<tr>
<td>Fe(_3)O(_4)-wastewater</td>
<td>WW</td>
<td>1.20</td>
<td>0.75</td>
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<td>Fe(_3)O(_4)</td>
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<td>0.66</td>
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<td>Fe(_3)O(_4)-WW</td>
<td>17.1</td>
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The parameters \(m\), \(D_m\) and \(r\) are the antilog of \(x\)-intercept, the slope and the linear correlation coefficient of the median-effect plot, signifying the shape of the dose-effect curve, the potency (EC\(_{50}\)), and the conformity of the data to the mass-action law, respectively. \(D_m\) and \(m\) values are used for calculating the CI values (Eq. 3, experimental section in this ESI).
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<th>Compound</th>
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<th>Species</th>
<th>Toxicological endpoint</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</th>
<th>Ref.</th>
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<td>Bioluminescence inhibition (30 min)</td>
<td>1304</td>
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<td>620</td>
<td>Cleuvers²</td>
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<td>Bioluminescence inhibition (15 min)</td>
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<td>178.73 (162.06–197.12)</td>
<td>Rosal et al.⁴</td>
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**NE=No effect at “x” (mg/L)**
References for Table S5


Fig. S3. Staining and visualization of lipid droplets. Representative confocal images of *Anabaena* sp. PCC 7120 CPB4337 cells (a) non exposed and cells exposed to (b) 451.5 mg/L of SiO$_2$, and (c) 442.6 mg/L of SiO$_2$-NH$_2$ nanoparticles. Images are (left to right) bright field, chlorophyll fluorescence (red), and Bodipy 505/515 fluorescence (green). Arrows indicate single cells detached from filaments.