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Enhanced removal of warfare agent tri-nitro-toluene by a *Methylophaga*-dominated microbiome

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ABSTRACT

Historical exposure of the marine environment to 2,4,6-trinitrotoluene (TNT) happened due to the dumping of left-over munitions. Despite significant research on TNT decontamination, the potential of marine microbiome for TNT degradation remains only little explored. In this study, TNT degradation experiments were conducted with sediment located near the World War I munition dumpsite – *Paardenmarkt* in the Belgian part of North Sea. A slow removal was observed using TNT as sole source of C and N, which could be enhanced by adding methanol. Degradation was reflected in nitro-reduced metabolites and microbial growth. 16S Illumina sequencing analysis revealed several enriched genera that used TNT as a sole source of C and N - *Colwellia*, *Thalossospira*, and *Methylophaga*. Addition of methanol resulted in increased abundance of *Methylophaga*, which corresponded to the rapid removal of TNT. Methanol enhanced the degradation by providing additional energy and establishing syntrophic association between methanol-utilizing and TNT-utilizing bacteria.

1. Introduction

2,4,6-trinitrotoluene (TNT) is a nitroaromatic compound widely used as an explosive during World War I and II. TNT and hexahydro-1,3,5-trinitro-1,3, 5-triazine (RDX) are still two of the most commonly used nitro-substituted explosives worldwide (Ek et al., 2007; Smith et al., n.d.; Rosen and Lotufo, 2007). For example, in the United States only, TNT is the most common explosive in the formulations of munitions produced in the order of 1 million kg annually (Hartter, 1985). Consequently, >1 million cubic yards of soil are contaminated with TNT at concentrations as high as 600,000 to 700,00 mg/kg (Griest et al., 1993). The marine environment is also exposed to TNT, primarily by dumping both surplus and damaged munitions, followed by the two World Wars (Commission, 1996). This practice was banned in 1972 by the introduction of the "Ocean Dumping Act," yet the marine environment is exposed to TNT by military training activities and detonation of unexploded ordnance (UXO) close to the coast (Smith et al., 2015). Several decades after the introduction of the act, TNT is still detected in oceans surrounding the dumpsites (due to leakage) and coastal areas close to military training sites (Darrach et al., 1998; Monteil-Rivera et al., 2004; Van Aken et al., 2004). This is alarming since TNT is a proven toxin causing disturbance to the food chain (Sanderson et al., 2010) and may lead to severe pollution of sediment (Smith et al., 2015) (incl. groundwater) and water column (Selim et al., 1995). Subsequently, TNT is among seven nitro-substituted explosives listed as priority pollutants by the US Environmental Protection Agency (EPA) (Keith and Telliard, 1979) and in the EU regulatory framework (Tornero and Hanke, 2016). Hence, TNT (bio)remediation is highly important to clean up contaminated sites.

There is a wealth of information on the biodegradation of TNT derived primarily from contaminated soil (Boopathy et al., 1994; Thiele et al., 2002; Serrano-González et al., 2018; Esteve-Núñez et al., 2005; Daun et al., 1998). Yet the mineralization of TNT using it as C and N source by a defined bacterial consortium remains a challenge. This is attributed to the structure of TNT – a highly oxidized trinitro-substituted aromatic ring that is resistant to oxidative microbial attack (Rieger and Knackmuss, 1995). Several pathways have been demonstrated (Esteve-Núñez et al., 2001); however, in both aerobic and anaerobic conditions,

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the degradation of TNT begins with the reduction of the nitro groups that can occur as a series of two-electron transfers by oxygen-independent nitroreductase enzymes (Type I). This reductive metabolism yields the nitroso, hydroxylamino, and amino derivatives of TNT, namely 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-4,6-dinitrotoluene (2-HADNT/4-HADNT), 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (2-ADNT/4-ADNT), and 2,4-diamino-6-nitrotoluene (2,4-DANT) (Esteve-Núñez et al., 2005; Smets et al., 2007). Degradation of these aminonitroaromatic compounds is necessary, as they are equally toxic as the parent compound TNT (Esteve-Núñez et al., 2001). The degradation in aerobic conditions is limited (Esteve-Núñez et al., 2001) and has been demonstrated to occur slowly in anaerobic conditions (Daun et al., 1998; Lenke et al., 1998). Complete reduction of nitro groups in anaerobic conditions results in 2,4,6-tri-aminotoluene (TAT) that can further mineralize to trihydroxytoluene, polyphenols, p-cresol, and acetate (Pichtel, 2012) to serve as C source for microorganisms. However, this transformation is only possible in the presence of additional substrates such as glucose, acetate, and citrate (Daun et al., 1998). Whether deriving C from a stable aromatic ring for assimilation remains a bottleneck for TNT degradation, deriving N can be relatively easy for microbial utilization. During the reductive metabolism of TNT, nitrite and ammonia are released and serve as N-source for microorganisms (Serrano-González et al., 2018; Esteve-Núñez et al., 2001). Due to limited degradation of TNT and accumulation of toxic metabolites in aerobic condition, research has been predominantly focused on degradation in anaerobic conditions, although most of the cases microorganisms capable of using TNT as N-source is the main outcome (Caballero et al., 2005; Esteve-Núñez and Ramos, 1998). Interestingly, recent studies on coastal areas showed significant mineralization and bacterial incorporation using C labeled TNT (Tornero and Hanke, 2016; Montgomery et al., 2011; Montgomery et al., 2020). Therefore, a variety of TNT degraders have been reported, yet, the potential of marine microbiome for the degradation has been overlooked. In particular microbiome near World War dumpsites might be well-adapted to the warfare agents due to slow leakage and hence, provides us with an opportunity to find novel taxa with degradation potential.

To fill this research gap, we conducted degradation experiments with sediment samples collected from the proximity of the munition dumpsite – *Paardenmarkt* in the Belgian part of the North sea. In 1919, mostly German World War I munition was dumped daily for six months on the sandbank. The dumpsite is approximately 4.5 km^2 in size and pentagon-shaped. Documentation regarding the exact amount of dumped munition is missing, but an estimated 35.000 tons of both conventional artillery shells and poisonous gas grenades were dumped (Missiaen, 2013). The sediment samples were incubated both at high and low concentrations of TNT in aerobic conditions. This is due to the fact that the drivers of degradation can differ at high and low concentrations (Kundu et al., 2019). Experiments showed that degradation of contaminant is fast at high concentration, which eventually becomes slow or even non-existent at low concentrations (Ehrl et al., 2019; Helbling, 2015; Kundu et al., 2022). In addition, microorganisms face a multitude of assimilable organic carbon in the environment. This provides metabolic and physiological flexibility to the microorganisms and often positively influences the degradation of contaminant by providing extra energy source for bacterial viability and activity (Egli, 2010). In this study, after the incubation with only TNT as sole C and N source, an additional C source – methanol, was used to boost the degradation. Biochemical analysis for TNT degradation and its metabolites was coupled to flow cytometry measurements and 16S rRNA gene sequencing analysis to monitor microbial growth and the shift in community structure. Our results showed the potential of the marine microbiome using TNT as C and N source at a slow rate, whereas the addition of an extra C source significantly boosted the degradation.

2. Materials & methods

2.1. Experimental design

Sediment samples near the *Paardenmark*t site (Supporting Information, Location ST5, Figure S1) were obtained (Sampling campaign by Flanders Marine Institute, VLIZ, 2020) and used as the inoculum (10 % *v*/v) for the degradation experiments. Experiments were conducted in triplicate in amber Duran bottles to inhibit photodegradation. The bottles were kept on a shaker with continuous agitation at 125 rpm and were closed with a perforated cap with 0.22 μm expanded polytetrafluoroethylene (ePTFE) membrane filter (VWR, Belgium) to allow aeration. Sediment slurry was added to filter-sterilized seawater (0.22 μm, Millipore, Germany) supplemented with nutrients (see Supporting Information, Table S1 for details). Two experiments were set up to study the biodegradation of TNT.

Experiment A was performed with a high concentration of TNT (80–100 mg/L) to mimic highly contaminated sites and subsequently enrich the indigenous TNT degraders present. A stock solution of TNT (20 g/L, dissolved in methanol) was obtained from Royal Military Academy, Belgium. A single pulse of TNT was planned in two ways: i) TNT stock solution was added to a dry, sterile Duran bottle and was evaporated by a gentle air stream. After complete evaporation, seawater supplemented with nutrients was added and kept on the shaker until complete dissolution. Subsequently, sediment slurry was added to initiate biodegradation. In this setting TNT was sole source of C and N ii) TNT stock solution was added and was not evaporated to dryness to provide methanol as an additional substrate for energy metabolism. Afterwards, seawater supplemented with nutrients and sediment slurry were added to the bottles. To investigate the influence of additional N on TNT degradation, one incubation was performed with addition of $(NH_4)_2SO_4$. after incomplete evaporation of TNT stock solution as described above. In this case, TNT degradation was studied in the presence of both additional C and N. The first incubation (phase 1) of the enrichment was performed for 45 days to provide sufficient time for adaptation of microorganisms to new external conditions. For subsequent phases, after every 30 days, the culture was transferred to a new bottle with fresh media supplemented with TNT. A total incubation time of 120 days and 136 days were implemented for conditions (i) and (ii), respectively.

Experiment B was performed with repeated spikes to sustain low concentrations of TNT, representing continuous and slow leakage of TNT from contaminated sites. The media used in this experiment had the same composition as Experiment A, with the exception that methanol was not evaporated. The same inoculum used in Experiment A was used. Two different low concentrations were studied - 300 μg/L (R1) and 2 mg/L TNT (R2). Two controls were included: i) C1 (no TNT, but with acetate 2 mg/L) to follow the time-dependent changes in the microbial community; ii) C2 (inoculated with autoclaved sediment and NaN3 (160 mM), blocking the metabolic activity (Otte et al., 2018)) to follow the adsorption of TNT and metabolites to the sediment. The experiment duration was four weeks, with four spikes of TNT each week.

2.2. Sampling for physical and chemical analysis

Samples were collected for flow cytometric and chemical analysis. Sampling was performed weekly for Experiment A and three times a week after (re)spiking for Experiment B. Samples were collected on the day of re-spiking, both before and after the re-spiking. Samples for flow cytometry were fixed in 2 % glutaraldehyde and stored at 4 °C, except for specific samples that were used for live-dead cell measurement. Samples for chemical analysis were filtered with a regenerated cellulose membrane filter (pore size 0.2 μm, diameter 47 mm; GE Healthcare ltd., UK) and were stored in brown vials at −20 °C. Sediment samples (1 g) were taken for the adsorption control (C2) after two weeks and at the end of the experiments and stored at −20 °C. Samples for 16S Illumina

sequencing were taken at the end of each phase of Experiment A and Experiment B in RNA/DNA-free vials. The vials were subsequently centrifuged (7 min, 18,213 g). The supernatant was discarded, and the cell pellet was stored at −20 °C.

2.3. Chemical analysis

TNT and its degradation products - 2-ADNT, 4-ADNT, 2,4-DANT and 2,6-DANT were analyzed by gas chromatography coupled to a triple quadrupole mass spectrometer (GC-TQMS, Thermo Scientific, Germany). 1–4 mL of the samples (depending on concentration) were extracted with dichloromethane (2 × 2 mL) and subsequently concentrated to 1 mL with gentle stream of N_2. 1,3,5-trinitrobenzene was added as internal standard. TNT and other metabolites were extracted from the sediment by specific accelerated solvent extraction (ASE) at a pressure of 10.3 MPa. For the sediment a clean-up is performed by first eluting the concentrate over an ALOx (10 % water) with 20 mL Dichloromethane. This concentrate is passed over GPC (Column ENVIROGEL. 19X300MM and ENVIROGEL COL. 19X150MM). 2,4,6-trinitrobenzene-C13-N15 was added as recovery standard. 1 μL of the extract was injected in the GC The chromatographic separation was performed on a RXI-5sil MS column (20 m length, 0.15 mm internal diameter, 0.18 μm film thickness, Restek, USA). The pressure value for high-pressure injection was 250 kPa, and the injection port temperature was set to 40 °C. The injection mode was splitless. Helium alphagas 2 (constant flow 1 mL/min) was used as carrier gas. The temperature program of the column was set to maintaining the initial temperature at 35 °C for 2 min, then increasing the temperature to 250 °C at a rate of 25 °C per minute, then increasing the temperature to 300 °C at a rate of 10 °C per minute, and finally holding for 4.5 min.

NH_4^+, NO_2^-, and NO_3^- were measured using MQuant® colorimetric strip kits (Supelco, Merck, Germany).

2.4. Flow cytometry

Sample preparation for flow cytometry was performed by diluting the samples 1/10 in phosphate buffer saline (PBS) and subsequently vortexing (30 s), sonication (20 min) and vortexing (30 s) again. Vortexing and sonication were used to separate the cells and the sediment as much as possible. After the second vortexing, samples were filtered over a Filcon filter (20 μm, BD Biosciences) to remove small sediment particles that could clog the flow cytometer. The background noise was minimized by letting the <20 μm sediment particles settle and using the supernatant for measurement. The samples were stained with SYBR® Green I (SG, 100× concentrate in 0.22 m-filtered dimethyl sulfoxide, Invitrogen, Belgium) for total cell analysis, and SYBR® Green I combined with propidium iodide (SGPI, 100× concentrate SYBR® Green I and 50 × 20 mM propidium iodide, in 0.22 m-filtered dimethyl sulfoxide, Invitrogen, Belgium) for live-dead analysis. Staining was performed with incubation for 20 min in the dark at 37 °C. Samples were analyzed immediately after incubation with an AttuneNxT flow cytometer (Fisher Scientific, Germany), equipped with two lasers (488 nm, 637 nm) and nine detection channels (FSC, SSC, BL-1: 530/30 nm, BL-2: 574/26 nm, BL-3: 695/40 nm, RL-1: 670/14 nm, RL-2: 720/30 nm, RL-3: 780/60 nm).

2.5. Illumina 16S rRNA gene sequencing

Genomic DNA was extracted from Experiment A and B sediment samples using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's protocol. PCR was performed using a recombinant Taq DNA Polymerase kit, Fermentas (Thermo Fischer Scientific, Waltham, MA, USA), in 25 μL reactions containing: 2.5 μL 10× Taq buffer (+KCl – MgCl2), 0.5 μL of 10 mM dNTP, 1.5 μL of 25 mM $MgCl_2$, 0.5 μL of 10 μM Primer 27f AGAGTTTGATCMTGGCTCAG, 0.5 μL of 10 μM Primer 1492r TACGGYTACCTTGTTACGACTT, 0.125 μL of 5 U/μL Taq polymerase, 0.065 μL of 20 mg/mL BSA (Roche Holding AG, Basel, Switzerland), 14.81 μL PCR-water and 1 μL sample.

Amplification was run, including initial denaturation for 7 min at 95 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C and 2 min extension at 72 °C. A final elongation step was included at 72 °C for 10 min. The PCR product was run along the DNA extract on a 2 % agarose gel for 30 min at 100 V. 10 μL of the original genomic DNA extract was sent to LGC Genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry with the primers mentioned above. The DADA2 R package was used to process the amplicon sequence data according to the pipeline tutorial (Callahan et al., 2016). In a first quality control step, the primer sequences were removed and reads were truncated at a quality score cut-off (truncQ = 2). Besides trimming, additional filtering was performed to eliminate reads containing any ambiguous base calls or reads with high expected errors (maxEE = 2,2). After dereplication, unique reads were further denoised using the Divisive Amplicon Denoising Algorithm (DADA) error estimation algorithm and the selfConsist sample inference algorithm (with the option pooling = TRUE). The obtained error rates were inspected and after approval, the denoised reads were merged. Subsequently, the ASV table obtained after chimera removal was used for taxonomy assignment using the Naive Bayesian Classifier and the DADA2 formatted Silva v138 (Quast et al., 2012). ASV's mapping back to anything other than 'Bacteria' were filtered out as they were considered technical noise. Singletons, reads occurring only once in all samples were considered noise and were removed as suggested in McMurdie and Holmes (2014) (McMurdie and Holmes, 2014). Visualization of the data was done using the phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Villanueva and Chen, 2019) packages.

3. Results and discussion

3.1. Presence of methanol as an extra C source boosted TNT degradation at high concentration

The incubation of sediment samples in the presence of TNT as the sole C and N source showed no growth of microorganisms over 30 days. After two transfers to new media supplemented with TNT, an adaptation in the bacterial community to TNT exposure was visible in terms of ~40 % transformation of the initial concentration of TNT (100 mg/L) after 15 days of exposure (Supporting Information, Figure S2). The degradation of TNT was reflected in the detection of two metabolites related to nitroreduction pathway – 2-ADNT and 4-ADNT, and bacterial growth. However, TNT removal is determined by two factors: adsorption and biodegradation, with comparable importance (Ariyarathna et al., 2017). Adsorption is dependent on grain size, water temperature, and organic carbon content (Smith et al., n.d.). Bacteria are shown to degrade adsorbed TNT, indicating the importance of sorption and surface-mediated bacterial transformation (Smith et al., n.d.; Smith et al., 2015). The dynamics of adsorption and desorption can also control the concentration detected in the liquid phase. The sorbed fraction of TNT and its degradation metabolites in the sediment (at the end of the experiment) contained 22 % of the total concentration in the experimental system. Hence, no accumulation of metabolites in the liquid phase coupled with slow bacterial growth indicates degradation of TNT by the indigenous microbial community at the *Paardenmarkt* site. Some studies have shown that during nitroreduction, some other metabolites are formed (Bai et al., 2015; Gupta et al., 2023). For example, reactive nitroso and hydroxyl amino-intermediates can react themselves to condense to azoxy-dimers. Hence, alternative scenario might be a fraction of TNT was transformed to these isomers besides 2ADNT and 4 ADNT. However, the azoxydimers are not further degraded and cannot be funneled to cellular metabolism. Here, slow growth was observed indicating that formation of different dimers was not significant. In contrast to the general observation of transforming TNT to its organic

derivatives, our results indicate the utilization of TNT as the sole source of C and N. This is in accordance with the earlier finding of TNT ring carbon utilization by natural sediment assemblages (Montgomery et al., 2011; Gallagher et al., 2010).

In anaerobic conditions, where the TNT degradation is faster, the presence of an additional substrate is crucial (Pichtel, 2012). Hence, to enhance the degradation in our aerobic system, an additional substrate–methanol was used. The ocean is believed to be a large reservoir for methanol (Howat et al., 2018; Jacob et al., 2005) as methanol has been experimentally measured both in sediment, water column and pore water (Zhuang et al., 2014; Williams et al., 2004; Yanagawa et al., 2016). Methylotrophic microorganisms consume methanol for aerobic respiration. In addition methanol degradation is associated with several other processes such as denitrification, iron reduction, sulfate reduction and methanogenesis (Yanagawa et al., 2016). Hence, methanol is an important compound in geochemical cycle. Furthermore, methylotrophic microorganisms are reported to play an important role in pollutant degradation (Mishamandani et al., 2014). Thus, methanol is an interesting model additional substrate to boost the degradation. With the addition of methanol, TNT degraded faster – 100 % removal was observed on the tenth day after the first exposure to TNT, whereas 40 % degradation was achieved with TNT as a sole source of C and N. The degradation of TNT was coupled to bacterial growth observed in the presence of extra C source methanol (1–2 log unit increase in cell concentration). Concerning the degradation products, both the first metabolites of TNT nitroreduction (2-ADNT and 4-ADNT, missing one nitro group) and the secondary metabolites of TNT nitroreduction (2,4-DANT missing two nitrogroups) were detected with the removal of TNT (Fig. 1). On day 0, trace concentrations of 2-ADNT and 4-ADNT were detected. This was due to the impurities present during the synthesis of TNT. Interestingly, these metabolites were detected in low concentrations (μg/L range) and accounted for 5.9 % of total TNT removal. As adsorption was not found significant, this suggests the metabolites were further degraded and might have been used for C and N assimilation. Several degradation mechanism has been proposed for C and N assimilation from TNT (Smets et al., 2007). After the nitroreduction of TNT molecules, ADNTs can be further transformed by ring hydroxylating dioyxygenases to produce catechols as well as bezyl alcohols (Johnson et al., 2001). Whether these metabolites can further be enzymatically degraded remains unclear. N assimilation from TNT happens by release of nitrite or ammonium by different routes of degradation. Aminodinitrotoluenes are oxidized by dioxygenases to form aminomethylnitrocatechol and release nitrite (Smets et al., 2007; Johnson et al., 2001). Some of the nitroreductase enzymes can reduce TNT aromatic ring by forming hydride-meisnheimer complex which can be subsequently denitriated to 2, 4, di-nitro-toluene and can release nitrite (Smets et al., 2007; Iman et al., 2017). Hydroxylamine products are also formed during the degradation to 2-ADNT and 4-ADNT. These can condensate with Meisenheimer complexes, resulting in re-aromatization of these complexes, formation of diarylamines, and the release of nitrite (Wittich et al., 2008). The hydroxylamine products can also undergo enzymatic Bamberger rearrangement in which the hydroxylamino group is converted to a range of amino and hydroxyl products and ammonium release (Smets et al., 2007; Caballero et al., 2005). N assimilation from TNT was suggested to occur via glutamine syntethase-glutamate synthase (GS-GOGAT) pathway (Caballero et al., 2005). While our experimental data do not provide evidence of using TNT as a C source in the presence of methanol, nitrite was detected in the experimental system indicating N assimilation from TNT. As no N source was present in the media, N assimilation from TNT successfully maintained the growth and viability. At the end of the experiment, 60 % of the cells were found to be alive. A similar observation of rapid removal of TNT with methanol has been reported, except that TNT was not used as a N source and no degradation of metabolites (2-ADNT and 4-ADNT) was observed (Van Aken et al., 2004). Comparing the removal rate observed with other easily assimilable organic C such as glucose, only 60 % removal was observed after 5 days with accumulation of degradation metabolites (Gupta et al., 2023).We tested the presence of additional N

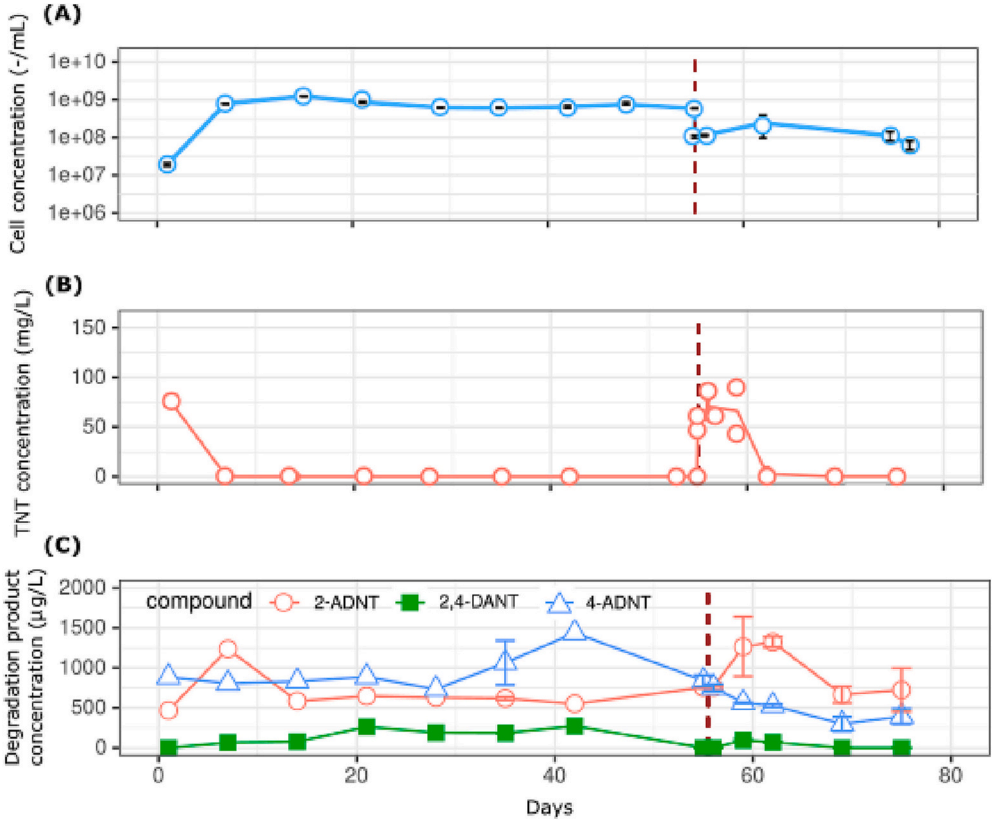

Fig. 1. Rapid degradation of tri-nitrotoluene (TNT) with the addition of methanol. A: Cell concentration measured during incubation experiment with a high concentration of TNT and methanol B: Concentration of residual TNT C) Concentration of different metabolites of TNT degradation. Brown dashed line denote two transfers of culture to fresh media supplemented with TNT and methanol. Error bar indicates the standard error of replicates (n = 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the TNT degradation rate. In the presence of additional N, the degradation of TNT was rapid (Fig. 2). Besides 2-ADNT, 4-ADNT, 2–4 DANT, another metabolite – 2,6 DANT was observed, indicating broad and diverse metabolism under N supplementation. Besides providing extra energy as a C source, methanol may also influence partitioning of TNT and degradation products between water and sediment. TNT and its degradation metabolites are known to adsorp to the sediment and have low solubility in water (Smith et al., 2015; Yost et al., 2007). Thus these compounds remain little bioavailable for bacterial metabolism (Robertson and Jjemba, 2005).Presence of methanol can enhance the dissolution of sorbed TNT to the aqueous phase and increase the bioavailability of TNT for bacterial metabolism.

The results at a high concentration of TNT (80–100 mg/L) are essential to understanding growth-coupled microbial utilization of TNT and subsequently enriching the degraders, however, degradation at environment-relevant concentration is of utmost importance. In the next section, the degradation of TNT observed in the low concentration regime is discussed.

3.2. Rapid degradation of TNT at low concentration

A single pulse of TNT (dissolved in methanol) to reach initial concentrations – 300 μg/L (R1) and 2 mg/L (R2) was used and monitored for 7 days. In total, 4 TNT spikes were added. The control with autoclaved sediment and NaN_3 addition (C2) showed only limited TNT removal after the first and second spike, and a step-wise increase in TNT concentration profile was observed throughout the experiment (Fig. 3). In contrast, after each TNT pulse, a rapid disappearance of TNT was observed in R1 and R2 (Fig. 4). TNT removal rates ranging 0.121–0.153 d^{-1} were observed in R2. At a low concentration (300 μg/L TNT, R1), TNT was removed after every spike with higher removal rates of 0.139–0.200 d^{-1}. With the disappearance of TNT, two degradation metabolites – 2-ADNT and 4-ADNT were observed, confirming the disappearance is due to microbial activity. The observed concentration range for 2-ADNT and 4-ADNT were low – (0.2–40 μg/L) and (1.2–45 μg/L), respectively. Metabolites accounted for 0.19 % (R1) and 0.71 % (R2) of spiked TNT in the experimental set-ups. This indicates the

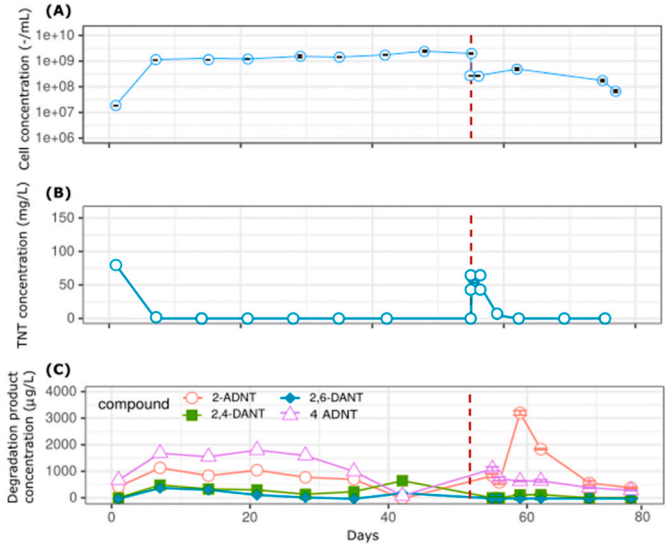

Fig. 2. The presence of additional C (methanol) and N (nitrite) sources along with TNT boosted the degradation of TNT. A: Increase of cell concentration during the incubation experiment. B: Degradation profile of TNT C: Concentration of degradation metabolites. Brown dashed line denote two transfers of culture to fresh media supplemented with TNT, methanol, and nitrite. Error bar indicates the standard error of replicates (n = 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

further transformation of these metabolites as observed at a high concentration. In contrast to the high concentration, significant microbial growth was not observed during the degradation of TNT. The cell count remained constant at 10^7 cells/mL in the control without TNT (C1) and the experiment with 300 μg/L TNT (R1). However, at the end of the experiment, an increase in cell count for R2 (2 mg/L, TNT) was observed, statistically greater than C1 for day 26 (Mann-Whitney *U* test, $\alpha = 0.002$) and day 29 (Mann-Whitney U test, $\alpha = 0.002$). The conditions R1 and C1 have low Food(F)/ Microorganism (M) ratios in the system, mimicking low energy availability in the natural environment (Hoehler and Jørgensen, 2013). Thus the available energy was used for biomass maintenance, not for growth. While rapid removal of TNT was reported at low concentrations due to the partitioning effect (Smith et al., 2015), microbial degradation is reported only at high concentrations (Boopathy et al., 1994; Thiele et al., 2002; Serrano-González et al., 2018; Esteve-Núñez et al., 2005; Daun et al., 1998). It is noteworthy that the indigenous microbial community at the munition dumpsite – *Paardenmarkt* has the intrinsic capacity to degrade TNT both at high and low concentrations. As a next step to explore the potential degraders, 16S rRNA gene sequencing analysis was performed.

3.3. 16S Illumina sequencing revealed Methylpophaga as an "enhancer" of TNT degradation

Microbial composition analysis of sediment samples in all conditions was performed based on 16S rRNA gene analysis. A clear difference in the microbial community composition in terms of relative abundance of different bacterial taxonomic groups is visible before and after TNT exposure at high concentrations (Fig. 5A, Supporting Information, Figure S3). The microbial community in the sediment sample used as an inoculum (Start condition, Fig. 5A) is highly diverse, and the relative abundance of members of genera belonging to *Pseudoalteromonas*, *Sulfurimonas*, and *Thiomicrorhabdus* were higher compared to other genera. Incubation of the sediment sample in the presence of TNT as a sole source of C and N (Condition TNT, Fig. 5A) resulted in a different distribution of taxonomic groups, with the most abundant genera being *Colwellia*, *Thalossospira*, and *Neptuniibacter*. Under the condition of an additional C source - methanol (Condition TNT + methanol, Fig. 5A), *Methylophaga* was the most abundant genus, followed by *Stenotrophomonas*, *Pseudomonas*, and *Thalossospira* in phase 1. In phase 3, the community composition was similar, with a much higher abundance of *Methylophaga* (~25 % in phase 1 vs. ~75 % in phase 3) and the emergence of *Halomonas*. The addition of both extra C and N (TNT + methanol + nitrite, Fig. 5A) caused a shift in microbial community structure, with the most abundant genera being *Methylophaga*, *Pseudoalteromonas*, and *Thalossospira*. Hence, the community composition was condition-specific in the individual experimental set-ups. Nevertheless, there were 9 ASVs common in the presence of methanol and without methanol (Fig. 5B), suggesting their role in the degradation of TNT under both conditions. Three of these ASVs were identified as members of the highly abundant genera detected (at least in one condition) - *Methylophaga*, *Thalassospira* and *Pseudoalteromonas*. While at high concentrations of TNT, the change in microbial community structure was visible, at low concentrations (R1–300 μg/L), little shift was observed compared to control without no TNT addition (Fig. 6). The relative abundance of *Pseudoalteromonas* was higher in R1 than the control condition. In R2, with relatively higher concentrations of TNT along with methanol, a clear shift in the community structure compared to the control was observed. *Methylophaga* was the most abundant genus, followed by *Thalossospira*, *Sulfurovum*, and *Pseudoalteromonas*.

External environmental factors select the relative abundance of taxa that can thrive under local conditions. As TNT degradation was observed in all conditions, these abundant bacterial taxa must play a role in TNT degradation. When TNT was present as the sole source of C and N, the most abundant taxa were reported to be aromatic hydrocarbon degraders. Recently, *Colwellia*, *Thalossospira*, *Pseudophaeobacter*, and

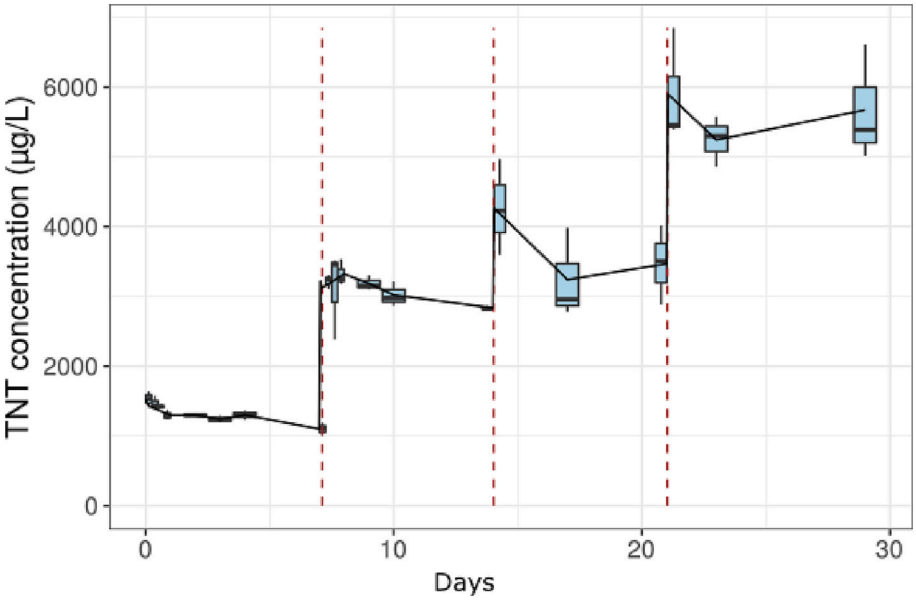

Fig. 3. Minimal adsorption of TNT in the sediment in the control set-up (C2). Microbial activity was inhibited by autoclaving the sediment and adding 150 mM NaN_3.

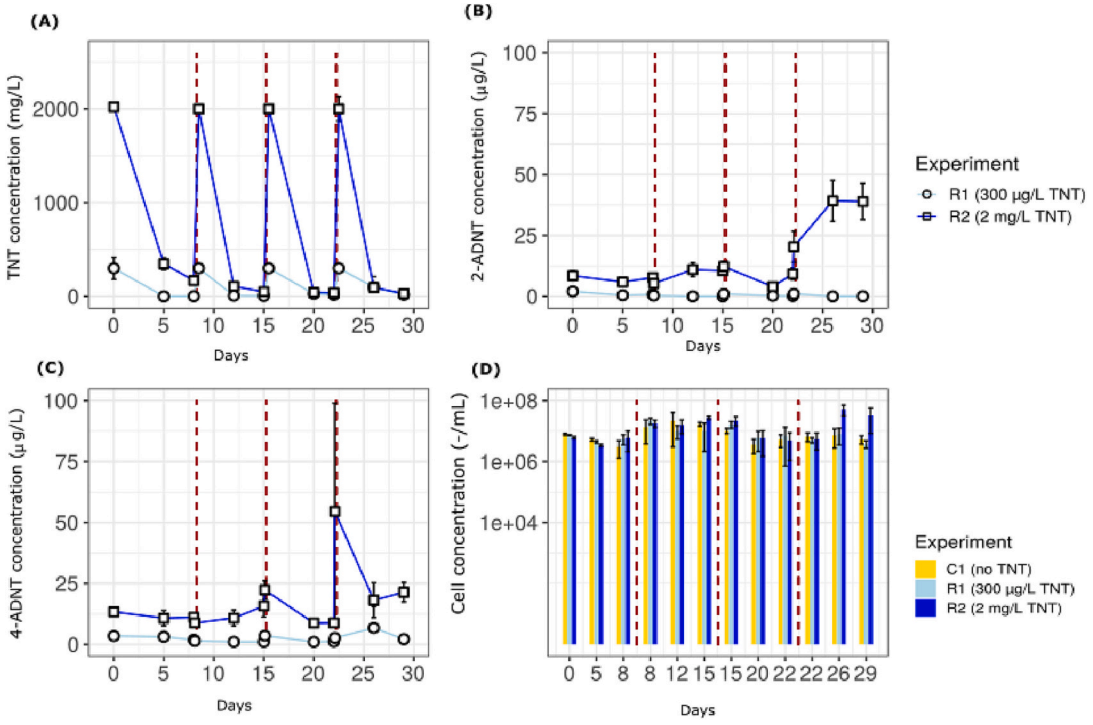

Fig. 4. Rapid degradation of TNT during experiments performed at low concentrations (R1–300 µg/L and R2–2 mg/L). A) Concentration of TNT detected in the seawater during repeated spikes of TNT. The degradation products of TNT – 2-ADNT and 4-ADNT are shown in B) and C) respectively. D) The cell concentration measured during repeated spikes of TNT (in R1 and R2) and in the control (C1) – served to reflect time-dependent changes in microbial communities.

Neptuniibacter have been reported to be involved in aromatic hydrocarbon degradation in marine environment (Mason et al., 2014; Santisi et al., 2022; Nikolova et al., 2021; Achberger et al., 2021). *Alcanivorax*, an obligate hydrocarbonoclastic bacteria (Xu et al., 2018) was also detected at low concentrations (Fig. 6). For the assimilation of C from TNT, aromatic ring cleavage is necessary. In an aerobic environment, bacteria can oxidize aromatic molecules with mono- or dioxygenase enzymes. Hydrocarbon degraders employ oxidizing reactions for ring cleavage and, thus, can assimilate C from the aromatic structure of TNT. However, TNT is resistant to oxidative attack due to the presence of three NO_2 groups; hence, reduction of NO_2 groups is a prerequisite prior to oxidizing reactions. The detected metabolites from the reduction pathway – 2-ADNT, 4-ADNT provide evidence that reductive metabolism was active, whereas slow microbial growth observed on TNT as sole source of C and N strongly suggests TNT degradation. It is not known whether the nitroreductase enzyme, responsible for TNT reduction, is present in hydrocarbon degraders. The potential of these genera has not been fully explored and novel taxa continue to be discovered. Hence, they might have intrinsic capacity for reducing TNT or other detected bacterial groups might also have nitroreductase enzyme to

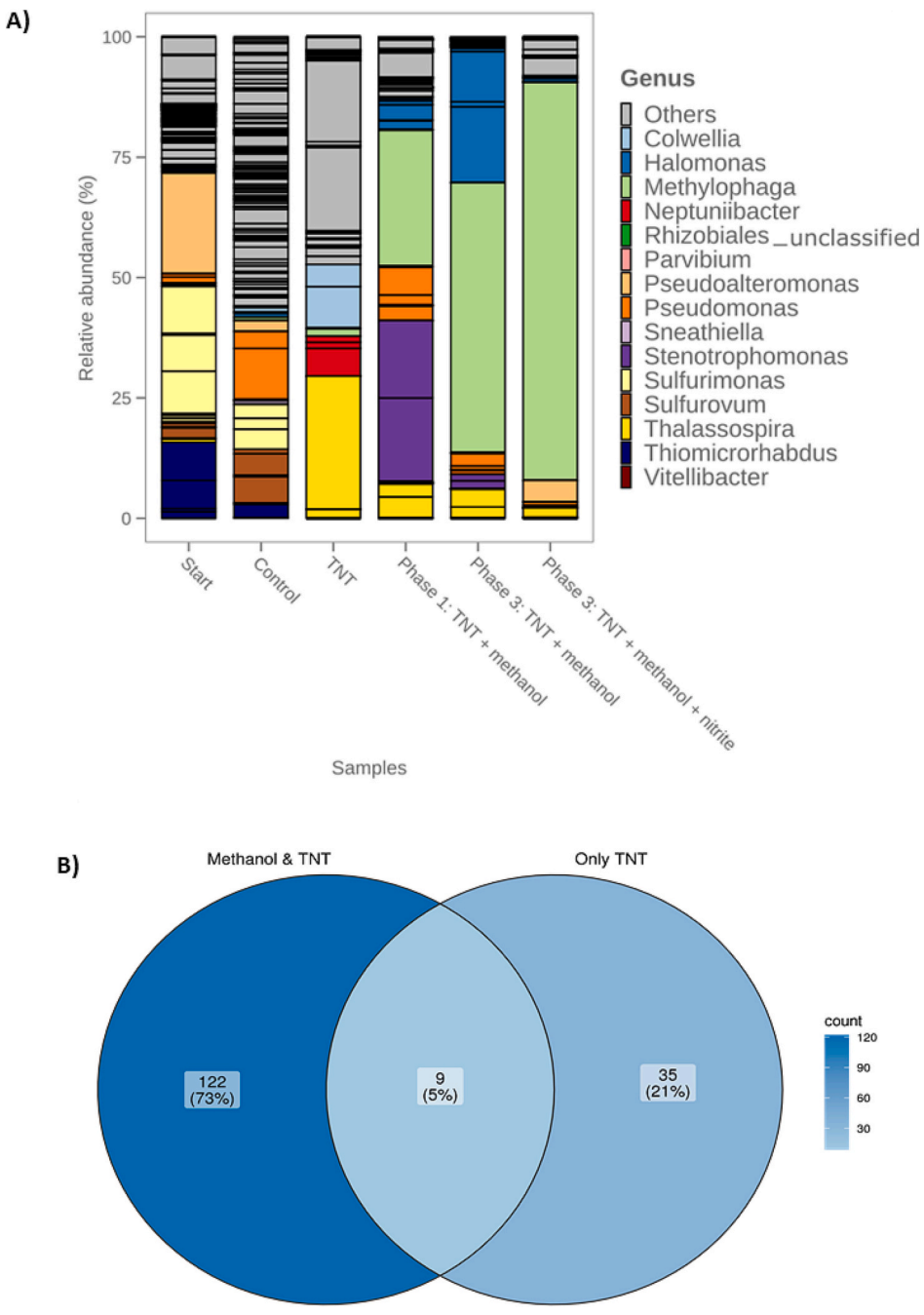

Fig. 5. Exposure to TNT resulted in a visible change in community composition. A) Community composition of TNT degrading enrichment culture under different conditions. Every segment of the bar corresponds to a single ASV. ASVs belonging to the top 15 abundant genera are colored, and the rest is mentioned as "other." A noticeable change in the community compositions is observed compared to the start inoculum and control (sampled at phase 1). In the presence of methanol and TNT, the community is dominated by *Methylophaga* followed by *Stenotrophomonas* and *Pseudomonas*. In the presence of only TNT *Colwellia*, *Thalossospira*, and *Neptuniibacter* were the most abundant genera. B) Venn-plot showing unique ASVs and shared between with methanol and methanol free condition. The average relative abundance of replicates are plotted. NMDS plot showing clustering of replicates under all conditions is included in Supporting Information (SI, Fig. S3).

initiate TNT reduction. Although less abundant than the hydrocarbon degraders, an ASV belonging to *Methylophaga* (relative abundance 1.63 %) is also among top 10 abundant genera. Members of *Methylophaga* genus belong to the *Piscirickettsiaceae* family in the *Gammaproteob*acteria and are methylotroph requiring C1 sources as growth substrate. Methylotrophs (*Methylobacterium* and *Methylopila*) has been shown to co-metabolize TNT to nitro-reduced metabolites, whereas *Methylobacterium* can even mineralize RDX (Van Aken et al., 2004; Avila-Arias et al., 2017). Hence, under the condition of TNT present as a sole source of C and N, the degradation of TNT would have been the result of syntrophic association of different bacterial groups – *Methylophaga* and hydrocarbon degraders.

With the addition of methanol, the abundance of *Methylophaga* increased. This is expected as methanol was present at 10 times higher concentration than TNT, and being a C1 compound supports the growth of methylotrophs. However, the addition of methanol and the higher abundance of *Methylophaga* corresponded to the rapid removal of TNT (Fig. 1, Fig. 5), indicating a role of *Methylophaga* in TNT degradation. Literature evidence suggests that methylotrophs can transform TNT into nitro-reduced compounds (Van Aken et al., 2004; Avila-Arias et al., 2017) but does not support the potential of further degradation of TNT metabolites. Interestingly, our data shows no accumulation of TNT metabolites in *Methylophaga*-dominated set-ups. It might be the case that *Methylophaga* only catalyze the TNT reduction steps, whereas aromatic ring cleavage is performed by other bacterial groups. Out of three other highly abundant genera, members belonging to *Pseudomonas* are widely known to be TNT degraders (Serrano-González et al., 2018), and *Thalassospira*, being hydrocarbon degraders (Santisi et al., 2022) can also perform aromatic ring cleavage. *Stenotrophomonas* can co-metabolize TNT (Avila-Arias et al., 2017). ASVs belonging to *Halomonas* increased

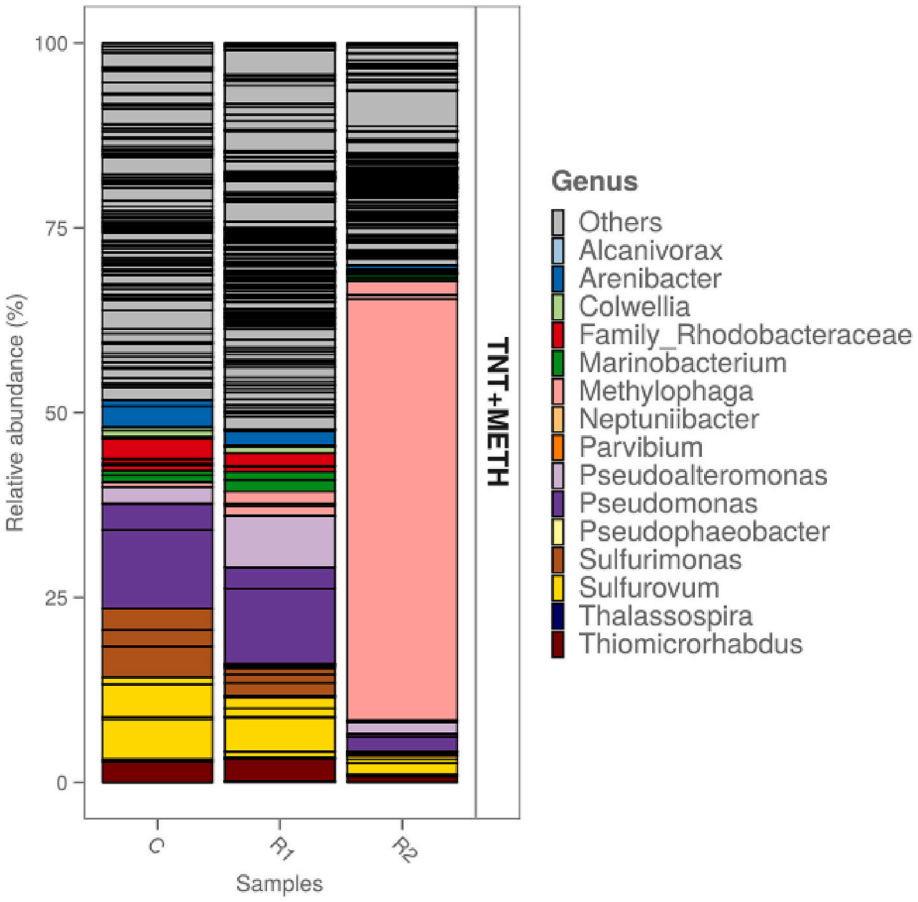

Fig. 6. Visual representation of the 15 most abundant genera divided in ASVs found in the exposure experiment, including the control (no TNT, C1), R2 experiment (2 mg/L TNT) and R1 experiment (300 μg/L) at the end of experiment B (day 28). The high diversity of genera in the control was replaced, by TNT exposure (2 mg/L), to a microbiome predominantly consisting of *Methylophaga*. Meth – methanol.

with time and this genus has also been suggested as aromatic hydrocarbon degraders (Hassanshahian and Boroujeni, 2016). However, although our knowledge of the diversity and potential of marine microorganisms has progressed, it is far from complete. Members of *Methylophaga* have been shown to grow and degrade polycyclic aromatic hydrocarbon (Mishamandani et al., 2014; Vila et al., 2010). Thus, aromatic ring cleavage of TNT can be performed by *Methylophaga* detected in this study. Hence, our results – higher abundance of *Methylophaga* and rapid removal of TNT without metabolite accumulation, together with literature data on co-metabolism of TNT and aromatic ring cleavage by methylotrophs - provide strong evidence that *Methylophaga* is a potential degrader of TNT. In the context of methanol utilization in marine environment, the extent of methanol oxidation by methylotrophic microorganisms is largely dependent on sediment characteristics. In case of deep sea sediment with fine grains, oxygen can slowly diffuse and it gets quickly consumed within the first millimeter of the sediment (Glud, 2008). The methanol oxidation will be restricted mostly to the upper layer of the sediment. In case of shallow sediment advection driven pore water movement introduces oxygen even in the deeper layer of sediment (Taubert et al., 2019) and oxidation process can be ongoing at different layers. Thus methylotrophic microorganisms can participate in TNT degradation in different settings of marine environment.

4. Conclusions

TNT, best known as an explosive, is a widespread contaminant in the marine environment. For example, in 2010, OSPAR's Quality Status Report listed 148 munition dumpsites in the North-East Atlantic Ocean and the North Sea alone, and TNT is the major explosive in the formulation of dumped munitions. Hence, contamination of TNT is a border transgressing problem. More recently, 'Munitions in the Sea' was launched as joint action in the context of the Joint Programming Initiative' Healthy and Productive Seas and Oceans' (JPI Oceans), highlighting the need to assess risks, define priorities and develop intervention options with regards to munitions in the marine environment. Although direct extrapolation of the results to munition dumpsites is difficult due to various geochemical conditions, our study holds promise in several aspects. First, it shows the degradation potential of indigenous microbial communities at munition dumpsites by using TNT as C and N source. Several novel genera were enriched and shown to be potential degraders. Second, it shows that adding the extra C source, methanol boosted the TNT degradation. Third, it is remarkable that TNT was rapidly degraded at high (copiotrophic) and low (oligotrophic) concentrations. However, our study does not provide mechanistic insight into the degradation process, highlighting the role of each enriched genera in terms of metabolite utilization and cellular metabolism. Future work linking directly the degradation activity to the enriched microbial strains by applying RNA-SIP (stable isotope probing) approach, and isolating enriched microbial strains and subsequent metabolomics study coupled with genome analysis will provide insight into the degradation pathway and syntrophic association.

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CRediT authorship contribution statement

Kankana Kundu – Conceptualization, Methodology, Validation, Writing – Original draft, Josefien Van Landuyt – Software, visualization, Writing – Editing and reviewing, Valrie Mattelin – Visualization, Writing – Editing and Reviewing, Bram Martin – Investigation, Marijke Neyts – Methodology, Investigation, Koen Parmentier – Resources, Writing – Editing and Reviewing, Nico Boon – Project administration, Funding acquisition, Supervision.

The manuscript was written with the contributions of all authors. All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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