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Note

Inhibitory effects of sodium azide on microbial growth in experimental resuspension of marine sediment

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ABSTRACT

Sodium azide (NaN_3) was evaluated as inhibitor of microbial growth and activity in marine sediment resuspensions by monitoring the abundance of free-living and sessile bacteria using both flow cytometry and qPCR methods. Results show that 50 mM of NaN_3 strongly inhibits bacterial growth under natural and enriched resource conditions.

Microbial communities play key roles in biogeochemical cycles of contaminants, affecting their fate by degradation, (de)sorption, redox transformations, albeit often in a still poorly understood way. In order to identify clearly the role of microbial communities in contaminant dynamics (e.g. trace metal cycling and mobility) and to distinguish biological and physicochemical contributions, it is crucial to develop experimental incubations under controlled conditions, to enable comparison of biotic microcosms with abiotic controls (i.e. where microbial growth and activity have been inhibited). However, in the complex matrices of contaminated ecosystems, the sterilization method must be chosen cautiously in order not to interfere with abiotic drivers of contaminant dynamics.

Several chemical and physical methods exist to sterilize various kinds of materials, based on heat, chemicals, antibiotics, filtration or radiations. Among physical methods, steam sterilization through autoclaving is the most widespread technique but it has several drawbacks when focusing on contaminant fate, such as: disruption of the physical structure of soils and sediments (Ramsay and Bawden, 1983; Trevors, 1996); release of dissolved metals and nutrients (Quéméneur et al., 2016; Senko et al., 2008); desorption of organic compounds acting as possible metal ligands and modification of metal speciation (Egli et al., 2006). γ-irradiation is considered as an alternative technique, but it is generally less easily available, requires long stabilization times (Ramsay and Bawden, 1983), and creates reactive redox agents that may induce abiotic reactions with metals (Trevors, 1996; Bank et al., 2008). Both γ-irradiation and autoclaving altered soil organic matter chemistry (functional group identity and abundance), thus affecting pollutant sorption, affinity and bioavailability (Brickett et al., 1995; Kelsey et al., 2010). Among chemical agents, methyl bromide, formaldehyde or mercuric chloride are efficient microbial inhibitors (Trevors, 1996), but they contaminate the matrix with toxic residues, including metals, complexing agents, and oxidizing/reducing compounds, making them unsuitable for the investigation of contaminant dynamics, especially trace metals (Brickett et al., 1995).

Among chemical poisons, sodium azide (NaN_3) is a strong metabolic inhibitor of the respiratory chain (Lichstein and Soule, 1944), used in various matrices to prepare abiotic controls avoiding physicochemical alterations, especially when studying metal-polluted matrices. NaN_3 is known to inhibit microbial growth and distinct microbial activities, such as MTBE degradation (Hanson et al., 1999), manganese-, iron-, and sulfur-oxidation in pure cultures and metal-contaminated river-sediments (Burton et al., 2009; Wang et al., 2009); PAH-degradation activity in wetland sediments (Beckles et al., 1998) and sulfate-reducing bacterial enrichment cultures (Tsai et al., 2009); mercury methylation and demethylation in lake sediments (Zhang and Planas, 1994). In these studies, NaN_3 concentration usually ranged between 1 and 150 mM. However, the efficiency of NaN_3 depends on its concentration and the environmental matrix properties, and few studies have quantified

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its inhibitory effect on microbial growth in marine matrices, especially in the presence of sediments.

The objective of this study is to evaluate the efficiency and the dose effect of NaN_3 in inhibiting microbial growth and activity during resuspension of marine sediment in seawater. The susceptibility of microbial communities was compared under natural- and enriched-resource supply conditions, representative of normal and nutrient rich environments. Moreover, both free-living and sessile microbes were targeted since both life forms are known to occur naturally in marine environments.

Surface seawater and surface (0–5 cm) marine sediments were collected in the northern part of the multicontaminated Toulon Bay (France). The sampling procedure and sampling site characteristics (MIS station) are detailed elsewhere (Dang et al., 2015). Chemical and biological characteristics of water and sediment samples from the same sites at a similar period of the year were previously described (Misson et al., 2016, Coclet et al., submitted).

Sediments and sea water were aerobically mixed at a solid/liquid ratio of 30 g L^{-1} (wet weight). Thirty milliliters of the sediment suspension were incubated in sterile 50 mL tubes at ambient temperature, in the dark, under continuous overhead shaking (15 rpm, Heidolph Reax 20) during 11 days. Tubes were frequently opened under sterile conditions to ensure sufficient oxygenation along the experiment. Two series of experiments were conducted. First, the inhibitory effect of a common NaN_3 concentration (50 mM) was evaluated under natural resource conditions, by comparison with unpoisoned control. Secondly, the inhibitory effect of low (5 mM) and high (50 mM) NaN_3 concentrations was evaluated under growth-promoting conditions (addition of 0.1× of Luria-Bertani (LB) broth and 1 g L^{-1} glucose), by comparison with unpoisoned control under the same nutrient-rich conditions. Each condition was done in triplicate. pH was measured in the suspension at the beginning (T_0), after 2 h, 4 days, and at the end (T_f) of the incubations using a Cyberscan 510 (Eutech) probe.

In the suspended phase, free-living prokaryote abundance and activity (esterase assay) were estimated at T_0, after 2 h, 4 days and at T_f by flow cytometry. One mL sediment suspension aliquots were centrifuged (2′, 800 g) to remove the largest particles. The supernatant was analyzed with an Accuri C6 flow cytometer (BD), total heterotrophic prokaryotes being enumerated after SYBR green staining (0.5× final concentration) and active prokaryotes being enumerated after CFDA staining (10 μM final concentration) according to previous work (Grégori et al., 2001).

For sedimentary microbial abundance quantification, 20 mL-samples of the sediment suspension were collected at initial and final days from all incubations, centrifuged at 10.000 rpm for 15 min, and the pellets were stored at −20 °C until DNA extraction. Total genomic DNA was extracted from the pellets (app. 325 mg) with PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, US). Bacterial abundances were quantified by real-time PCR (CFX96, Bio-Rad Laboratories) of 16S rRNA gene fragments, with GoTaq qPCR Master Mix (Promega), using GML5F and Univ516 primers, as described previously (Duran et al., 2015).

Under natural resource conditions, and without addition of NaN_3, the abundance of free-living prokaryotes in the suspended phase showed an 8-fold increase compared to T_0 during the first four days, prior to returning to the initial level by T_f (Fig. 1A), probably as a result of resource depletion or predator-prey succession (Sauret et al., 2015). The number of CFDA-degrading (active) free-living prokaryotes followed a similar pattern, although their transient increase was limited to 4-fold (Supplementary information SI.1A). In the sedimentary phase, the number of bacterial 16S rRNA gene copies increased by a factor of 9 between T_0 and T_f (Fig. 2), confirming global bacterial growth and indicating different dynamics between free-living and sessile prokaryotes.

When NaN_3 was added at 50 mM, the growth of free-living prokaryotes was inhibited throughout the experiment (Fig. 1A) and free-living active cells were less numerous (4-fold decrease compared to T_0) as soon as 2 h after poisoning, maintaining this low level until the end of incubation (Fig. SI.1A). Moreover, sessile bacterial 16S rRNA gene copy number demonstrated a 4-fold decrease between T_0 and T_f (Fig. 2). Therefore, under natural resource conditions, NaN_3 at 50 mM was found to efficiently inhibit microbial growth and activity in both suspended and sediment phases.

However, NaN_3 efficiency required further evaluation under rich substrate conditions favoring microbial growth. Depending on the matrix properties, high NaN_3 concentration may induce physicochemical interactions with contaminant dynamics, through -for example- abiotic CO_2 and volatile acid evolution as well as pH rise, as evidenced in soil when NaN_3 concentration exceeded 77 mM (Rozycki and Bartha, 1981). It is thus important to assess if lower NaN_3 concentrations have sufficient inhibitory effect, even under rapid growth-promoting conditions.

Under simulated nutrient rich conditions (LB and glucose addition), microbial growth was strongly enhanced in both suspended and sediment compartments, in absence of NaN_3 poisoning. A continuous increase was observed for both total (Fig. 1B) and active (Fig. SI·1B) free-living cell abundance, exceeding 1000-fold enrichments between T_0 and T_f. Sessile bacteria 16S rRNA gene copy number also increased by a factor larger than 1000 between T_0 and T_f (Fig. 2).

Under high resource availability, the use of 5 mM NaN_3 (i.e. in the low range of reported concentrations in the literature) reduced the planktonic and sessile biomass growth (by a factor 100 compared to the unpoisoned condition) but was not sufficient to completely inhibit

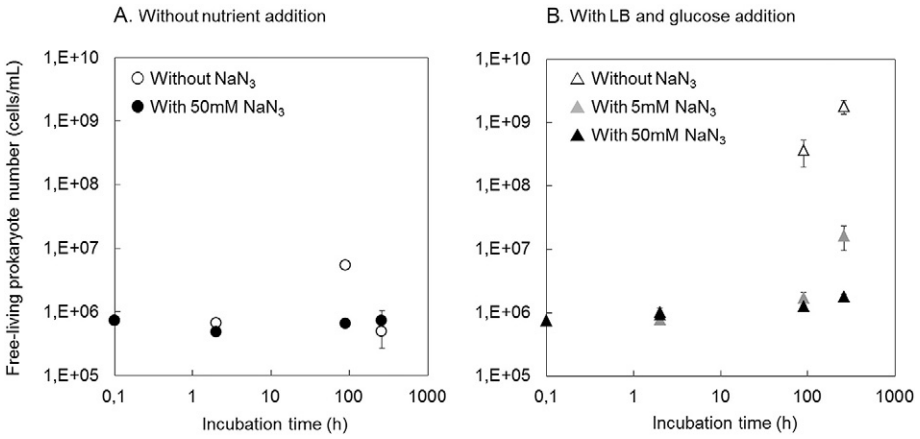

Fig. 1. Abundance of free-living prokaryotes in the suspended phase, quantified by flow-cytometry along incubation time, under (A) natural resource conditions and (B) nutrient-rich conditions (supplemented LB and glucose medium), in absence of NaN_3, and in presence of NaN_3 at 5 mM and 50 mM. Each point represents the average of triplicate incubations and error bars represent standard deviations.

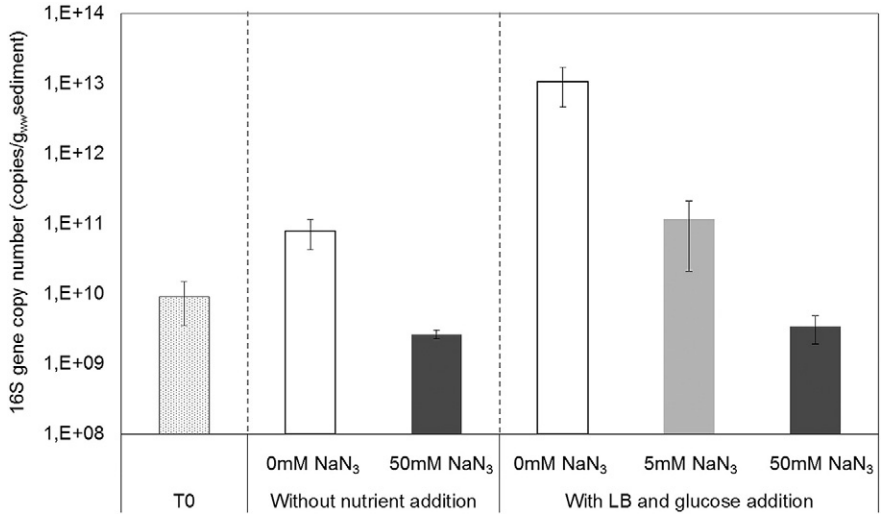

Fig. 2. Bacterial abundance in the sediment phase, at the initial time (T0) and at the end of incubations, with or without nutrient addition, in absence of NaN_3, and in presence of NaN_3 at 5 mM and 50 mM. The abundance was estimated through the quantification of 16S rRNA gene copies per gram of sediment (ww: wet weight) by qPCR. Each point represents the average of triplicate incubations and error bars represent standard deviations.

microbial growth and activity in either suspended or sediment phases. Indeed, growth was only slowed down and delayed by the addition of NaN_3 at 5 mM, which still allowed a 22-fold increase of free-living prokaryote abundance (Fig. 1B), a 50-fold increase in active free-living cell number (Fig. SI·1B), and a 12-fold increase of bacterial 16S rRNA gene copy number, between T_0 and T_f (Fig. 2). The efficiency of NaN_3 is reported to be dose-dependent: it was for example less efficient (at 20 mM) than other chemicals (acetonitrile) to inhibit dehydrogenase activity in river sediments (Mosher et al., 2003), while it had only a partial inhibition effect (at 1 mM) on manganese reduction in pure bacterial cultures, lower than other metabolic poisons (formaldehyde, antimycin, CCCP) under anaerobic conditions (Myers and Nealson, 1988).

By contrast, the addition of 50 mM NaN_3 resulted in a 1000-fold reduction of planktonic and sessile bacterial growth compared to the unpoisoned condition. Despite growth-favoring conditions, the addition of 50 mM NaN_3 led to a stable number of free-living active cells during the whole incubation (Fig. SI·1B) and a slight (3-fold) decrease of sessile bacterial 16S rRNA gene copy number between T_0 and T_f (Fig. 2). However, a minor (2-fold) increase of free-living prokaryote abundance was still observed during the incubation (Fig. 1B). The fact that the abundance of sessile bacteria not only remained constant (indicating impediment of growth), but in some cases decreased during the incubation (in presence of NaN_3 at 50 mM, independently of the substrate conditions) suggests that NaN_3 not only suppressed microbial growth but may also have lysed a fraction of the initial indigenous cells. When considering the active fraction of free-living prokaryotes, NaN_3 (at both tested concentrations) seemed to have a stronger transient inhibitory effect at short term, i.e. resulting in a loss of active cells (by a factor 4 to 14) during the first two hours, before further increase (Supplementary Fig. S1).

In absence of NaN_3, pH dropped from 8.3, at T_0, to 7.3 ± 0.2 and 7.6 ± 0.2, at T_f (respectively with and without nutrient enrichment), as a result of microbial activity. Under growth favoring conditions, the addition of 5 mM NaN_3 did not modify the amplitude of pH drop (decreasing to 7.2 ± 0.1 at T_f), while the addition of 50 mM NaN_3 resulted in a less marked pH decrease compared to the unpoisoned control (down to 7.7 ± 0.0 at T_f), thus confirming the partial efficiency of 5 mM NaN3 and the better reduction of microbial activity in presence of 50 mM NaN_3. The difference of pH between T_0 and 50 mM NaN_3 condition may be due to (i) residual microbial activity or (ii) abiotic effect of NaN_3.

Thus, in nutrient rich environments, 50 mM NaN_3 appears efficient for complete inhibition of growth of sessile bacteria over 11 days (contrary to 5 mM NaN_3), but failed to totally inhibit free-living prokaryote growth, as a small fraction of free-living cells remained active and able to grow. Therefore, the use of NaN_3 to perform abiotic controls in microcosm experiments appears feasible when employed at sufficiently high concentration (≥50 mM) for short term incubations, depending on resources availability. NaN_3 poisoning is considered as relatively benign in terms of metal interactions and abiotic oxidation processes (precipitation, leaching…) even in metal-contaminated matrices (Neubauer et al., 2002; Rentz et al., 2007). Although the present study demonstrates the inhibition efficiency of NaN_3 on microbial growth and activity, its potential physicochemical effects on the geochemistry of the samples should be further evaluated according to the targeted contaminant, the poison concentration, and the matrix properties. For example, NaN_3 reacts chemically with some organic compounds, such as atrazine, and thus should not be used to prepare abiotic controls in atrazine degradation studies (Chefetz et al., 2006).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.12.021.

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