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# Environmental context determines the impact of titanium oxide and silver nanoparticles on the functioning of intertidal microalgal biofilms†

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Coastal environments are receiving habitats for most nanoparticle (NP) waste. Coastal sediments, into which NPs accumulate, support microalgal biofilms that provide important ecosystem processes: primary production, enhanced sediment stabilisation, and nutrient recycling. We assessed the impact of realistic concentrations of titanium oxide (TiO<sub>2</sub>) and silver (Ag) NPs on marine microalgal biofilms and associated ecosystem processes in simulated natural conditions, by exposing natural biofilms to TiO<sub>2</sub> and Ag-NPs for one-month periods in outdoor tidal mesocosms under three contrasted environmental contexts (seasons). Ag-NPs had no significant effects on microalgal biomass, sediment biostabilisation potential and sediment-water oxygen and nutrient fluxes, even at concentrations (25 µg l<sup>-1</sup>) higher than current estimated levels (25 ng l<sup>-1</sup>). TiO<sub>2</sub>-NPs had no significant effect at current expected concentrations (25 µg l<sup>-1</sup>), but higher concentrations (25 mg l<sup>-1</sup>) resulted in decreased microalgal biomass; decreased ability of biofilms to biostabilise sediment, therefore limiting their coastal protection potential; reduced primary production and modified nutrient recycling. TiO<sub>2</sub>-NPs impacts were dependent on the environmental context: most effect was seen in winter, while no toxicity on biofilms was demonstrated in early spring. Our findings demonstrate that while Ag-NPs, being liable to dissolution into Ag<sup>+</sup> ions under the conditions tested, are not expected to have an environmental impact if current predictions of environmental loading prevail, TiO<sub>2</sub>-NPs may have ecological consequences in coastal environments in addition to direct impacts on microbial biomass.

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## Environmental significance

Ag and TiO<sub>2</sub> nanoparticles are emerging contaminants of coastal waters. Their toxicology has been well studied in laboratory conditions, but their real impact on coastal ecosystems is poorly characterised. We demonstrated that under environmentally relevant conditions TiO<sub>2</sub>-NPs can have a negative effect on multiple coastal processes, such as coastal protection potential, oxygen fluxes and nutrient recycling. These effects were dependent on environmental context (season of year) and measurements of biomass alone are not sufficient to evaluate impacts on ecosystem functioning. Our research therefore both provides a first step towards the comprehension of NP impact in coastal environments, and guides future research by demonstrating the importance of assessing impacts in a variety of contexts, and directly on ecosystem processes.

## Introduction

The production of engineered nanomaterials has increased since the early 1990s. Titanium oxide (TiO<sub>2</sub>) nanoparticles (NPs) are produced in great quantities,<sup>1,2</sup> possess photocatalytic and anti-fouling properties and are widely

used in paints, sunscreens and other cosmetics, and food packaging.<sup>3–5</sup> Silver NPs (Ag-NPs) are used in many products;<sup>6</sup> for example, in textiles, medical materials and household appliances because of their anti-microbial properties.<sup>6,7</sup>

A consequence of the development and use of products containing NPs is the production of “nano-waste” and the release of NPs to the environment.<sup>4,7–9</sup> The efficiency of sewage treatment works (STW) in removing NPs from their waste water streams can be high, reaching 50–90%.<sup>10,11</sup> For instance, sulfidation of Ag-NPs in STW will limit, but not suppress, the release of Ag-NPs into surface waters.<sup>10–12</sup> TiO<sub>2</sub>-NPs do not dissolve or transform easily and can be released in the environment through STW discharge.<sup>13</sup> Through these

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STW discharges, rivers and runoff waters, coastal habitats are the recipient of large amounts of NPs.<sup>4,15</sup> In addition to these inputs, TiO<sub>2</sub>-NPs can be released directly in coastal waters as they are an important component of sunscreens, which are currently the dominant source of TiO<sub>2</sub>-NPs in the marine environment.<sup>16,17</sup>

The high ionic strength of seawater may promote NP aggregation, as NPs become less stabilised by electrical repulsive force in seawater.<sup>5,18,19</sup> Aggregation can then lead to increased deposition into sediments.<sup>20</sup> Ag-NPs fate in seawater will depend on their size, concentrations, and concentrations of other ions, such as Cl<sup>-</sup> and S<sup>2-</sup>.<sup>6</sup> Up to 90% of Ag-NPs can deposit from seawater to sediment in less than 24 h.<sup>20</sup> TiO<sub>2</sub>-NPs can also be incorporated into natural organic matter aggregates (marine snow), increasing their deposition onto sediments.<sup>18,19</sup> These processes mean that sediment-inhabiting (benthic) organisms may have the highest exposure to NPs arriving in coastal waters.<sup>4,21</sup>

In coastal environments, photosynthetic microbial biofilms dominated by microalgae (predominantly benthic diatoms) are the main primary producers of unvegetated mudflats.<sup>23–25</sup> These microphytobenthic biofilms are of key importance for the functioning of coastal systems, where they influence the recycling of nutrients,<sup>26</sup> form the basis of food webs<sup>27,28</sup> and become a potential entry point of NPs in marine trophic networks.<sup>21,29</sup> Biofilms stabilise sediments, thus promoting coastal protection by limiting sediment erosion.<sup>30,31</sup> In culture, microalgal growth can be reduced at Ag-NP concentrations from 200 µg l<sup>-1</sup>,<sup>6</sup> and at TiO<sub>2</sub>-NP concentrations from 5 mg l<sup>-1</sup>.<sup>32</sup> In periphytic biofilms, concentrations between 1 to 5 mg l<sup>-1</sup> of TiO<sub>2</sub>-NPs reduced microalgal development and enhanced the biofilm metabolism,<sup>33</sup> through an alteration of the composition of the microbial community.<sup>33,34</sup> Impacts of Ag and TiO<sub>2</sub>-NPs on coastal microphytobenthic biofilms have the potential to substantially influence the functioning of coastal systems, and decrease the ecosystem services they provide to human populations.<sup>31,35</sup>

The toxicity of TiO<sub>2</sub>-NPs and Ag-NPs towards aquatic and marine microorganisms (both microalgae and bacteria) is clearly demonstrated in cultures under laboratory conditions. However, experiments conducted in more complex environments to determine if current levels of exposure to NPs are endangering microorganisms or microbial processes are more equivocal. Ag-NPs appear to have limited toxicity on the abundance of, and processes driven by, microorganisms in freshwater or estuarine biofilms,<sup>15,36–39</sup> except at concentrations (1000 µg l<sup>-1</sup>) far higher than modelled environmental concentrations (1–100 ng l<sup>-1</sup>).<sup>40–42</sup> Shifts in species composition of bacterial communities have been recorded at Ag-NP concentrations of 200 µg l<sup>-1</sup>.<sup>36,39,43,44</sup> The link between alteration of community composition by NPs and modification of associated processes is not straightforward; for instance, bacterial activity to degrade hydrocarbons can be maintained in presence of Ag-NPs, despite significant modification of the composition of the

bacterial communities.<sup>36</sup> Toxicity of NPs to microorganisms has also been shown to be taxon specific,<sup>37</sup> and influenced by environmental parameters, such as presence of UV light for TiO<sub>2</sub>-NPs<sup>2</sup> and Ag-NPs,<sup>45</sup> and temperature for Ag-NPs.<sup>46</sup>

In this study, we used environmentally relevant experimental conditions<sup>47</sup> to determine if TiO<sub>2</sub>-NPs and Ag-NPs affect coastal biofilms and their associated ecosystem processes. TiO<sub>2</sub> were chosen as the most produced NP,<sup>1,2</sup> and Ag-NPs as the NP used in the highest diversity of products.<sup>6</sup> Both have also been identified as NPs “of concern” regarding environmental degradation,<sup>48</sup> and are likely to behave differently in the environment, given that Ag-NPs are soluble and redox sensitive, whereas TiO<sub>2</sub>-NPs are relatively inert and biopersistent.<sup>5</sup> We performed experiments on natural intertidal biofilms, maintained in outdoor tidal mesocosms subject to natural weather conditions during three contrasted periods of the year. We simulated the constant inputs of NPs into coastal waters by dosing the mesocosms with close to expected natural concentrations of NPs (based on published modelled and measured concentrations) at regular intervals over a 28 day-period. In addition, we used a 1000 times higher concentration of TiO<sub>2</sub> and Ag-NPs, to allow for comparison with other laboratory and mesocosms studies, as well as a combined TiO<sub>2</sub> + Ag-NP treatment to look for potential synergistic or antagonistic effects.<sup>49</sup> We analysed the effects of NPs on microphytobenthic biomass, and also on biofilm-associated ecosystem processes: the ability to biostabilise sediment (as assessed by biofilm extracellular carbohydrate matrix content); primary production, organic carbon fluxes; and the cycling of inorganic nutrients across the sediment–water interface.

We hypothesised (1) that at current concentrations NPs will have little impact on biofilm biomass or ecosystem processes, while higher concentrations would have acute and chronic impact on both biomass and processes. (2) That changes in ecosystem processes will be dependent on changes in microalgal biomass. Finally, as microphytobenthic biofilms undergo a seasonal cycle of species-changes<sup>50,51</sup> and altered ecophysiology throughout the year,<sup>52</sup> we hypothesised (3) that the impact of NPs will depend on the physiological condition of the biofilm throughout the seasonal cycle, especially as such dynamics are associated with seasonal variations in light and temperature. Based on the above hypothesis, we proposed that TiO<sub>2</sub>-NPs would be more toxic to biofilms in summer, when light and UV levels are highest.<sup>2</sup>

## Materials & methods

### Experimental set-up

Multiple cores (6.8 cm in diameter, 10 cm deep) of surface sediment supporting microphytobenthic biofilms were sampled from an intertidal mudflat on the Essex coast (51°46'45.7"N 1°02'49.0"E, UK) and placed in experimental tanks with natural seawater (ESI† Fig. S1). Microphytobenthic biofilms from this site have been characterised,<sup>53,54</sup> and are



dominated by diatoms, with a minimal contribution from cyanobacteria. Such biofilms are characteristic of diatom-dominated intertidal biofilms found widely across NW European coastal habitats.<sup>24,25</sup>

For each NP treatment (see below), 4 independent tidal mesocosms were established. Mesocosm are particularly well suited to understand the impact of NPs on complex systems such as microbial biofilms.<sup>55</sup> Each mesocosm consisted of an experimental tank containing 4 sediment cores, and a reservoir tank. Seawater was pumped in and out of the experimental tanks twice per 24 h, and advancing 1 h per day to match the natural rhythm of tides on the Essex coast. During low tide, the top of cores was exposed to air, and cores were covered with a few centimetres of water at high tide (ESI† Fig. S1).

Three 28 day long experiments were implemented, to capture different environmental contexts (Tables 1 and 2): in November to December 2016 (TiO<sub>2</sub>-NPs only – termed *winter*); one in February to March 2017 (*early spring*) and in June to July 2017 (*summer*).

The mesocosms were located outside in an unshaded area, exposed to natural light and temperature conditions (recorded every 10 minutes throughout the experiment with a HOBO logger set at the same height as the top of a core). Salinity of system water was monitored daily and kept constant by addition of seawater (after heavy rain events) or distilled water (to compensate evaporation). To prevent depletion of nutrients, all seawater was replaced with fresh natural seawater in experimental and reservoir tanks at weekly intervals.

## NP preparation and addition

We established 6 experimental treatments:

- (i) no added NP (control treatment);
- (ii) and (iii) two TiO<sub>2</sub>-NP treatments: target concentrations of 25 µg l<sup>-1</sup> and 25 mg l<sup>-1</sup>; 25 µg l<sup>-1</sup> was chosen to

approximate the estimated current concentration of TiO<sub>2</sub>-NP in wastewater treatment plant effluents;<sup>3,16,42</sup>

(iv) and (v) two Ag-NP treatments: target concentrations of 25 ng l<sup>-1</sup> and 25 µg l<sup>-1</sup>; 25 ng l<sup>-1</sup> was chosen to approximate the estimated current concentration of Ag-NP in wastewater treatment plant effluents;<sup>11,14,41</sup>

(vi) one treatment with TiO<sub>2</sub> and Ag-NP together (target concentrations of 25 µg l<sup>-1</sup> of TiO<sub>2</sub>-NP and 25 ng l<sup>-1</sup> of Ag-NP).

Uncoated TiO<sub>2</sub>-NPs (anatase:rutile (80:20 vol%), sold as 20 nm diameter) were purchased from American Elements (Los Angeles, USA). The mixture of both forms was chosen to mimic natural conditions where NPs will originate from diverse sources.<sup>3,56,57</sup> There are indeed numerous applications that do not require a specific phase of TiO<sub>2</sub> (e.g. painting, photocatalytic applications...).<sup>56,58,59</sup> PVP-coated silver nanospheres (sold as 25 nm in diameter) were bought from NanoComposix (San Diego, USA). PVP-coated NPs, which disperse easily in water, are likely to be found on coasts where most NPs will arrive from rivers and run-off water. The size of NPs in water was assessed by TEM and DLS and found different from the manufacturer's specifications for both NPs (ESI† Fig. S2a and b): TiO<sub>2</sub>-NPs were larger than expected (most individual particles had length and width between 50 and 200 nm), while Ag-NPs exhibited two peaks in the size distribution, one around 15 nm and another around 40 nm (ESI† Fig. S2 for more details on the size and shape of NPs).

NP target concentrations (see treatments above) were obtained by diluting NP stocks into the experimental mesocosms. For each TiO<sub>2</sub>-NP or Ag-NP treatment, a stock solution was prepared at the beginning of the experiment in ultra-high purity water. Immediately before each addition into the mesocosms, the stocks were sonicated for 30 min and then 9 ml of sonicated stock solution was added in each 9 l mesocosm under that treatment. The same stock solution was used for the whole experiment. The release of Ag<sup>+</sup> ions

**Table 1** Environmental conditions in the tidal tanks and seawater throughout the three experiments (mean ± standard error, measurements taken throughout the experiment), as measured by HOBO logger (temperature & light intensity), Winkler titrations (O<sub>2</sub> concentrations), Skalar Formacs<sup>HT</sup> TOC analyser (total organic carbon concentrations) and SEAL analytical AACE auto-analyser (nutrient concentrations). Please note that nutrient concentrations are reported here, whereas nutrient fluxes between water and sediment were analysed as ecosystem processes in the rest of the paper. Mean nutrient fluxes per season can be found in Table S1†

| Name                                                                 | <i>Early spring</i> | <i>Summer</i>            | <i>Winter</i>  |
|----------------------------------------------------------------------|---------------------|--------------------------|----------------|
| Month                                                                | Feb./Mar. 2017      | Jun./Jul. 2017           | Nov./Dec. 2016 |
| Temperature (°C)                                                     | 8.0 ± 0.1           | 22.3 ± 0.1               | 7.5 ± 0.1      |
| Temperature variations (°C)                                          | 0.1 to 25.8         | 9.6 to 48.8 <sup>a</sup> | −4.2 to 17.0   |
| Light intensity (µmol s <sup>-1</sup> m <sup>-2</sup> )              | 168.3 ± 5.4         | 541 ± 13                 | 61.0 ± 3.0     |
| Photoperiod (h d <sup>-1</sup> )                                     | 11.2 ± 0.7          | 17.7 ± 0.4               | 8.8 ± 0.0      |
| O <sub>2</sub> concentration (µmol l <sup>-1</sup> )                 | 228 ± 1             | 138 ± 1                  | 209 ± 4        |
| TOC (mg l <sup>-1</sup> )                                            | 14.5 ± 2.4          | 10.1 ± 0.5               | 26.3 ± 1.6     |
| NO <sub>2</sub> <sup>-</sup> concentration (µmol l <sup>-1</sup> )   | 0.42 ± 0.02         | 0.43 ± 0.02              | 0.47 ± 0.05    |
| NO <sub>3</sub> <sup>-</sup> concentration (µmol l <sup>-1</sup> )   | 19.9 ± 0.4          | 12.4 ± 0.5               | 17.3 ± 1.0     |
| NH <sub>4</sub> <sup>+</sup> concentration (µmol l <sup>-1</sup> )   | 1.81 ± 0.18         | 2.6 ± 0.15               | 1.89 ± 0.42    |
| SiO <sub>4</sub> <sup>4-</sup> concentration (µmol l <sup>-1</sup> ) | 6 ± 0.1             | 2.1 ± 0.0                | 11.4 ± 0.3     |
| PO <sub>4</sub> <sup>3-</sup> concentration (µmol l <sup>-1</sup> )  | 0.83 ± 0.04         | 0.78 ± 0.03              | 1.62 ± 0.22    |

<sup>a</sup> Temperatures over 40 °C reached around noon for a couples of hours, on 11 occasions.



**Table 2** Biological variables measured in intertidal sediments at the start of each experiment (mean  $\pm$  standard error). Chl *a*: chlorophyll *a*. Phaeophytin is a degradation form of Chl *a*

| Variable                                                                                  | Early spring     | Summer           | Winter           |
|-------------------------------------------------------------------------------------------|------------------|------------------|------------------|
| Month                                                                                     | Feb./Mar. 2017   | Jun./Jul. 2017   | Nov./Dec. 2016   |
| Chl <i>a</i> concentration ( $\mu\text{g g}^{-1}$ sed dw)                                 | 86 $\pm$ 4       | 24 $\pm$ 1       | 88 $\pm$ 8       |
| Chl <i>a</i> /phaeophytin ratio                                                           | 1.56 $\pm$ 0.04  | 1.22 $\pm$ 0.07  | 1.63 $\pm$ 0.06  |
| Colloidal carbohydrate concentration ( $\mu\text{g g}^{-1}$ sed dw)                       | 54.7 $\pm$ 2.0   | 69.8 $\pm$ 2.4   | 175 $\pm$ 147.5  |
| Carbohydrate/Chl <i>a</i> ratio                                                           | 0.71 $\pm$ 0.04  | 3.07 $\pm$ 0.13  | 2.34 $\pm$ 0.27  |
| Photosynthetic potential $F_v/F_m$                                                        | 0.45 $\pm$ 0.00  | 0.47 $\pm$ 0.00  | 0.41 $\pm$ 0.01  |
| Benthic respiration ( $\text{mmol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ ) <sup>a</sup>      | -0.99 $\pm$ 0.10 | -2.34 $\pm$ 0.23 | -1.59 $\pm$ 0.32 |
| Net primary production ( $\text{mmol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ ) <sup>a</sup>   | 0.08 $\pm$ 0.21  | 0.90 $\pm$ 0.24  | 0.65 $\pm$ 0.25  |
| Gross primary production ( $\text{mmol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ ) <sup>a</sup> | 1.21 $\pm$ 0.23  | 3.30 $\pm$ 0.36  | 2.16 $\pm$ 0.39  |
| Production/respiration ratio                                                              | 1.05 $\pm$ 0.17  | 1.42 $\pm$ 0.12  | 1.45 $\pm$ 0.24  |

<sup>a</sup> A positive flux is a net flux from sediment into the overlaying water.

from Ag-NP meant that the stock solution contained a mixture of Ag-NP and Ag ions (see ESI† S2 for more details on Ag-NP dissolution).

Most NPs settle in the first 24 h when in contact with seawater,<sup>60,61</sup> so that without regular addition of NPs, the concentrations of NPs in the water of the mesocosms would have declined. The mesocosm systems were also subjected to weekly water changes with fresh seawater, to prevent nutrient limitation and simulate estuarine flushing processes. To accommodate these factors, NPs were added into mesocosms after every water change, then every two days until the next water change in order to maintain the concentration of NPs in water close to the target concentration. NPs could precipitate on the top of the sediment core (*i.e.* on the biofilm), but also on the bottom of the tank and in the reservoir.

## Measurements

Sampling took place before any NP addition (T0), then after 4 and 28 days; the sampling design is summarized in Fig. S3†. Briefly, each independent replicate mesocosm (4 replicate mesocosm per treatment) contained 4 cores: one core was used for sediment-water flux measurement throughout the experiment (repeated measures design), while the 3 remaining cores were used for mini-core sampling and lens-tissue extractions of biofilms throughout the experiment. Mini-cores were never taken in the same spot twice during an experiment.

Sediment mini-cores (1.4 cm diameter, 2 mm deep) were kept frozen at -20 °C until analysis for chlorophyll *a*, phaeophytin and carbohydrate concentrations. Chlorophyll *a* and phaeophytin concentration were determined spectrophotometrically,<sup>62</sup> and colloidal carbohydrate concentration was measured with colorimetry.<sup>54,63,64</sup> Colloidal carbohydrate concentration was measured as this is directly related to biostabilisation potential through the production of mucilaginous matrices containing colloidal EPS.<sup>30,54,64</sup>

Lens tissue extractions were used to isolate epipellic diatoms from the sediment.<sup>65</sup> Lens-tissues were placed on the surface of the sediment after emersion of the cores

(falling tide), and left for 1 h under illumination (light used for flux measurements as described below). Lens-tissue extracts were then resuspended in filtered natural seawater by gentle mixing and dark-adapted 30 min at a seasonally relevant temperature. Photosynthetic potential ( $F_v/F_m$ , the maximum PSII photochemical efficiency) of microalgae was then measured using a FRR fluorimeter.<sup>66,67</sup>

On each sampling day, one core per tank was used for oxygen, carbon and nutrient sediment-water exchange measurements as described in Thornton *et al.*<sup>50</sup> Each sediment core was enclosed within a long core tube with seawater from the site, and maintained at *in situ* temperature during two sequential 3 hour incubations: one incubation in the dark (during the *in situ* immersion period) followed by one in the light (during the *in situ* emersion period). Overlaying water was sampled before and after each incubation to measure oxygen, total organic carbon (TOC, *i.e.* particulate organic carbon + dissolved organic carbon) and nutrient (nitrate, nitrite, ammonium, silicate and phosphate ions) concentrations. Oxygen concentrations were measured using Winkler titration;<sup>68</sup> TOC concentrations with a Skalar Formacs<sup>HT</sup> TOC analyser, and nutrient concentrations using SEAL Analytical AACE auto-analyser. Fluxes between sediment and water were determined for each core from the concentration changes, and normalised (based on surface core area) to flux per square meter per hour. A technical problem during the *winter* experiment meant that flux measures were made on day 7 instead of day 4.

All cores used for flux measurements were sieved (1 mm mesh size) at the end of the experiment, and macrofauna preserved in 100% ethanol. All individual animals were counted and identified and width or length measurements taken. Biovolume of individuals were calculated using geometric shapes and published morphometric relationships for *Hydrobia*, *Macoma* (mollusca) and *Hediste* polychaete annelid worms.<sup>69–71</sup>

## Statistical analyses

Differences in the measured variables between experimental treatments (control and NP treatments) were tested at two



time points: after 4 days (short term, acute toxicity) and 28 days (longer term, chronic toxicity), using multiple comparison Kruskal-Wallis tests (also called non-parametric ANOVA; R statistical framework<sup>72</sup>). When significant differences between treatments were found, we performed pairwise *post hoc* Mann Whitney tests with Bonferroni correction. This correction for multiple comparison meant that our statistical power was too low to determine significant differences between two individual treatments.

To compare inorganic nutrient fluxes, non-metric multi-dimensional scaling analyses (nMDS, 500 iterations; Primer 5 software<sup>73</sup>) were performed for each experiment, with similarity matrix constructed with normalised Euclidian distances using the dark and light flux values for all nutrients. ANOSIM was then used to test the difference in nutrient fluxes between treatments. When relevant, SIMPER analyses were used to determine which fluxes were responsible for the most difference between NP treatments (control included).

Non-metric multi-dimensional scaling (nMDS, Bray Curtis similarity with square root transformation and Wisconsin double standardisation; R version 3.6.1 with the package *vegan*<sup>72,74</sup>) was used to visualise differences in macrofauna community structure. nMDS was based on total biovolume of each taxa to provide a better reflection of the potential impact of the taxa on the overall assemblage functioning.

## Results

### Nanoparticles and environmental context

TiO<sub>2</sub>-NPs ranged in size between 50 and 200 nm (ESI† Fig. S2c), and with a zeta potential at  $-17.5 \pm 0.9$  mV, aggregated in seawater and were deposited on the biofilm surface (ESI† Fig. S4a). Ag-NPs showed no sign of aggregation and deposition in seawater, despite a zeta potential of  $-9$  mV, due to their PVP coating (ESI† Fig. S2d). Ag-NPs were more consistent in size, with a median size of 12 nm (ESI† Fig. S2a and d). Analysis of dissolution of Ag-NPs in seawater revealed greater dissolution of Ag-NPs into Ag<sup>+</sup> ions in *summer* seawater than in *early spring* seawater (ESI† Fig. S2e).

A number of point measurements of Ag concentrations in the surface sediments were taken at the end of our *early spring* and *summer* experiments with ICP-MS (1 measurement per treatment per experiment; sediment pooled from the 4 cores of each treatment). Concentrations were around 0.02 µg Ag g<sup>-1</sup> sediment (dry weight) in control cores and between 0.02 and 0.17 µg g<sup>-1</sup> in cores treated with silver. These low numbers confirm a low deposition of Ag-NPs onto the sediment cores. There was a trend for sediment Ag concentrations to be lower in *summer* compared to *early spring*.

The environmental conditions in the three seasonal periods (contexts) were well contrasted (Tables 1 and 2), with the experiments in November to December 2016, February to March 2017 and June to July 2017 representing the physical, chemical and biological conditions in the Colne estuary in

*winter*, *early spring* and *summer*, respectively.<sup>25</sup> To mirror the natural annual cycle of microphytobenthic biofilms which start growing in early spring, then decline in winter,<sup>52</sup> results from the three contexts are presented in the sequence, *early spring*, *summer*, *winter*.

Microscopic identification of microalgae in lens-tissue extracts confirmed the presence of living benthic diatoms in our sediment cores. The sediment cores supported healthy microphytobenthic biofilms, with chlorophyll *a*/phaeophytin ratio above 1 and  $F_v/F_m$  measurements above 0.4 on all lens-tissue extracts of the epipellic diatoms (Table 1).  $F_v/F_m$  measurements did not show any sign of decrease throughout the experiments in control tanks, showing that the mesocosm conditions were appropriate to support the healthy development of microalgal biofilms.

Macrofaunal communities were significantly different between seasons (ESI† Fig. S4b), with *Macoma balthica* and *Hediste diversicolor* being more abundant in *summer* compared to other seasons (ESI† Table S1). Dolicopodid larvae were absent from cores in the *summer* experiment. In *early spring*, *H. diversicolor* were significantly less abundant in treatments with TiO<sub>2</sub>-NPs (KW test,  $p < 0.05$ ), but otherwise there were no significant effects of experimental treatments on the densities of macrofauna.

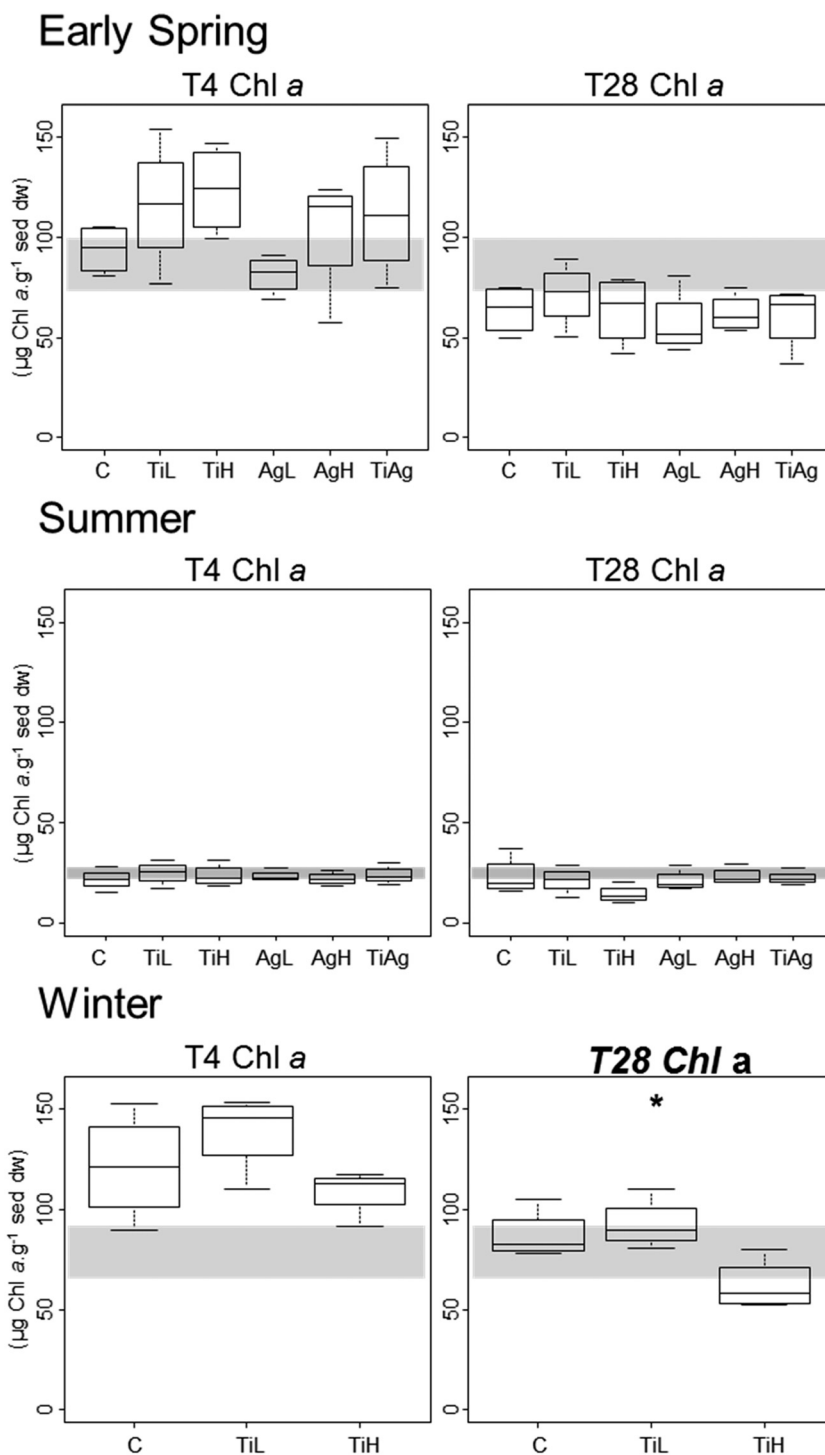
### Experimental treatments

Microphytobenthic biomass on our sediment cores was measured using the proxy of chlorophyll *a* content in the first 2 mm of the sediment, after 4 and 28 days of experiment (Fig. 1). No significant differences between treatments were found after 4 days regardless of the season (Kruskal-Wallis,  $p > 0.05$ ). After 28 days in *winter*, significant differences were observed (Kruskal-Wallis,  $p = 0.026$ ), with chl *a* content 28% lower in the presence of high concentrations of TiO<sub>2</sub>-NPs compared to control treatment.

Colloidal carbohydrate content of the surface sediment (first 2 mm), a proxy for sediment biostabilisation potential, was measured after 4 and 28 days of experiment (Fig. 2). No significant effects of the NP treatments were found after 4 days (Kruskal-Wallis,  $p > 0.05$ ). However, after 28 days in both *summer* and *winter*, TiO<sub>2</sub>-NPs significantly impacted biofilm development (Kruskal-Wallis,  $p = 0.023$  in *summer*,  $p = 0.024$  in *winter*). At high concentrations TiO<sub>2</sub>-NPs caused a 44% reduction of colloidal carbohydrate content in sediment in *summer*, and 40% reduction in *winter* (compared with the control treatment). A similar trend, although not statistically significant, was seen in *early spring* ( $p > 0.1$ , 28% reduction). These indicate a potential for TiO<sub>2</sub>-NPs to decrease the capacity of microalgal biofilms to stabilise sediments.

Neither TiO<sub>2</sub>-NPs nor Ag-NPs showed any influence on biofilm photosynthetic potential of diatoms in the biofilms, regardless of the experimental conditions, with no significant differences between treatments for either  $F_v/F_m$ , or gross primary production (calculated from oxygen fluxes between sediment and water in the dark and in the



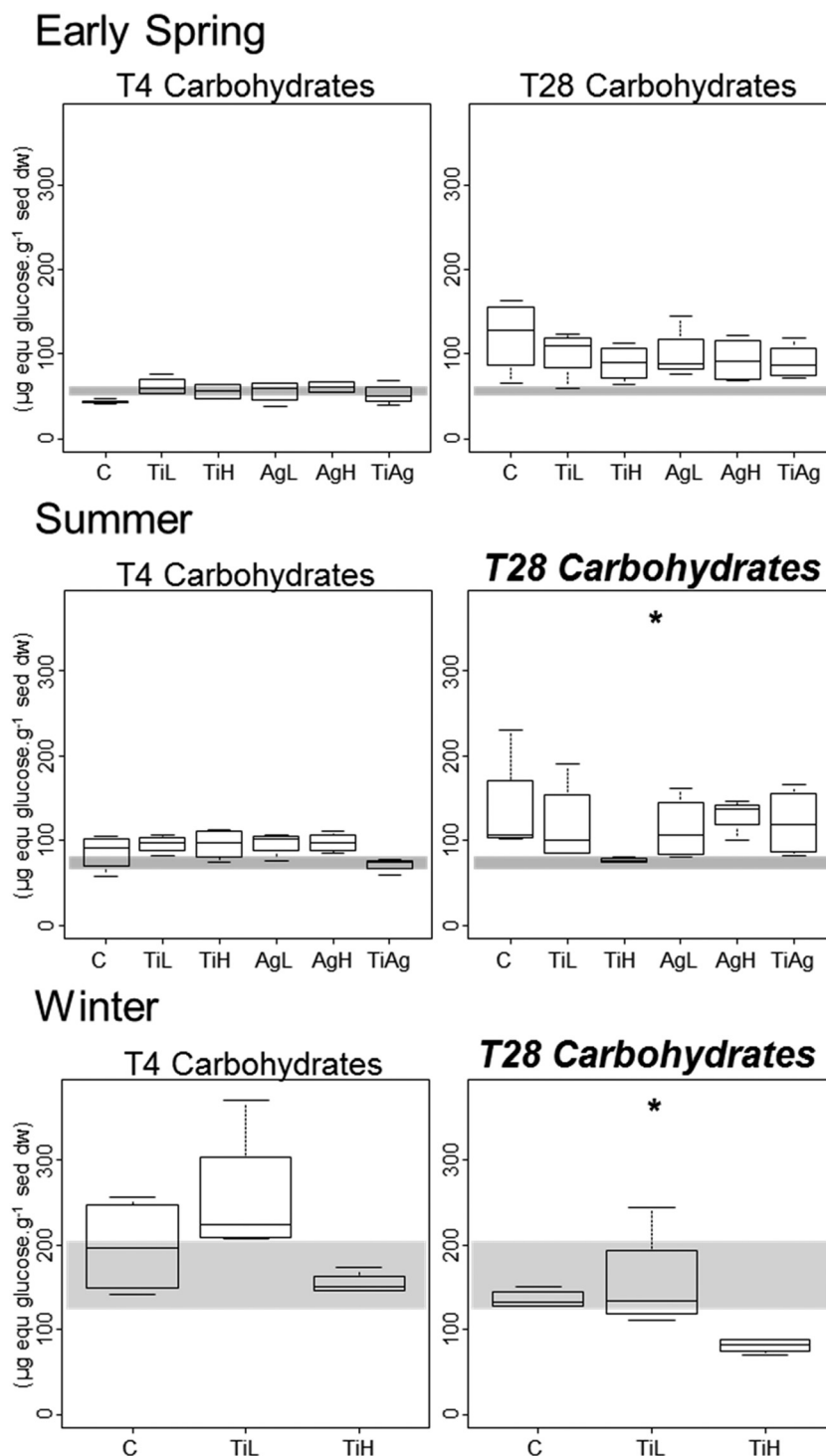


**Fig. 1** Boxplot of microphytobenthic biomass (sediment chlorophyll a concentration) after 4 and 28 days of experiment. Box limits represent lower (Q1) and upper quartiles (Q3), midline represents sample median (Q2) and whiskers represent the smallest and largest observations. C: control, no NP. TiL: target concentration  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2\text{-NP}$ . TiH: target concentration  $25 \text{mg l}^{-1}$  of  $\text{TiO}_2\text{-NP}$ . AgL: target concentration  $25 \text{ng l}^{-1}$  of Ag-NP. AgH: target concentration  $25 \mu\text{g l}^{-1}$  of Ag-NP. TiAg: target concentrations  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2\text{-NP}$  and  $25 \text{ng l}^{-1}$  of Ag-NP. Graph title italicised and in bold and the asterisk indicate the presence of significant differences between treatments ( $p < 0.05$ , Kruskal Wallis test, taking into account all treatments). The shaded areas highlight values between the first and third quartiles of measures at the start of the experiment (T0 days).

light; Kruskal-Wallis,  $p > 0.05$ ; data not shown). In *winter*, net primary production (NPP, *i.e.* oxygen fluxes from sediment to water in the light; Fig. 3) was significantly

different between treatments after 7 days of experiment (Kruskal-Wallis,  $p < 0.05$ ): the sediment switched from a net oxygen production to a net oxygen consumption in



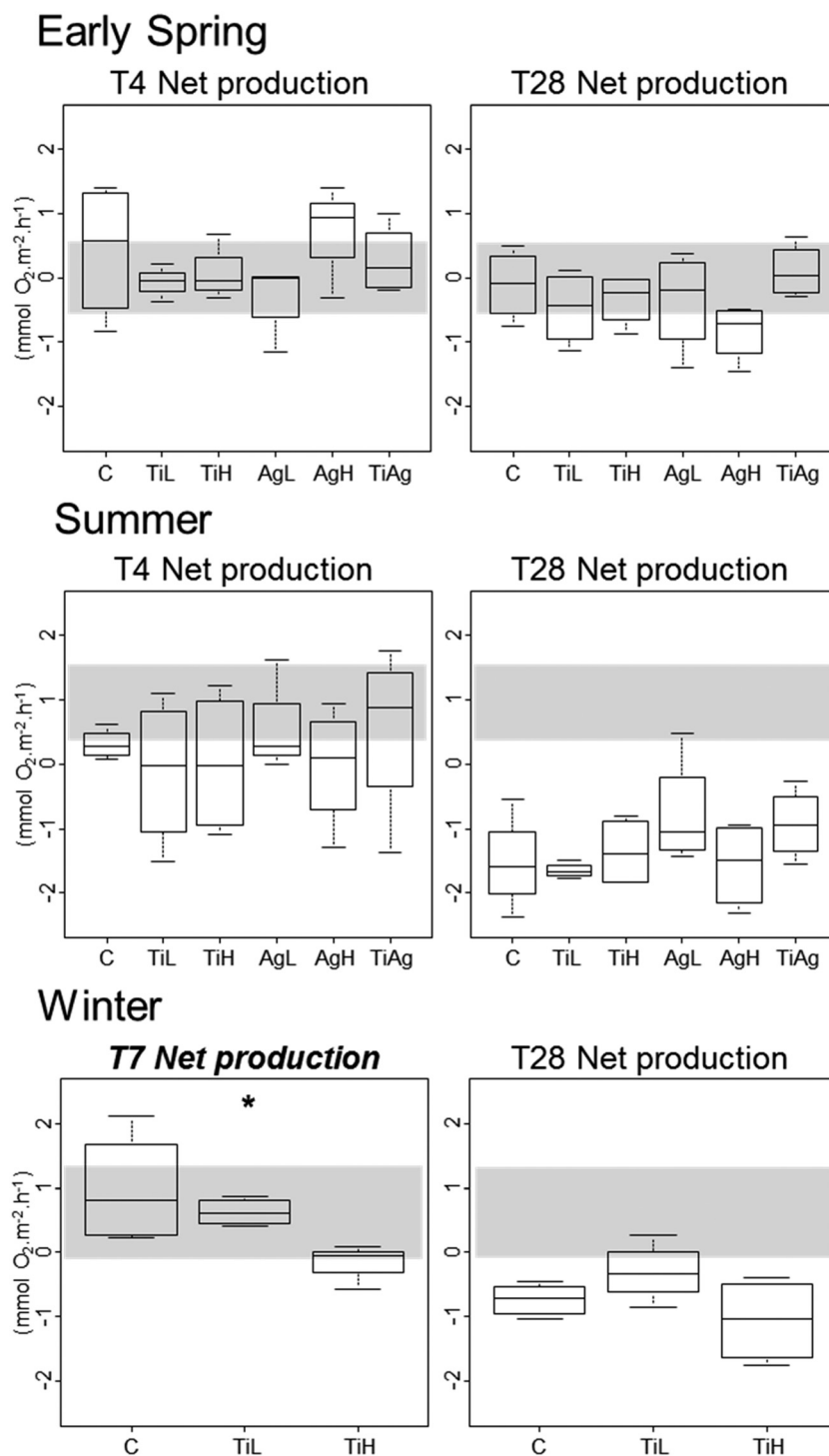


**Fig. 2** Boxplot of sediment biostabilisation potential, as measured by carbohydrate concentrations in sediment, after 4 and 28 days of experiment. Box limits represent lower (Q1) and upper quartiles (Q3), midline represents sample median (Q2) and whiskers represent the smallest and largest observations. C: control, no NP. TiL: target concentration  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2$ -NP. TiH: target concentration  $25 \text{mg l}^{-1}$  of  $\text{TiO}_2$ -NP. AgL: target concentration  $25 \text{ng l}^{-1}$  of Ag-NP. AgH: target concentration  $25 \mu\text{g l}^{-1}$  of Ag-NP. TiAg: target concentrations  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2$ -NP and  $25 \text{ng l}^{-1}$  of Ag-NP. Graph title italicised and in bold and the asterisk indicate the presence of significant differences between treatments ( $p < 0.05$ , Kruskal Wallis test, taking into account all treatments). The shaded areas highlight values between the first and third quartiles of measures at the start of the experiment.

presence of high concentrations of  $\text{TiO}_2$ -NPs. However, no significant influence of NPs on NPP was observed at the end of the *winter* experiment, or in other seasons (Kruskal-Wallis,  $p < 0.05$ ).

Other carbon-related sediment–water fluxes, *i.e.* benthic respiration and TOC fluxes, did not show any differences between treatments in any of the experiments (data not shown; mean TOC fluxes per season given in Table S2†).





**Fig. 3** Boxplot of net primary production (oxygen flux from sediment to water in the light), after 4 and 28 days for *early spring* and *summer* experiments, after 7 and 28 days for the *winter* experiment. Box limits represent lower (Q1) and upper quartiles (Q3), midline represents sample median (Q2) and whiskers represent the smallest and largest observations. C: control, no NP. TiL: target concentration  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2$ -NP. TiH: target concentration  $25 \text{mg l}^{-1}$  of  $\text{TiO}_2$ -NP. AgL: target concentration  $25 \text{ng l}^{-1}$  of Ag-NP. AgH: target concentration  $25 \mu\text{g l}^{-1}$  of Ag-NP. TiAg: target concentrations  $25 \mu\text{g l}^{-1}$  of  $\text{TiO}_2$ -NP and  $25 \text{ng l}^{-1}$  of Ag-NP. Graph title italicised and in bold and the asterisk indicate the presence of significant differences between treatments ( $p < 0.05$ , Kruskal Wallis test, taking into account all treatments). The shaded areas highlight values between the first and third quartiles of measures at the start of the experiment.

The influence of NPs on inorganic nutrient sediment–water fluxes was analysed considering all nutrient fluxes together: nitrate, nitrite, ammonium, phosphate and silicate

fluxes in both dark and light conditions (mean fluxes per season, Table S2†). Nutrients fluxes were not significantly different between NP treatments and controls in any season



after 4 days of treatment. At the end of the experiments, nutrient fluxes were different between treatments in *winter* only (Fig. 4 for *winter*, ANOSIM,  $R = 0.22$ ,  $p = 0.043$ ; Fig. S4,†  $p > 0.05$  for other seasons). SIMPER analysis in *winter* showed that alterations of fluxes of ammonium, nitrate and silicate in the dark, and flux of ammonium in the light, explained most of the difference between treatments in *winter* (Fig. 4).

## Discussion

### Lack of observed toxicity of silver nanoparticles under environmentally relevant conditions

The PVP-coated Ag-NPs used showed no sign of aggregation or sinking even at high concentrations, but some of them dissolved in seawater (ESI† Fig. S2), and greater dilution could be expected at the low concentrations used in our experimental mesocosms.<sup>75</sup>  $\text{Ag}^+$  ions originating from the NP could form complexes with  $\text{Cl}^-$  or  $\text{S}^{2-}$  ions, or link with microbial biofilms at the surface of the sediment.<sup>21</sup> AgCl complexes can then act as Ag-NP precursor, but such formation remains low in seawater with high  $\text{Cl}^-$ ,<sup>76</sup> especially at low Ag concentrations.<sup>77</sup> Ag-NPs have been found to accumulate in biofilms,<sup>55</sup> in particular in simulated marine environments,<sup>7</sup> but our point measurements of Ag content in sediment suggest such accumulation was limited in the experimental set up deployed.

Using environmentally relevant concentrations ( $25 \text{ ng l}^{-1}$ ) or higher concentrations ( $25 \text{ } \mu\text{g l}^{-1}$ ), our experiments showed no significant impact of silver (Ag) NPs on marine biofilms or associated ecosystem functions, regardless of the environmental context. This result did not support our original hypothesis 1, which was based on the evidence that Ag-NPs are used mainly for their anti-microbial properties (they were registered as biocide in the USA in 1954) and on the large body of literature investigating the toxicity of Ag-NPs.<sup>6</sup> Ecological risk assessments indicate that Ag-NPs are the NPs of most concern at current estimated concentrations

(higher risk characterisation ratio, *i.e.* concentrations in environment *vs.* estimated safe concentration).<sup>78</sup> Several reasons might explain our findings.

Seawater can increase agglomeration and sedimentation of Ag-NPs with a charged surface,<sup>20</sup> therefore decreasing their toxicity, though this process was not apparent in our experimental tanks (Fig. S2†). The dissolution of PVP-coated Ag-NPs into ionic form, including  $\text{Ag}^+$ ,  $\text{AgCl}_2^-$  and  $\text{AgCl}_3^{2-}$  has been shown to increase in seawater compared to ultra-pure water.<sup>79</sup> Our data (Fig. S2e†) does suggest a higher dissolution of Ag-NPs under *summer* conditions than in *early spring* conditions, indicating a temperature dependence of dissolution.<sup>46</sup> In freshwater, PVP-coated AgNPs don't exhibit such seasonal differences in dissolution.<sup>80</sup> The precipitation of  $\text{Ag}^+$  with  $\text{Cl}^-$  or  $\text{S}^{2-}$  in seawater and sediments lowers the bioavailability of silver ions, which are partly responsible for Ag-NP toxicity.<sup>4,81–84</sup> The toxicity of Ag-NPs can however be greater than  $\text{Ag}^+$  ion toxicity at equivalent concentrations:<sup>38,85,86</sup> a combination of dissolution followed by precipitation would overall lessen the potential toxic impact. PVP coated Ag-NPs, as used in our study, may have limited toxicity compared to uncoated NPs<sup>87–89</sup> but coatings can also increase NP toxicity<sup>37,90</sup> so that the overall effect of coatings on the environmental effect of Ag-NP is difficult to forecast.

Most studies demonstrating Ag-NP toxicity towards microorganisms use concentrations that are higher than those used in our study ( $25 \text{ ng l}^{-1}$  as environmentally relevant, and  $25 \text{ } \mu\text{g l}^{-1}$  as 1000 times higher). Growth limitation of marine biofilms was found at concentrations from  $200 \text{ } \mu\text{g l}^{-1}$ ,<sup>43</sup> in estuarine plankton from  $500 \text{ } \mu\text{g l}^{-1}$ ,<sup>91</sup> in freshwater biofilms at  $10 \text{ mg l}^{-1}$ ,<sup>87</sup> and marine benthic bacteria at  $50 \text{ mg l}^{-1}$ ,<sup>37</sup> though no effect on freshwater microorganisms was found at  $500 \text{ } \mu\text{g l}^{-1}$  (ref. 92) or on estuarine bacteria at  $1000 \text{ } \mu\text{g l}^{-1}$ .<sup>15</sup> In wetland mesocosms, Ag-NPs at  $2.5 \text{ mg l}^{-1}$  showed a very significant impact on aquatic plants and carbon chemistry in the water.<sup>93</sup> Other studies showed short-term toxic effects (for one or a few days) at lower or similar concentrations (tens of  $\text{ } \mu\text{g l}^{-1}$  to  $\text{mg l}^{-1}$ ), such as increased stress for freshwater bacteria at  $10 \text{ } \mu\text{g l}^{-1}$ ,<sup>94</sup> but microbial assemblages can then recover.<sup>20,82,86</sup>

Our study demonstrated that a regular exposure to low but environmentally realistic concentrations of Ag-NPs for one month had little effect on microphytobenthic biofilms, in terms of biomass, photosynthesis, and nutrient exchange processes. This is likely due to a combination of biological protection mechanisms and resistance, and chemical transformations of Ag-NPs in the environment. Microorganisms in coastal biofilms are embedded in a matrix of extracellular polymeric substances (EPS),<sup>95</sup> which can provide protection from toxicants.<sup>96</sup> Microbial EPS can trap and accumulate Ag-NPs and  $\text{Ag}^+$  ions efficiently,<sup>29,38,85</sup> limiting their access to the cells.<sup>85</sup> As a result, cells protected with EPS are less susceptible to Ag-NP toxicity than cells without EPS.<sup>85,97</sup> Longer-term exposure to Ag-NPs could however potentially alter coastal biofilms and associated

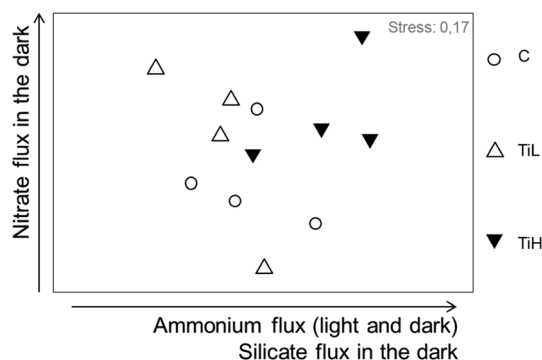


Fig. 4 nMDS plot based on nutrient fluxes from sediment to water after 28 days of experiment in *winter*. C: control, no NP. TiL: target concentration  $25 \text{ } \mu\text{g l}^{-1}$  of  $\text{TiO}_2$ -NP. TiH: target concentration  $25 \text{ mg l}^{-1}$  of  $\text{TiO}_2$ -NP. The arrows indicate the fluxes responsible for most difference between treatments (SIMPER analysis). For *early spring* and *summer* data, see Fig. S5.†



processes through modification of the microbial community.<sup>44</sup>

### Toxicity of titanium oxide nanoparticles on coastal biofilms

In our experiments TiO<sub>2</sub>-NPs did not remain permanently in suspension in seawater and many were deposited on the sediment cores or the bottom of the tanks within a day of NP addition (visual observation, see Fig. S4a†). TEM images and zeta potential measurements showed that our the TiO<sub>2</sub>-NPs did aggregate rapidly in seawater (Fig. S2c†), behaviour which is consistent with other studies.<sup>19</sup> TiO<sub>2</sub>-NPs are also known to associate with “marine snow” which also leads to sedimentation in natural seawater.<sup>18</sup> Based on the doses of TiO<sub>2</sub>-NPs added, the surface area of our tanks and cores, and an estimated 90% of NPs sinking after each addition, we can estimate a final sediment load at 56 mg TiO<sub>2</sub> m<sup>-2</sup> for TiL and 56 g TiO<sub>2</sub> m<sup>-2</sup> for TiH treatments at the end of 28 days in our experiments.

TiO<sub>2</sub>-NPs displayed toxic effects on microphytobenthic biofilms at high concentrations (25 mg l<sup>-1</sup>), with decreased microphytobenthic biomass and affected nutrient recycling in *winter*. Unlike the behaviour of Ag-NPs, TiO<sub>2</sub>-NPs are chemically stable under environmental conditions; and thus their toxicity is not associated with their dissolution into Ti ions, but with the NPs themselves.<sup>98</sup> No such effect was observed at low concentrations (*i.e.* current expected concentration in STW effluents; 25 µg l<sup>-1</sup>).<sup>3,16</sup>

TiO<sub>2</sub>-NPs limited the biostabilisation potential of biofilms in both *summer* and *winter*, with concentrations of colloidal carbohydrate being 40–44% lower in presence of TiO<sub>2</sub>-NPs at high concentrations compared to controls. This result was surprising as colloidal carbohydrates, and EPS in general, can act as a protection mechanism for cells,<sup>34</sup> and EPS production increases under a variety of stresses.<sup>99,100</sup> Other studies showed that diatoms in periphytic biofilms exposed to TiO<sub>2</sub>-NPs and CuO-NPs showed an increased production of EPS<sup>33,87</sup> protecting cells by mitigating ROS-associated stress.<sup>34</sup> Different classes of EPS do not display the same response to NP exposure:<sup>33</sup> Miao *et al.*<sup>87</sup> showed a higher content of protein than carbohydrates in the EPS produced following such stress. In our experiment, TiO<sub>2</sub>-NPs might have affected cells by direct shading effect, therefore limiting light-dependent colloidal carbohydrate and EPS production.<sup>64,101</sup> Also, NPs may disrupt EPS biochemical production pathways, which have been shown to alter under temperature and salinity stresses,<sup>102</sup> and could show similar responses in presence of NPs.

TiO<sub>2</sub>-NPs occur commonly as two different crystalline structures, anatase and rutile; a mix of both phases of titanium was chosen in this experiment to take into account the diversity of TiO<sub>2</sub>-NPs arriving on coasts from different sources,<sup>3,56,57</sup> for instance: coatings, paints, sunscreens. Toxicity of TiO<sub>2</sub>-NPs is twofold: a direct physical effect on cells, associating with and shading them, interrupting energy transduction along the membrane and limiting cells access

to light.<sup>103</sup> TiO<sub>2</sub>-NPs also lead to the production of reactive oxygen species (ROS) when in contact with cells,<sup>2,5,103</sup> especially 'OH that damage membranes and proteins. Anatase has a higher photocatalytic activity<sup>103,104</sup> than rutile: it produces more ROS in presence of light. It is suggested that a mixture of both forms is more toxic that exposure to each form separately.<sup>103</sup>

The concentrations at which we observed toxic effects (25 mg l<sup>-1</sup>) were consistent with literature data on the toxicity of TiO<sub>2</sub>-NPs: significant toxicity was observed from one or a few mg l<sup>-1</sup> in cultures<sup>1,2,32</sup> and on river periphyton,<sup>98</sup> to 50 mg l<sup>-1</sup> in freshwater biofilms.<sup>103</sup> Current expected concentrations of TiO<sub>2</sub>-NPs in the water (around 25 µg l<sup>-1</sup>) did not impact coastal biofilm growth or ecosystem functioning in our experiments. However, these findings must be nuanced by two facts: Binh *et al.*<sup>104</sup> showed toxic effects of TiO<sub>2</sub>-NPs on freshwater biofilms at similarly low concentrations (30 µg l<sup>-1</sup>), but only after 22 weeks of experiment. Similar long-term chronic impacts may occur for coastal biofilms, which our 4 week experiments would not have detected. This view is consistent with high concentrations of TiO<sub>2</sub>-NPs showing toxicity mainly at the end of the experiments, when exposure time was longest. There is also uncertainty concerning the actual levels of environmental exposure; currently available measures of several types of NP concentrations in sediments are on average 1000 times higher than those predicted by modelling.<sup>40</sup> Therefore our “high concentration” treatment might not be so far from current concentrations of TiO<sub>2</sub>-NPs in coastal sediments.

Mixtures of Ag-NPs and TiO<sub>2</sub>-NPs at low concentrations did not impact significantly biofilm biomass or associated biogeochemical processes. No “cocktail effect” was therefore demonstrated; different results may however be obtained at higher concentrations. TiO<sub>2</sub>-NPs can reduce Ag-NP toxicity towards bacteria in dark conditions, but increase the toxicity in the light.<sup>105</sup> The interaction also seems to be dependent on light quality and cycle,<sup>49</sup> but also on salinity, as chloride (Cl<sup>-</sup>) concentration in seawater limits the availability of Ag<sup>+</sup> ions.<sup>21</sup> NPs also interact with other types of pollutants: the disruption of membranes by TiO<sub>2</sub>-NPs can facilitate the entry of other pollutants, such as heavy metals, in the cells, therefore increasing their toxicity.<sup>106</sup> Approaches to determine the active concentrations of mixtures of NPs, other toxicants, and physical and chemical cofounding variables at the site of biological impact at scales relevant to microbial processes *in situ* are needed to better understand environmental impacts of NPs in combination with other stressors.

### Influence of environmental context on the toxicity of nanoparticles

The toxicity of TiO<sub>2</sub>-NPs was dependent on environmental context: no effects were seen in *early spring*, with most effects demonstrated in *winter*, thus supporting the first part of our third hypothesis. Differences in water chemistry (ionic or



organic carbon content for instance) will influence the interactions between NPs and their abiotic environment, the fate of potential ions they release and therefore both NP bioavailability and toxicity.<sup>5,6</sup> Higher temperatures increased the dissolution of Ag-NPs, increasing the release of potentially toxic ions (see Fig. S2e†);<sup>46</sup> however, no toxicity was observed for Ag-NPs in our experiments, regardless of the temperature. Environmental conditions will influence how NPs aggregate and sink onto the sediment.<sup>5,6,18,94,105</sup> In sediments as well as in the water, chemical parameters such as sulphide content will affect toxicity.<sup>88</sup> Furthermore, TiO<sub>2</sub>-NPs are photoactive compounds, which means that the levels and wavelength range of light will influence their toxicity.<sup>2,103,107</sup> For instance, TiO<sub>2</sub>-NPs are toxic to phytoplankton only in the presence of UV radiation.<sup>2</sup>

In our experiments, we expected the greatest toxicity of TiO<sub>2</sub>-NPs to be in *summer*, where light and UV levels were higher.<sup>2</sup> Some significant effects were observed in *summer*, but greatest toxicity to biofilms was found in *winter* when light and temperature were the lowest. In the *summer* and *winter* experiments where significant toxicity was observed, biofilms were characterised by high production to respiration ratios, and high amounts of colloidal carbohydrate per unit Chl *a* (Table 2). High production to respiration ratios, and high colloidal carbohydrates to Chl *a* ratios, indicate physiologically-active populations of microalgae,<sup>52</sup> with different biogeochemical processes active compared with other seasons.<sup>52</sup> We can hypothesise that these differences explain the observed differences in toxicity. For instance, degradation processes are preponderant in winter,<sup>52</sup> and may be enhanced in the presence of ROS produced when NPs are present; this could explain the increased toxicity seen on this season. It is also interesting to note that chronic exposure to silver NPs in a wetland mesocosm also showed a higher sensitivity of prokaryotes in winter,<sup>44</sup> while winter communities of freshwater diatoms were more resistant to herbicides.<sup>108</sup> Our results highlight the necessity to repeat experiments under different environmental contexts encompassing different seasons and physiological states of the biological community under investigation, if we are to understand NP toxicity on the environment.

### Environmental impact of titanium oxide nanoparticles

Our experiments investigated the effect of NPs on coastal microphytobenthic biofilms, and on the ecosystem processes that they influence. Mesocosm approaches allow for controlled experimental manipulation of intact intertidal sediment communities while maintaining as close as possible natural environmental conditions.<sup>54,55,109,110</sup> The impact of grazing fish<sup>111</sup> and wave action on biofilm resuspension<sup>112</sup> were not simulated, but our approach simulated important environmental drivers of sediment-biofilm ecology: tidal cover, natural daylight quality and periodicities, rainfall, temperature, and nutrient resupply. Microphytobenthic activity (Chl *a*:phaeophytin ratio,  $F_v/F_m$ )

indicated healthy biofilm functioning was sustained throughout the experimental periods, permitting conclusions about the environmental impacts of addition of NPs on biofilm related ecosystem functions to be drawn.

Sediment biostabilisation potential, as measured by colloidal carbohydrate content in the sediment,<sup>30,31</sup> was significantly reduced in both *summer* and *winter* at high concentrations of TiO<sub>2</sub>-NPs. Further research may help determine the threshold at which such toxicity is observed, its longer-term effects and whether shifts of microbial assemblages occur. Similar reductions of sediment biostabilisation potential have been demonstrated at high concentration of Ag-NPs for freshwater biofilms, where the structure of the biofilm was affected and sediment adhesiveness was largely reduced.<sup>113</sup> Experiments in stream mesocosms with Ag-NPs showed reduced mechanical stability of freshwater biofilms despite unaffected microbial viability and biofilm architecture being unaffected at concentrations of 600  $\mu\text{g l}^{-1}$ .<sup>114</sup> By reducing the colloidal carbohydrate content of microphytobenthic biofilms, TiO<sub>2</sub>-NPs have a potentially significant environmental effect in reducing biostabilisation of sediment, leading to increased resuspension of biofilms and sediment in sheltered estuaries by wind driven waves.<sup>112</sup> Coastal sediment protection through biostabilisation is an important ecosystem service provided by microbial biofilm,<sup>31</sup> as coastal erosion has been estimated to cost about 500 million dollars per year in the US only.<sup>115</sup>

In our experiments, NPs had no effect on biofilm photosynthetic maximum potential ( $F_v/F_m$ ), or respiration, but a short-term negative influence of TiO<sub>2</sub>-NPs on net primary production was observed in *winter*. Some studies in cultures or mesocosms have shown deleterious effect of Ag-NPs<sup>81,87,91,97</sup> and TiO<sub>2</sub>-NPs<sup>103</sup> on the photosynthetic activity of microalgae, but usually at high concentrations compared to current expected environmental concentrations; other studies have shown limited impact.<sup>38</sup> One long-term study on the effect of TiO<sub>2</sub>-NPs on freshwater biofilms at environmentally relevant concentration showed a decrease in photosynthetic activity after 4 to 6 months.<sup>104</sup>

We found that TiO<sub>2</sub>-NPs influenced nutrient recycling, especially increasing ammonium fluxes from sediment into the overlaying water. This is to our knowledge the first demonstration of the effects of TiO<sub>2</sub>-NPs on nutrient recycling in coastal systems. Ag-NPs at high concentrations limit the growth of nitrifying bacteria in culture, limiting nitrification in estuarine sediments,<sup>37,90</sup> and limit the growth of ammonia-oxidising bacteria.<sup>37</sup> Reduced ammonia-oxidation and nitrification will increase sediment ammonium concentrations, therefore increasing efflux of ammonium from sediment;<sup>116</sup> this cascading effect is consistent with what we observed in our experiment with TiO<sub>2</sub>-NPs treatments. Reductions in microphytobenthic biomass will also reduce microphytobenthic demand for ammonium in surface sediments,<sup>116</sup> increasing ammonium fluxes from sediment to water. The next step will be to assess if these



effects are the result of the modification of the algal, bacterial or archaeal assemblages, or result from direct influence of TiO<sub>2</sub>-NPs on physiological processes. NPs can modify the composition of microbial assemblages,<sup>21,22,36,46,98</sup> and therefore the ability of the community as a whole to drive ecosystem processes, noting that redundancy between microbial groups means that some processes, such as hydrocarbon degradation, may stay unaffected despite shifts in microbial assemblages.<sup>36</sup>

The link between reduction of biomass by NPs and modification of ecosystem processes is far from straightforward. We found that the biostabilisation potential of biofilms and primary production was affected by NPs while photosynthetic biomass was not (in *summer* and after 4 days in *winter*, respectively), and that a decrease of microphytobenthic biomass following TiO<sub>2</sub>-NP addition (at the end of the experiment, in *winter*) did not result in any alteration of oxygen fluxes. These observations do not support our hypothesis 2; and therefore, evaluating the environmental impact of NPs therefore requires not only the undertaking of experiments in environmentally relevant contexts (this study),<sup>47</sup> but also a measure of the impact of NPs on both communities and ecosystem processes.

## Conclusion

This study has shown that TiO<sub>2</sub>-NPs can limit the growth of coastal microphytobenthic biofilms in simulated natural conditions, while PVP coated Ag-NPs do not appear to be toxic in these conditions. TiO<sub>2</sub>-NPs have the potential for ecological impacts on the functioning of coastal systems, as they limit the coastal protection potential of biofilms, temporarily limit the primary production and alter nutrient recycling. Effects on biogeochemical processes were not directly dependent on changes in microalgal biomass, and all toxic effects varied between environmental contexts such as seasons. Our research therefore both provides a first step towards the comprehension of NP impact in coastal environments, and guides future research by demonstrating the importance of assessing impacts in a variety of contexts, and directly on ecosystem processes.

## Conflicts of interest

There are no conflicts of interest to declare.

## Acknowledgements

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