Evaluation of the transformation of solid pentolite by TNT and PETN degrading bacteria.

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Abstract

Pentolite, a mixture of 2,4,6-trinitrotoluene (TNT) and pentaerythritol tetranitrate (PETN) (1:1 w:w) is a solid explosive with military and civilian uses as the main component of blasting charges and ordnance. Chronic exposure to pentolite produces health problems such as cancer, liver damage and irritations to the upper airways. Furthermore, leakage of these explosives from unexploded ordnance can contaminate groundwater and soils. Although the transformation of solid pentolite has not been studied in depth, it is known that partially reduced byproducts of the transformation of TNT may inhibit the transformation of other explosives such as PETN. In the present study, pure strains, and mixed consortia of TNT or PETN degrading bacteria were used to establish defined consortia, and their ability to degrade solid pentolite was evaluated in a microcosm study, using chunks of pentolite (0.2 g) with a minimal salts media (MT2M). Thirty-nine bacteria and mixed cultures, along with nineteen defined consortia, and 3 microcosms without inoculum were evaluated for ~ 2,700 days. Explosive transformation was monitored by HPLC and the bacterial density was measured by plate count. Solid explosive transformation (up to 69%) was observed by 3 mixed cultures, 2 bacteria and 3 defined consortia, additionally one of the microcosms without inoculum showed transformation of the explosives. (29 % PETN, 21 % TNT). Mixed cultures showed a higher transformation (23 % PETN, 19 % TNT) of the explosives than that obtained by the defined consortia (20 % PETN, 17 % TNT). The presence of TNT and PETN transformers on the surface of the solid pentolite and their tolerance the explosive is worth noticing, as their ability to degrade explosives could also be exploited in the bioremediation of explosive contaminated sites.

Introduction

Pentolite is a 1: 1 mixture of 2,4,6-trinitrotoluene (TNT) and pentaerythritol tetranitrate (PETN) used in the civil industry as part of detonation charges, "blasting caps" and in the military as a component of ammunition (Agrawal and Hodgson, 2007). The industrial production of explosives produces toxic byproducts such as partially nitrated compounds (i.e., 2,4-dintrotoluene,DNT) and other nitrated products with potential to contaminate the environment nearby (Meyers et al., 2007). Explosives like TNT and 1,3,5-Trinitro-1,3,5-triazinane (RDX) can last up to 50 years in the environment without significant transformation, due to the highly recalcitrant nature of these xenobiotic compounds (Amaral et al., 2009; Meyers et al., 2007). The amount of unexploded ordnance in the world is unknown, but unexploded ordnance represents a persistent source of contamination. Out in the environment the exposure to the elements may compromise the outer casing or shell of the explosive and allow groundwater to seep into the explosives, which could contaminate the surrounding soil (Taylor et al., 2015).

Microorganism commonly found in soil are able to transform explosives, especially those in explosive contaminated sites (Juhasz & Naidu, 2007; Kalderis et al., 2011). These microbes use explosives as a carbon or nitrogen source, or as part of co-metabolic processes under aerobic or anaerobic conditions. There are multiple metabolic pathways used by bacteria to transform, albeit partially the TNT and PETN molecules (Stenuit & Agathos, 2019; Binks et al., 1996). In the transformation of TNT , some of these pathways produce secondary products such as hydroxyaminodinitrotoluenes (HADNT) and different azoxy compounds, that become dead end products that can accumulate in the cell (Duque et al., 1993; Stenuit & Agathos, 2019; Stenuit et al., 2005). Although it has been demonstrated that consortia of bacteria cooperate in the transformation of these secondary metabolites, which reduces their toxicity (Gunnison et al., 1993). PETN transformation is not as widely reported. Binks et al. (1996) reported the transformation of this explosive by *Enterobacter cloacae* PB2 using a PETN reductase enzyme that which intervenes in the transformation of TNT (Stenuit et al., 2005). This transformation was only partial, as it did not mineralize the explosive. Explosive transformation by consortiums of bacteria obtained from contaminated sites have been widely reported in the literature (Boopathy et al, 1998; Robertson & Jjemba, 2005; Khan et al, 2015) However, those studies report transformation of a single explosive at low concentrations. With pentolite, Arbeli et al (2016) showed that the transformation of PETN can be affected by the transformation of TNT, suggesting that the lack of an efficient degradation pathway of these explosives leads to the accumulation of toxic byproducts in the cell.

There are not many studies that test the degradation of explosives in their solid form, but under laboratory conditions, dissolved in liquid media (50- 100 mg/L) bacteria obtained from a TNT contaminated site have transformed TNT (Avellaneda, 2020; Avila-Arias et al., 2017; Erkelens et al., 2012; Nyanhongo et al., 2008), but engineering a defined consortium brings certain challenges as the competition for nutrients and antagonism between the different bacteria are factors to be considered (Jawed et al., 2019). Consortium of bacteria may be more equipped to handle the transformation of complex compounds, and there are multiple tools that enable the selection of different bacteria based on their metabolism to make a consortium with multiple applications in biotechnology and bioremediation. (Brenner et al.,2008; Shong et al., 2012) however, there is not much information regarding their use in the transformation of pentolite as well as how to create defined consortia using explosive transforming microorganisms. In this study we evaluated the ability of TNT and PETN degrading bacteria, and defined consortia made with these bacteria, to transform solid pentolite using “chunks” of pentolite. To the best of our knowledge this is the first report of transformation of a solid explosive by bacteria and mixed cultures.

Materials and methods.

TNT and PETN transforming microorganisms.

TNT and PETN transforming microorganisms were isolated from environmental samples collected from an explosive manufacturing plant near Bogota, Colombia, Bacteria were

isolated using enrichment cultures with the environmental samples under aerobic (Avila-Arias et al., 2017) and anaerobic conditions (García, 2014). Additionally, activated carbon with explosives (biobaits) were used to isolate bacteria from soil (Rojas, 2010)

Explosive transforming microorganisms were capable of growing >0.125+-0.025 $_{O.D\ 600nm}$ in <14 days and transformed over 50% of explosive in MT2 with TNT or PETN (100 mg/L) as sole nitrogen source.

For the present study, TNT, PETN, and pentolite transforming bacteria were, grouped as follows: TNT: 9 pure strains and 11 mixed cultures, PETN; 1 strain and 12 mixed cultures and 3 pentolite degrading bacteria (supplementary table 1). During isolation, we found that some of the explosive transforming bacteria were groups of two or more isolates, that when grown in groups were able to transform the explosive, but on their own they lost this ability, it was decided to use them as a mixed culture (e.g: CMP231).

Identification of the explosive transforming bacteria.

The TNT, PETN, and pentolite transforming bacteria were all sequenced following the methodology used by Avila et al. (2017). This same method was used to identify the solid explosive transforming microorganism selected in this study. Briefly, an enzymatic method was used for DNA extraction and for 16S rRNA gene amplification and bacterial identification, we used the universal primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3 ') and 1492R (5′-TACGGYTACCTTGTTACGACTT-3'). The forward and reverse sequences were sequenced at Macrogen Inc. (Seoul, Korea) using the Sanger technique. The consensus sequence was obtained using the CLC Main Workbench 8.2 (CLC bio) program (Aarhus, Denmark). A BLAST was performed on the results, using the sequence data obtained from the RDP database (Ribosomal Database Project 10; http://cme.msu.edu/). The identity percentage (> 99%) was used to perform the taxonomic affiliation at the species level.

Establishment of defined consortia

In order to find the best combination of explosive transforming bacteria, we defined consortia using the results from the screening of explosive degrading bacteria, using four parameters to group them:

1. **Key member.** Based on the results of the transformation of each explosive and the growth obtained by each bacterium and mixed culture selected, considering the amount of explosive transformed, the transformation rate, the growth, the growth rate and the production of surfactants
2. **Origin.** Grouping the bacteria and mixed cultures according to the place where the environmental samples were taken.
3. **Categories.** Bacteria and mixed cultures were grouped according to the explosive they transform (TNT or PETN), the production of surfactants, and 7 random groups (A-F) created with a random number generator (Excel) and a group with all bacteria.

4. **Biobaits and anaerobic cultures.** Bacteria obtained using activaded carbon with explosives (biobaits) and in anaerobic enrichment culture approaches.

Pentolite transformation study in microcosms

Pentolite based explosive that failed to detonate could be found underground on an aqueous environment (groundwater) at low temperatures (< 21 ° C). To simulate these conditions, microcosms with MT2 media and the solid chunks of explosive were used. Each microcosm consisted of a clear glass 20 ml flask with 5 ml of a modified MT2 media and pentolite “chunks” (~ 0.1 g). MT2 media (Avila-Arias et al., 2017) was modified by supplementing with $(NH_4)_2\ SO_4$ (1% w/v) and yeast extract (1% w/v) with the aim of increasing bacterial biomass. The bacteria or defined consortium was added at 0.6 ± 0.025 $O.D_{600nm.}$ and then sealed.

Microcosm were incubated for 2500 days (~ 7 years) at ~ 21 ° C in the dark without shaking. For each bacteria, mixed culture and defined consortia, six sampling events were used. On each sampling event, 3 microcosms of each were removed (the whole microcosm was removed from the study) to determine the bacterial density (plate count) and the transformation of TNT and PETN by high performance liquid chromatography (HPLC).

Extraction of explosives.

“Sacrifice” sampling of the microcosms was performed by adding 10 mL of acetonitrile and shaking with a vortex for 1 minute to completely dissolve the pentolite. 50 µL of the sample was taken and diluted in 2,450 µL of acetonitrile and 2,500 µL of type I water in a 10 mL amber bottle. The bottle was shaken 10 times with a sterile 5 ml syringe and 1.5 mL of the solution was filtered through a nylon filter (0.22 µm) in a chromatography vial.

Analytical methods

The explosive concentration of the microcosms was analyzed by HPLC, using a Shimadzu chromatograph (Prominence 20A) coupled with a diode array detector (PDA) The injection volume of each vial was 10 µL. The reading wavelength was 210_{nm} for PETN and 254_{nm} for TNT. A Pinnacle DB C18 column (Restek; Bellefonte, PA) with a mobile phase composed of acetonitrile and water (47:53 v:v) was used at a constant flow of 1.5 ml min^{-1}; Oven temperature was 40°C with a total run of 27 minutes.

Bacterial count

To monitor the bacterial count over time, the drop plate technique was used with nutrient agar. Each microcosm was shaken (vortex) to loosen the biomass adhered to the pentolite “chip”, later the microcosm was opened in a laminar flow cabin and 100 µL were taken in order to perform serial dilutions (10^{-1} to 10^{-3}) in sterile saline solution (0.85% w / v). Subsequently, three droplets (20 µL) of each dilution were plated in nutrient agar. In the same way, direct plating (100 µL) of the microcosm was carried out. The agar plates

were incubated at room temperature (~ 21 ° C) for 48 h and then the colonies were counted.

Monitoring the transformation of solid pentolite in the microcosm

The weight of the pentolite “chips” on each microcosm was recorded before addition in the microcosm. With this weight, the initial concentration of explosives (equation 1 and 2) was determined. Concentration at each sampling event was obtained by HPLC using the initial weight of the explosive and correcting by the dilution (concentration in mg/L in 5 mL of media) (Equation 3). Based on previous tests carried out in the laboratory, the TNT: PETN ratio in the pentolite batch used in this study was 43.75:56.25, respectively. Equation 4 was used to calculate the transformation of the explosives, where the concentration obtained by HPLC of each explosive is used to calculate a recovery percentage and with it, calculate the total transformation of the explosive.

Equation 1.

- Mass (mg) of the pentolite chip: Weight (g) x R x 1000

R= Relation: 0.4375 for TNT and 0.5625 for PETN

Equation 2.

$$Initial\ concentration\ of\ the\ explosive\ mg/L\ = \frac{Explosive\ \ massWeight\ (mg)\ of\ the\ explosive\ \times 1000}{5}$$

Equation 3.

$$\text{Recuperation}\ (\%\mathbf{R}) = \frac{\text{Fc}}{\text{Ic}} \times 100$$

Ic: Initial concentration of TNT or PETN (from eq 1)

Fc: Final concentration of TNT or PETN (from HPLC)

Equation 4:

$$\text{Transformation}\ (\%) = 100 - \%\mathbf{R}$$

To define which of the bacteria, mixed cultures and defined consortia transformed the solid pentolite, the following parameters were followed

1. Constant transformation in various sampling events: explosive transformation should increase over time.
2. Transformation percentage: Transformations above 20 % achieved on the microcosms at the end of the study was considered.

Results

Transformation of solid pentolite

In total, 64 bacteria capable to transform TNT and PENT were used in this study, grouped in 31 bacteria capable to transform TNT, 30 bacteria capable of transforming PETN and 3 bacteria capable to transform TNT and PETN. These bacteria were gram negative, ubiquitous, and non-sporulated, as expected from soil samples (supplementary table 1). Some of the TNT degrading bacteria were previously identified by Avila-Arias et al (2017) and included bacteria capable to produce surfactants, which may improve the transformation of a solid explosive by increasing its bioavailability and transformation of pentolite (TNT: PETN). Transformation of PETN throughout the study was higher (21%) but not significantly different than the transformation of TNT (18%) (Figure 1).

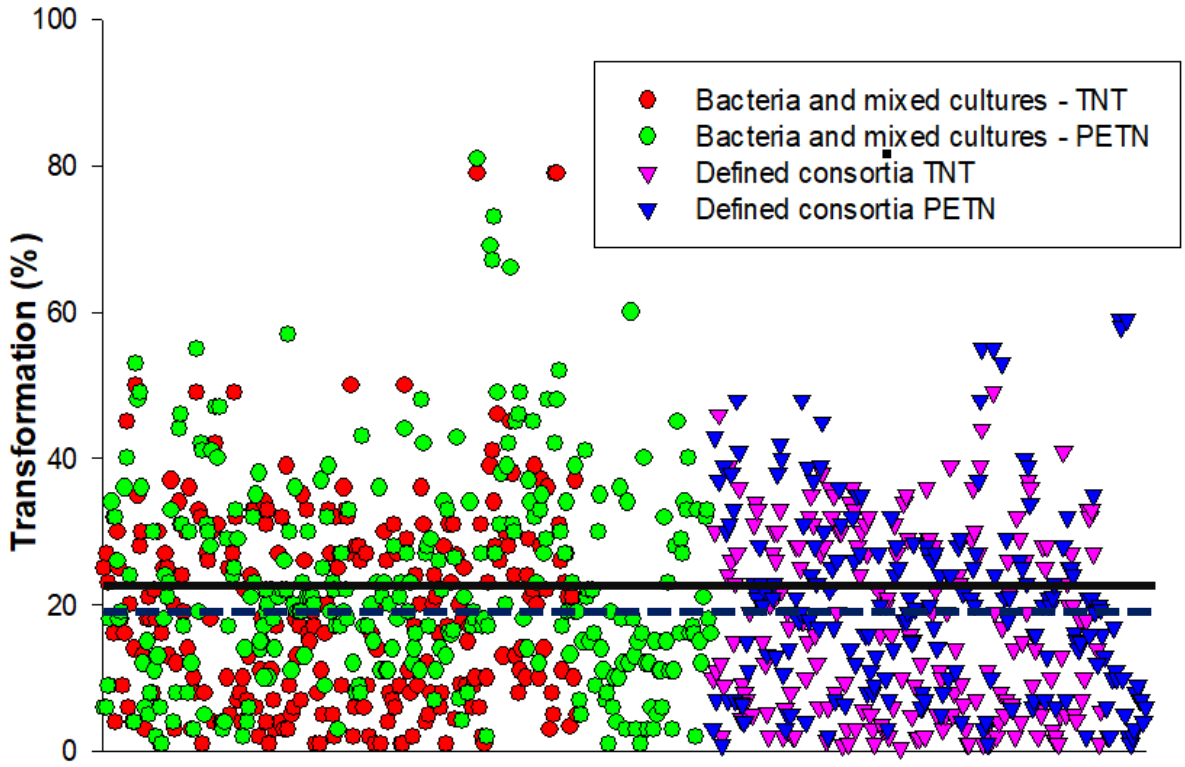


Figure 1. Transformation of TNT and PETN by bacteria, mixed cultures and defined consortia, contiuous line represents transformation of PETN, non continuous line represents transformation of TNT

Abiotic microcosms

There was transformation on the 3 microcosms without inoculum used in this study, however only microcosm without inoculum 1 (MS1) showed a cumulative transformation of TNT and PETN in the last two sampling events. (**Figure 2**) Interestingly it presented bacterial recount (5 ± 3 log CFU / mL) in the first 800 d of the study, but no recount was observed in the last sampling events. Despite the lack of an inoculum, the transformation of both explosives was above 20 % at the end of the study, albeit with a high variation on the results (TNT 21% ± 23 and PETN 29% ± 14). With the interest of identifying the

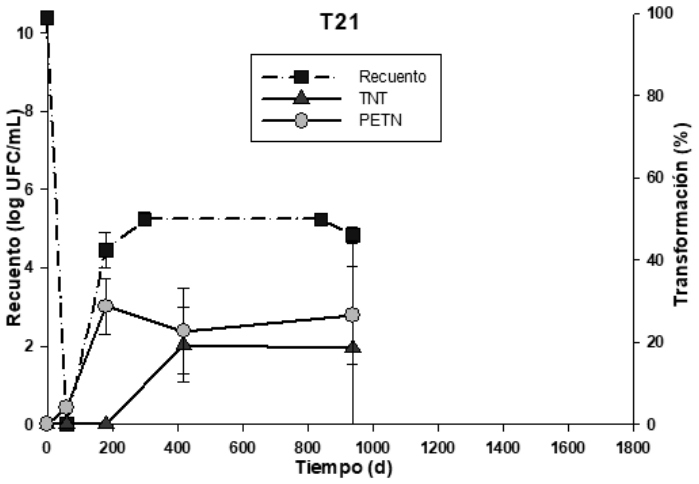
bacteria involved in the transformation of pentolite by the abiotic microcosms. We selected MS3, which showed growth throughout the study and presented a bacterial count of 3.7 (log CFU / mL) at the last sampling event. The bacteria isolated from MS3 were *Pseudomonas veronii*, *Stenotrophomonas maltophilia*, and *Citrobacter freundii.*

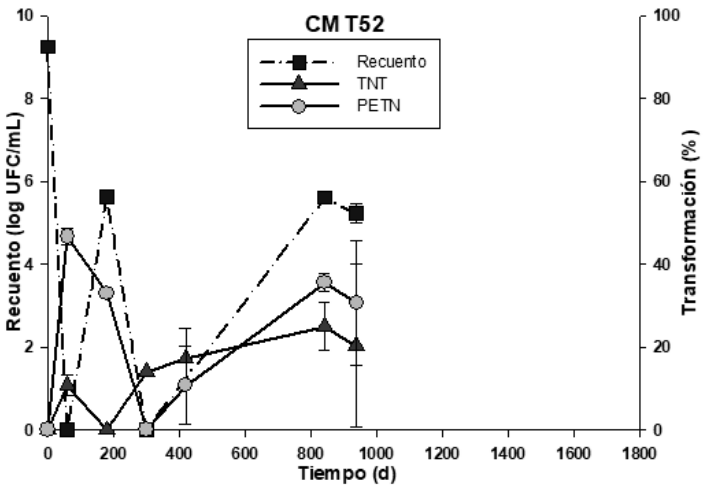
Bacteria and mixed cultures

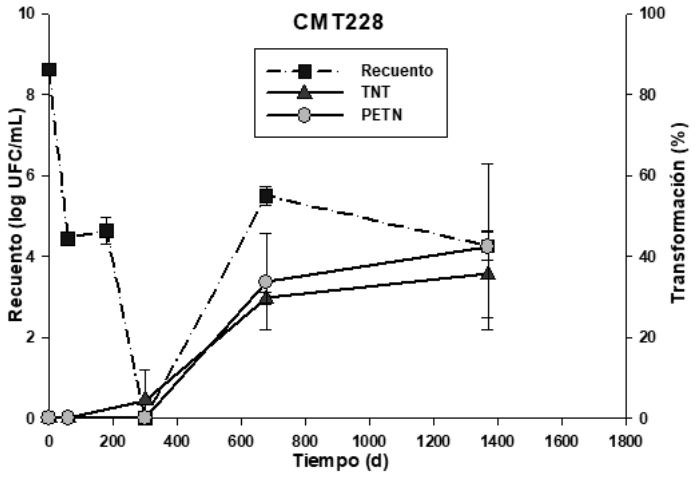
We selected 1 bacteria and 4 mixed cultures with a transformation of the explosives: T21, CMT52, CMT228, CMP231 and CMP227. In the mixed culture CMP231 (supplementary table 1) the highest transformation of TNT and PETN was observed in the last sampling event (**Figure 2**). This mixed culture is made up of *Rhodococcus ruber* and *Cupriavidus basilensis* but at the end of the study the bacteria identified from the bacterial counts were *Stenotrophomonas rhizophila* and *Citrobacter freundii.* In the mixed culture CMT228 transformations of 35% ± 11 of TNT and 42% ± 21 of PETN were observed. This mixed culture is made up of two strains: Serratia marcescens and S. maltophilia and at the end of the study a bacterial recount of 4.3 (log CFU / mL) was obtained. The two strains were identified as *S. maltophilia* and *Citrobacter freundii.* The mixed culture CMP227 comprised of 3 bacteria: *R. ruber*, *Mycobacterium fluoranthenivorans* and *Pseudoxanthomonas mexicana*. And a transformation of 36% ± 10 of the TNT and 39% ± 9 20% of the PETN was achieved, no bacterial recount was obtained in any of the sampling events (**Figure 2**)

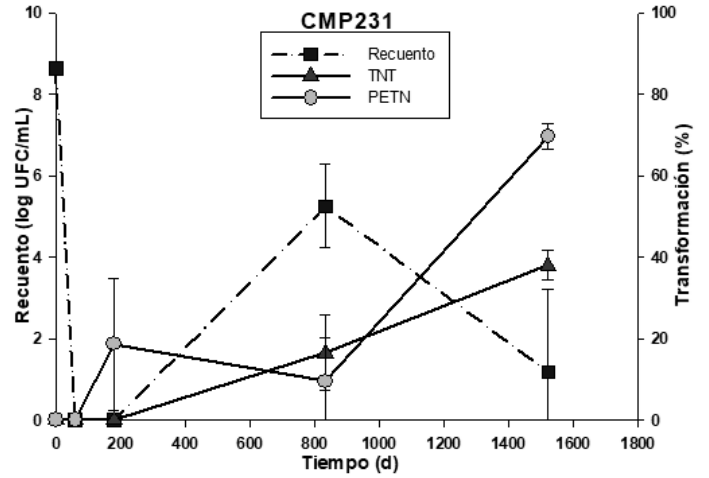
Defined consortia

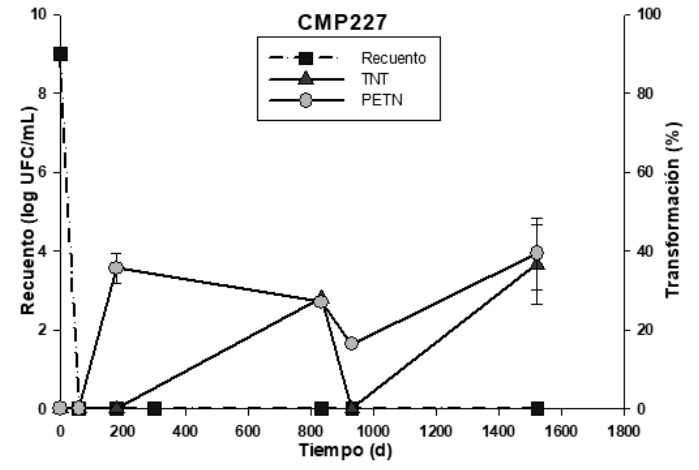
The defined consortia obtained less transformation (20% PETN, 17% TNT) compared to that of the mixed cultures by themselves. Only the defined consortia C1 and C4 showed a steady transformation of the TNT and PETN (**Figure 2**) The defined consortia C1 transformed 14% TNT and 13% of PETN and was inoculated (Table 2) with surfactant producing T17 (Achromobacter spanius), T94(Pseudomonas veronii), CMT5 (*A. spanius, S. maltophilia*) and the PETN transforming mixed culture CMP174 (*R. radiobacter, S. maltophilia*). At the end of the study only *S. rhizophila, S. maltophilia* and *S. marcescens* were identified. The defined consortia C4 transformed 18% of TNT and 17% of PETN, and was inoculated with the TNT transforming mixed cultures: **CMT41**(*S. scionense, P. nitroreducens)*, **CMT43** (*P. koreensis, P. nitroreducens*), **CMT30** (Avellaneda, 2020) (*S.yanoikuyae, R. planticola)*, CMP208 (*V. boronicumulans, P. Mexicana)*, CMP214 (*S. liquefaciens, S. maltophilia*), CMP215 (*R. jialingiae, R. radiobacter, S. maltophilia*), CMP231 (*R. ruber, C. basilensis*) the TNT transforming mixed cultures included in C4 included those described by Avila et al. (2017) as surfactant producing bacteria, in addition CMT30 was also described by Avellaneda et al. (2020) as being able to transform TNT, DNT and PETN. At the end of the study only *C. freundii* was identified from the isolates.

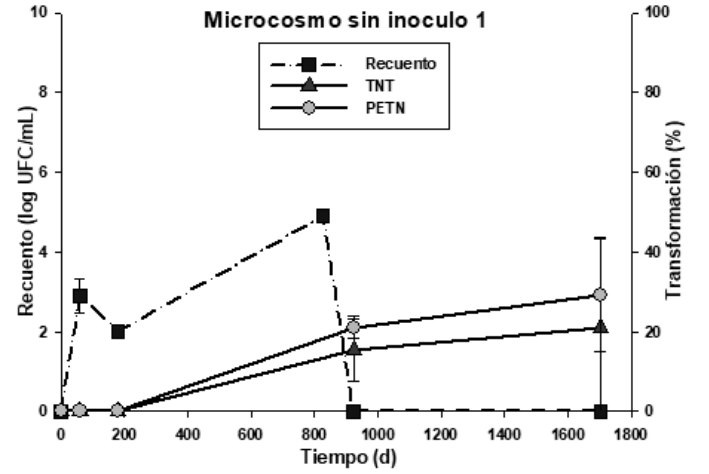

T21
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)


CM T52
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)


CMT228
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)


CMP231
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)


CMP227
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)


Microcosmo sin inoculo 1
Recuento
TNT
PETN
Recuento (log UFC/mL)
Transformación (%)
Tiempo (d)

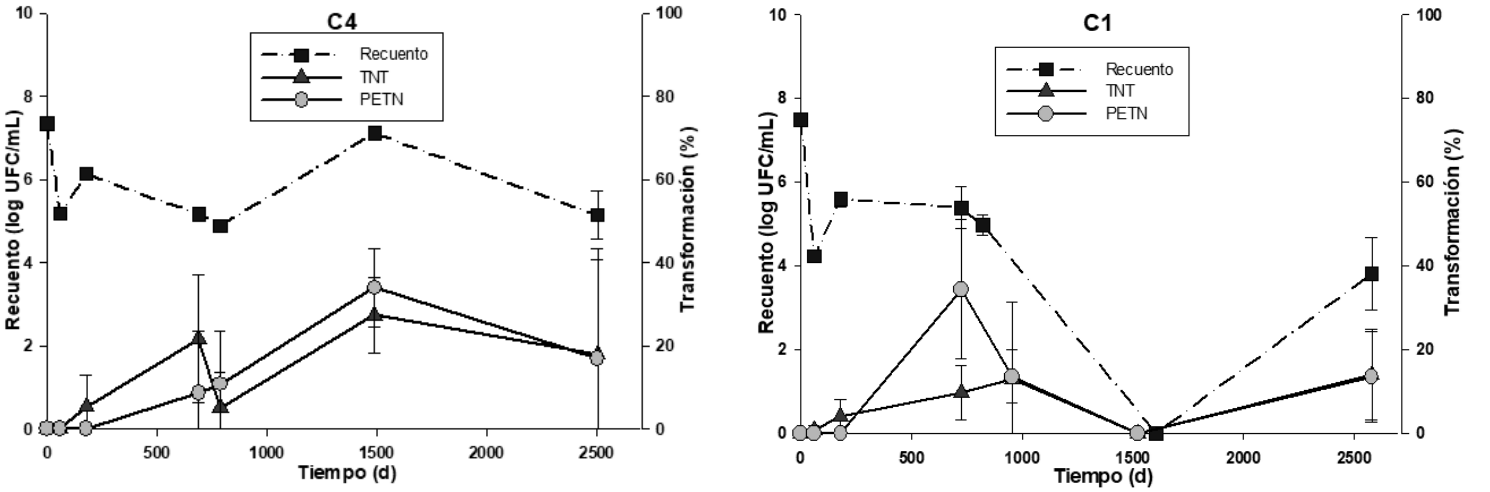


Figure 2. Transformation of TNT, PETN and bacterial recount of the bacteria, mixed cultures (CM) and defined consortia capable of transforming solid pentolite. Vertical lines are the standard deviation of 3 replicates. Squares (■) with pointed lines indicate bacterial recount in log (UFC/mL). ▲ Transformation of TNT. ● Transformation of PETN

Discussion

Evaluation of the transformation of solid pentolite

The variation observed in the transformation of TNT and PETN could be due to analytical variability, operator error, interactions with bacteria present in the pentolite “chips”, or prolonged contact with explosives. Transformation of the explosives did not result in a higher bacterial count, as there were cases (CMP227) were a constant transformation of TNT and PETN was observed with no recount throughout the study.

Under aerobic conditions, with TNT dissolved in MT2 a high transformation of TNT has been observed (>90%) (Avila-Arias et al., 2017), the same goes for solid TNT chips (70%) (Erkelens et al., 2012) however its worth mentioning that undetonated pentolite may find itself on an aqueous low oxygen environment, which may hinder its transformation rate. In addition, TNT and PETN have a low solubility in water (130 mg/L TNT, 0.1 mg/L PETN) wich may limit its bioavailability. In this study the transformation was significantly lower, reaching up to 69% of transformation of only one explosive (**Figure 2**, CMP231) High concentration of TNT and PETN (20,000 mg of pentolite in 5 ml of modified MT2), scarcity of nutrients, aerobic conditions and accumulation of toxic byproducts of the transformation of TNT (Esteve-Núñez et al., 2001; Stenuit & Agathos, 2019) may have contributed to the overall lower transformation, however, to the best of our knowledge this is the first study were significant (20 %) transformation of both solid explosives was observed.

Microcosms without inoculum

Species of the bacteria identified (16S rRNA) at the end of the study from MS3 (*P. veronii, S. maltophilia, and C. freundii*) were also reported by Ávila et al (2017) as bacteria capable of transforming TNT, with production of surfactants, which may have influenced on the transformation obtained by the abiotic control (MS1). To the best of our knowledge, this is the first time that bacteria have been isolated from solid pentolite, and the transformation obtained by these strains show that not only they are able to survive in direct contact with the explosives, but they are also able to transform both of them, which make these bacteria potential candidates for environmental remediation of explosive contaminated sites and as part of a biodegradable explosive.

Bacteria and mixed cultures

A greater transformation of TNT and PETN was observed by bacteria and mixed cultures than that obtained by the defined consortia. Both bacteria and mixed cultures with the capability to degrade TNT or PETN were originally isolated from aerobic enrichment cultures of environmental samples (Avila-Arias et al., 2017). The bacteria used in this study are capable of growing under aerobic/anaerobic conditions, and the ability of bacteria to transform explosives under aerobic and anerobic conditions have been widely reported (Binks et al., 1996; Fleischmann et al., 2004; Mercimek et al ., 2013; Stenuit et al ., 2006; Zhuang et al ., 2014). In the literature, the use of consortia or mixed cultures

of bacteria isolated from explosive contaminated soils have shown the ability to transform explosives such as TNT (Gunnison et al., 1993; Muter et al., 2012) RDX (Khan et al., 2015) 2.4 DNT (Snellinx et al., 2003) and PETN (Zhuang et al., 2012; Zhuang et al., 2014). In TNT transformation studies, consortia were found to be able to mineralize the explosive more efficiently than single bacteria, probably because they work together to transform both the explosive and the toxic by-products of the transformation (Gunnison et al., 1993). Arbeli et al. (2016) found that TNT transformation exerted a negative impact on PETN transformation. This contrasts with the results found were in CMP231 reached a higher transformation of PETN (69% ± 3) than that of TNT (38% ± 4) (**Figure 2**). At the end of the study, a bacterial count of 1.2 ± 2 log CFU / mL was observed. The low count is likely due to nutrient depletion in these microcosms, and prolonged exposure to high concentrations of both explosives, which may have left the bacteria in a viable but non-cultivable state (Bergkessel et al, 2016). It is interesting to note that the bacteria identified (16S rRNA) at the end of the study in the bacterial counts of CMP231 did not correspond with what was originally inoculated (*C. ruber, C. basiliensis*) but rather matched with *S. rhizophila* and *C freundii*, these genera were also found in the abiotic microcosm MS3, indicating that they may have been carried over from the pentolite “chips”.

Stenotrophomonas sp. are capable of transforming different xenobiotic compounds such as nitroaromatics and pesticides (Ryan et al., 2009; Verma et al., 2010). Especially *S. rhizophilia*, which shares many metabolic pathways with *S. maltophilia*, but not a pathogen, is of special interest in biotechnological applications (Alavi et al., 2014). Within this study, many bacteria and mixed cultures were identified with *Stenotrophomonas* sp. capable of transforming TNT and PETN (Avila-Arias et al., 2017). So, *S. rhizophilia* could have played a vital role in the transformation of these explosives. Regarding *C. freundii*, strains of *Citrobacter* sp. have been isolated from soils contaminated with TNT and are capable of transforming TNT (Kao et al., 2016; Kitts et al., 1994; Liang et al., 2017). An analysis of the transcriptome and proteome of Citrobacter sp. indicates that this species is capable of expressing a nemA reductase (Liao et al., 2018) , an enzyme that intervenes in the transformation of TNT. Liang et al., (2017) found that the addition of an amino nitrogen amendment (yeast extract 0.07 % and tryptone hydrolysate 0.035%) to a TNT-contaminated slurry increased TNT transformation but also increased toxicity in the medium, it is possible that in CMP231 the combination of the inoculated bacteria and those found at the end of the study had a role in transforming both explosives, as well as the toxic by-products of the TNT transformation (Gunnison et al., 1993) so the PETN transformation was not affected.

CMT228 obtained a high transformation of TNT and PETN (35% ± 11 and 42% ± 21, respectively) and *S maltophilia* and *C freundii* were found in the microcosms at the end of the study, *S. maltophilia* is part of this mixed culture and was also found in the microcosm without inoculum 3 together with *C. freundii*. The transformation of TNT and PETN by this mixed culture was greater than that found in the microcosm without inoculum 3, so the inoculated bacteria may have enhanced the transformation of explosives within these microcosms.

The bacteria that were inoculated into CMP227 (*R. ruber*, *M fluoranthenivorans* and *P Mexicana)* are capable of transforming xenobiotic compounds such as pesticides and TNT (Cycoń et al., 2017; Martínková et al., 2009; Serrano-González et al., 2018) although the transformation of the TNT was very erratic, with sampling events where there was no transformation of TNT (**Figure 2, CMP227**) it is likely that, as in other microcosms, exposure to contaminants contributed to the lack of counts. In the mixed culture CMT52 and the bacteria T21 Ávila et al. (Avila-Arias et al., 2017) found that CMT52 (P. japonica, P. monteilii, R. radiobacter) and the T21 bacteria (S. maltophilia) were capable of transforming > 90% of TNT and had a high degradation rate (> 7 mg / L-1.d-1). These factors played a role in the transformation obtained. It is striking that T21 comes from a sample of soil not contaminated with explosives and yet was able to transform both explosives. (Avila-Arias et al., 2017).

Defined consortia

In the literature, the use of defined consortia for the transformation of sites contaminated with toxic compounds such as hydrocarbons has shown promising results (Poddar et al., 2019; Tahhan., 2011). However, despite comprising different combinations of bacteria and mixed cultures capable of transforming both explosives, in this study the transformation obtained with these bacteria was lower (Figure 1) than those obtained by the mixed cultures. It is possible that in the defined consortia, factors such as the compatibility of the bacteria used in the consortia, competition for substrate, metabolite exchange (Jawed., 2019) and the recycling of nutrients between the bacteria in each microcosm (Sangeetha., 2017) Influenced the survival and explosive transformation capability. In nature, consortia are more resilient to changes in the environment, but the long-term behavior of the bacteria that make up the defined consortia made in the laboratory are very difficult to predict, and it is one of the great challenges facing the design of defined consortia (Brenner et al., 2008; Shong et al., 2012).

An analysis of the metabolic networks that can occur between bacteria within the microcosm can be a useful tool for predicting the behavior of bacteria in defined consortia (Shong et al., 2012) and other independent culture tools such as comparative genomics can be used to study possible nutritional interactions between bacteria in a consortium (Gude y Taga, 2020).

Lastly, C4 showed no significant decrease in counts at the start of the transformation assay and it managed to keep a bacterial recount of ~ 5-6 log CFU / ml (**Figure 2**) There are no reports in the literature where the bacteria used in C4 are used for the transformation of explosives, however, the bacteria of this defined consortium showed promising results in the TNT transformation tests (Avila-Arias et al., 2017) and in this study of transformation of solid pentolite. CMT41, CMT43 and CMT30 are transforming mixed cultures of TNT, producing surfactants (Avila-Arias et al., 2017) . CMT30 was also evaluated by Avellaneda (Avellaneda, 2020) and was able to transform TNT and PETN. the remaining mixed cultures: CMP208, CMP214, CMP215 are capable of transforming PETN and CMP231 was the one that obtained the highest transformation of TNT and

PETN at the end of this study. However, the transformation obtained at the end of the study by this consortium was not significantly different from any of the other defined consortia. At the end of the study and despite a high count (5 log CFU / ml) only *C. freundii* was found in these microcosms, which probably comes from the pentolite chips, and was also found in the mixed cultures CMP231 and CMT228 that obtained a high transformation of the explosive.

Concluding remarks

During this study in spite of the difficult conditions encountered by the bacteria, we were able to determine the biotransformation (~ 60 %) of solid pentolite (TNT: PETN). The mixed cultures (CMT228, CMP231, CMP227) presented a greater transformation, but not significantly different than the pure bacteria and the defined consortia. This suggest that these mixed cultures could be a better option in the degradation of solid explosives and should be studied in more detail.

throughout the study, transformation of PETN (21%) was observed in the presence of TNT (18%) which indicates the bacteria used in this study are capable of transforming both explosives.

The bacteria found in the abiotic microcosms (*C. freundii, S. maltophilia, S. rhizophila, S. marcescens* and *P. veronii*) may have been bacteria that colonized the pentolite “chips” and have a potential to transform pentolite.

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Supplementary material

Table 1. Identification of the TNT and PETN transforming bacteria

Explosive used as nitrogen source	Isolation method	Code [a]	Id.
TNT		CMT5	*Achromobacter spanius* *Achromobacter spanius* *Stenotrophomonas maltophilia*
		CMT7	*Pseudomonas veronii* *Pseudomonas koreensis* *Stenotrophomonas maltophilia*
		CMT41	*Sphingobium scionense* *Pseudomonas nitroreducens*
		CMT43	*Pseudomonas koreensis* *Pseudomonas nitroreducens*
		CMT52	*Pseudomonas japonica* *Pseudomonas monteilii* *Rhizobium radiobacter*
		CMT62	*Pseudomonas migulae* *Raoutella planticola* *Pseudomonas nitroreducens* *Stenotrophomonas maltophilia* *Rhizobium radiobacter*
		CMT71	*Pseudomonas poae* *Rhizobium radiobacter* *Achromobacter spanius*
		CMT109	*Achromobacter spanius* *Pseudomonas knackmussii* *Rhizobium radiobacter* *Stenotrophomonas maltophilia*
		CMT160	*Pseudomonas monteilii* *Methylopila capsulate*
		CMT228	*Serratia marcescens* *Stenotrophomonas maltophilia*
		CMT30	*Sphingobium yanoikuyae* *Raoultella planticola*
		T12	*Sphingobium yanoikuyae*
		T17	*Achromobacter spanius*
		T21	*Stenotrophomonas maltophilia*
		T94	*Pseudomonas veronii*
		T217	*Pseudomonas sp.*
		BT301	*Bosea vestrisii*
		BT302	*Agrobacterium sp.*
		BT303	*Stenotrophomonas maltophilia*
		BT304	*Xanthomonas citri*

PETN	CMP2	*Bradyrhizobium pachyrhizi* *Stenotrophomonas maltophilia*
	CMP6	*Arthrobacter defluvii* *Stenotrophomonas chelatiphaga*
	CMP10	*Bosea minatitlanensis* *Brevundimonas bullata*
	CMP174	*Rhizobium radiobacter* *Stenotrophomonas maltophilia* *Achromobacter spanius*
	CMP208	*Variovorax boronicumulans* *Pseudoxanthomonas mexicana*
	CMP214	*Serratia liquefaciens* *Stenotrophomonas maltophilia*
	CMP215	*Rhodococcus jialingiae* *Rhizobium radiobacter* *Stenotrophomonas maltophilia*
	CMP227	*Rhodococcus ruber* *Mycobacterium fluoranthenivorans* *Pseudoxanthomonas mexicana*
	CMP229	*Methylobacterium goesingense* *Leifsonia naganoensis* *Serratia nematodiphila* *Rhizobium radiobacter* *Stenotrophomonas maltophilia*
	CMP231	*Rhodococcus ruber* *Cupriavidus basilensis*
	CMP233	*Gordonia sputi* *Stenotrophomonas maltophilia*
	CMP234	*Stenotrophomonas chelatiphaga* *Rhizobium radiobacter*
	BP301	*Bosea vestrisii*
Pentolite	PT1	*Pseudomonas sp.*
	PT2	*Pseudomonas sp.*
	PT3	*Pseudomonas sp.*

[a] CM: Mixed cultures, B: biobaits, PT: anaerobic enrichment cultures.

Table s2. Bacterial strains used on each defined consortium

Classification	Code	Strains
Sampling origin	C3	CMT5, CMT7, T12, T17, T21, CMP6, CMP10, CMP2
	C4	CMT41, CMT43, T30, CMP208, CMP214, CMP215, CMP231
	C5	CMT71, CMP227, CMP229, CMP233
Categories	C6	CP227, CMT43, CMP234, T17, CMT160, CMP6, CMP215
	C7	CMP214, P215, CMT7, BP301, PT1, CMP10, CMT109
	C10	CMP208, CMP214, CMP215, CMP234, CMP227, CMP231, CMP233, CP229, CMP2, CMP6, CMP10, CMP174, BP301
	C9	CMT52, T21, T17, CMT109, CMT7, CMT160, CMT62, CMT5, T12, CMT41, CMT71, CMT43, CMT30, T94, BT301, BT302,BT303, BT304, T217, CMT228
	C11	CMT5, CMT41, CMP10, PT1, T21
	C12	CMT7, CMT62, CMP208, PT2, CMT52
	C8	PT1, CMT109, CMP229, T17, CMT5
	C19	P208, P214, P215, CP234, CP227, CP231, CP233, CP229, P2, CP6, CP10, P174, P301, CT52, T21, T17, CT109, CT7, CT160, CT62, CT5, T12, CT41, CT71, CT43A, CMT30, T94, T301, T302, T303, T304, T217, T228c, PT1, PT2, PT3
Key member	C13	CMT52, CT62, CT71, T94, T17, CP233, P208, CT160, CT7, CP234, P6, CP10, P215, P229, P301, CP234
	C14	P174, CT62, CT71, T12, CP229, P214, P215, P208, CT41, CT43a, CT52, BP301, CP234, T94
	C15	TC160, P214, CP234, CP229
	C1	P174, T17, T94, CT5
	C2	P174, T17, T94, CT5, P2
	C18	CT52, CT41, T94
Isolating method	C16	PT1, PT2, PT3
	C17	P301, T301, T302, T303, T304

618 **Table 3.** Identification of the isolated strains

Nomenclatura	Inoculated	Last sampling event	%ID
CMP231	*R. ruber/C. basilensis*	*Stenotrophomonas rhizophila*	99,4
		Citrobacter freundii	99,8
CMT228	*S. marcescens, S. maltophilia*	*Stenotrophomonas maltophilia*	96,4
		Citrobacter freundii	99,2
Microcosms without inoculum	N/A	*Stenotrophomonas maltophilia*	96,3
		Pseudomonas veronii	99,4
		Stenotrophomonas maltophilia	99,6
		Stenotrophomonas maltophilia	99,7
		Citrobacter freundii	99,2
C1T2 / C1	*R. radiobacter,S. maltophilia, A. spanius, P. veronii*	*Citrobacter freundii*	99,4
		Citrobacter freundii	99,4
		Stenotrophomonas rhizophila	96,5
		Stenotrophomonas maltophilia	99,6
		Serratia marcescens	99,1
C4	*P. koreensis,P. nitroreducens,S. yanoikuyae,S. scionense ,V. boronicumulans, P. mexicana ,S. maltophilia,S. liquefaciens, R. jialingiae,R. radiobacter,R. ruber, C. basilensis*	*Citrobacter freundii*	99,2
		Citrobacter freundii	99,4

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