**ANA ROBERTA LIMA DE MIRANDA**

# COMUNIDADE BACTERIANA DO SOLO APÓS SETE ANOS DE APLICAÇÃO DE LODO DE CURTUME COMPOSTADO

**TERESINA-PI**
**2018**

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Tese apresentada à Universidade Federal do Piauí, como parte das exigências do Programa de Pós-Graduação em Agronomia- Agricultura Tropical, para obtenção do título de Doutora em Ciências com área de concentração em Agricultura Tropical.

Orientador
Dr. Ademir Sérgio Ferreira de Araújo

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**TERESINA-PI**

**2018**

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*“Não basta fazer coisas boas - é preciso fazê-las bem”*

*Santo Agostinho*

## SUMÁRIO

## RESUMO GERAL

Oriundo de indústrias de processamento de pele animal em couro, o lodo de curtume é rico em compostos orgânicos e inorgânicos capazes de promoverem fertilização, nutrição de vegetais e de microrganismos presentes no solo. Contudo, o uso excessivo deste resíduo, pode gerar distúrbios ecológicos e interferências na microbiota, principalmente devido à concentração de cromo (Cr) proveniente dos processos de curtimento. Por ser um elemento químico pertencente ao grupo dos metais pesados capaz de ser absorvido por organismos vivos, alcançando diversos níveis da cadeia trófica, tornam-se preocupante elevadas concentrações deste metal no solo. O efeito do uso de lodo de curtume compostado (LCC) após sete aplicações anuais sucessivas, foi avaliado por meio de relações entre as propriedades químicas e valores de Unidade Taxonômica Operacional (OTUs) da comunidade bacteriana do solo, tendo por base valores de abundância relativa à nível de filo, classe, ordem, família e gênero, com intuito de observar os efeitos causados na estrutura da microbiota do solo após aplicação de diferentes quantidades de LCC. O experimento foi conduzido em blocos casualizados, com quatro repetições e cinco tratamentos correspondentes a doses de LCC, sendo 0 (controle), 2,5, 5, 10 e 20 t/ha (base seca). Aos 0, 45, 75, 150 e 180 dias de experimento, avaliou-se os níveis de P, K, Ca, Mg, Na, pH, condutividade elétrica (CE) e o teor de carbono orgânico total (COT) no solo, bem como, a composição bacteriana presente no solo, pela técnica de sequenciamento de nova geração, correlacionando-a aos atributos químicos do solo após uso do resíduo. Foram cultivadas em períodos distintos plantas de milho e feijão-caupi durante o experimento para observações paralelas sobre o comportamento vegetal às condições de estresse e fertilização. Os valores de pH, C orgânico, P, Ca, Mg e Cr aumentaram com o aumento das doses de LCC. Grupos específicos de bactérias como Actinobacteria, Acidobacteria, Firmicutes, Proteobacteria e Chloroflexi, tornaram-se abundantes após a aplicação do resíduo ao longo do tempo, sugerindo interpretações sobre a capacidade adaptativa ao incremento de atributos químicos e resistência ao metal pesado. Análises de Redundância (RDA) e Análises de Resposta de Curva Principal (PRC), mostraram estreitas relações dos níveis de pH, cromo, condutividade elétrica e C orgânico entre a comunidade bacteriana e as influências na dinâmica estrutural da comunidade. Os resultados mostraram efeitos rizosféricos nas culturas de milho e feijão-caupi cultivados no decorrer do experimento, sobre a comunidade bacteriana, ocasionando diferenciação de grupos de bactérias nos diferentes períodos de florescimento e senescências das plantas. Houve efeito significativo do tempo de amostragem e das doses de LCC nas propriedades químicas do solo que por sua vez, interferem na estrutura da microbiota bacteriana, selecionando organismos com capacidade adaptativa e de resistência ao cromo.

**Palavras-chave**: Diversidade microbiana, atributos químicos do solo, metagenômica, cromo.

## ABSTRACT

The tannery sludge is derived from industries that transform the animal's skin to leather and has rich organic and inorganic composition capable of promoting fertilization, nutrition of plants and microorganisms present in the soil. However, the excessive use of this residue causes ecological disturbances and interferences in the microbiota, mainly due to the concentration of chromium (Cr) from the tanning processes. Because it is a chemical element belonging to the group of heavy metals capable of being absorbed by living organisms, reaching several levels of the trophic chain, it becomes worrisome the high concentration of this metal in the soil. The effect of the use composited tannery sludge (CTS) after seven successive applications was evaluated through the relationship between chemical properties and operational taxonomic unit (OTUs) values of the soil bacterial community, based on values of abundance relative to the phylum level, class, order, family and genre, in order to observe the effects caused in the soil microbiota structure after the application of different amounts of CTS. The experiment was conducted in randomized blocks, with four replicates and five treatments corresponding to CTS doses, being 0 (control), 2.5, 5, 10 and 20 t / ha (dry basis). The values of P, K, Ca, Mg, Na, pH, electrical conductivity (EC) and the total organic carbon (TOC) in the soil were evaluated at 0, 45, 75, 150 and 180 days of experiment, as well as, by the new generation sequencing technique, the bacterial composition present in the soil, correlating it to the soil chemical attributes after the use of the residue. Maize and cowpea plants were cultivated at different periods during the experiment for parallel observations on the vegetal behavior to stress conditions and fertilization. The values of pH, organic C, P, Ca, Mg and Cr increased with increasing doses of CTS. Specific groups of bacteria such as Actinobacteria, Acidobacteria, Firmicutes, Proteobacteria and Chloroflexi, were abundant after the application of the residue over time, suggesting interpretations on the adaptive capacity to increase chemical attributes and resistance to heavy metal. Redundancy Analyzes (RDA) and Principal Curve Response Analyzes (PRC) showed close relationships of pH, chromium, electrical conductivity and organic carbon levels between the bacterial community and the influences on its community structural dynamics. The results also showed rhizospheric effects of maize and cowpea plants grown during the experiment, under the bacterial community, causing differentiation of groups of bacteria in the different periods of flowering and senescence of the plants. There was a significant effect of sampling time and CTS doses on soil properties that in turn interfere with the bacterial microbiota structure, selecting organisms with adaptive capacity and resistance to chromium.

**Key words**: Microbial diversity, soil chemical attributes, metagenomic, chromium.

## 1 INTRODUÇÃO GERAL

O volume indiscriminado de resíduos gerados pelas atividades antrópicas mal destinados no meio ambiente, geram perturbações e desequilíbrios nos mais diversos ecossistemas. (Sánchez, 2008), e encontrar medidas eficazes de destinação de resíduos, que minimizem ou eliminem os impactos ao meio ambiente, tornou-se uma grande preocupação para as indústrias atualmente (Alves e Barbosa, 2013)

A atividade curtumeira apresenta-se como grande geradora de resíduos impactantes à natureza. Apesar de promotora de empregos e de contribuições significativas à economia, tem-se registrado, grandes contaminações decorrentes do descarte irregular dos resíduos gerados por estas industrias durante o processo de transformação da pele animal em material mais durável, o couro (Pacheco, 2005).

Lodo de curtume é o nome dado aos resíduos sólidos ou semi-sólidos produzidos nas indústrias curtumeiras com presença de compostos orgânicos e inorgânicos em quantidades elevadas. Seu potencial tóxico está atrelado ao uso de compostos químicos como por exemplo, ácidos, sulfatos e hidróxidos para cumprimento das etapas dos processos de curtimento do couro, porém, um dos principais componentes desses resíduos que contribuem para o desequilíbrio ambiental, são os metais tóxicos, mais especificamente, o cromo, capaz de ser bioacumulado, causando sérios danos aos organismos, como mostram estudos sobre acúmulo de Cr nos tecidos vegetais e solo após adição sucessiva de LCC (Santos et al., 2014; Oliveira; Araújo; Melo, 2015; Sousa et al., 2017).

O descarte e utilização deste resíduo pode ser feito em ambientes agrícolas como fonte de material orgânico e inorgânico, capaz de fertilizar os solos e por consequência, nutrir os vegetais. Outra característica relevante dos lodos de curtume que o faz ser indicado para uso agrícola, é a capacidade de neutralização da acidez do solo, proporcionado pelos teores de carbonato de cálcio provenientes do processo de industrialização. Além disto, sua fração orgânica pode conferir melhorias diretas na estrutura dos agregados do solo, tornando-os menos sensíveis à erosão e ainda, sob o ponto de vista microbiológico, reequilibrar as populações e atividades microbianas no solo.

Uma forma interessante e consideravelmente mais segura vem sendo estudada para utilização do lodo de curtume na agricultura por meio da compostagem (Miranda

et al., 2014; Guimaraes et al., 2015; Oliveira; Araújo; Melo, 2015; Araújo et al., 2017; Sousa et al., 2017). A compostagem é um processo de decomposição aeróbia, durante o qual há desprendimento de vapor de água, gás carbônico e energia, devido à ação de microrganismos aeróbicos. Por ser um processo estritamente biológico, os microrganismos convertem resíduos orgânicos em material estável, conhecido como húmus. A compostagem dessa forma, se mostra um processo eficiente de reciclagem de resíduos industriais, devido a sua capacidade de estabilização de compostos químicos, além de uma forma garantida de eliminação de patógenos.

Apesar da existência de controvérsias sobre a aplicação dos lodos ao solo e principalmente a sua utilização, o solo constitui o mais seguro local de descarte de poluentes, quando comparados a atmosfera, pois possuem capacidades de melhor oxidação e quelação, sendo o estado oxidável a forma química menos tóxica, podendo ser removido mais fácil e seguramente da cadeia alimentar. Entretanto, se fazem necessários estudos contínuos sobre esta prática e as suas reais consequências ao meio-ambiente, descrevendo os efeitos do uso de LCC com o tempo e as quantidades utilizadas, devido aumento dos teores de cromo, alterações dos atributos químicos do solo e as possíveis interferências na microbiota.

Neste sentido, questões cientificas podem ser construídas:

1) A estrutura e diversidade dos microrganismos são influenciadas pela aplicação de LCC ao longo do tempo?
2) As mudanças nas propriedades químicas do solo influenciam o comportamento da comunidade bacteriana do solo?
3) Há parâmetros químicos específicos que interfiram na estrutura da comunidade bacteriana do solo em resposta à aplicação de LCC?

Com base no exposto, este estudo foi dividido em dois capítulos com o objetivo de responder as questões:

- Capítulo I **-** Responses of soil bacterial community after seventh yearly applications of composted tannery sludge**;**
- Capítulo II - Less abundant bacterial groups are more affected than those most abundant in composted tannery sludge treated soil

# 2 REFERENCIAL TEÓRICO

## 2.1 Lodo de curtume compostado e seu uso na agricultura

Indústrias curtumeiras fazem parte do setor industrial voltado ao processamento de couro bovino, grande gerador de resíduos sólidos, conhecidos por lodo de curtume (Malafaia et al., 2016). O lodo de curtume apresenta características desejáveis para ser utilizado na agricultura como insumo agrícola por apresentar elevado potencial de neutralização da acidez do solo, altas composições orgânicas e inorgânicas (Martines, 2009) que contribuem para melhor desenvolvimento das plantas (Araújo et al, 2007; Souza et al, 2017) e de alguns microrganismos presentes no solo (Miranda et al, 2018)

Em função dos elevados teores de elementos traços nos lodos de curtume (Patel; Patra, 2015), as formas de disposição são motivos de preocupação constante. Dentre as principais alternativas de tratamento e destino de lodos de curtume no Brasil, incluem sua disposição em aterros sanitários e várias formas de disposição no solo, tais como a recuperação de áreas degradadas e uso como fertilizantes em culturas como milho, feijão e pastagens (Silva, 2012).

A reutilização de resíduos como fertilizantes do solo oferece uma série de vantagens sobre alternativas de gestão ambiental, porque reduz o uso de outros fertilizantes e elimina a necessidade de tratamento subsequente. A compostagem consiste em uma técnica de processamento de resíduos orgânico com objetivo de transforma-los em húmus a serem utilizados na agricultura de forma viável (Silva et al., 2010; Santos et al., 2011; Gonçalves et al., 2014) como adubos.

Berilli et al. (2014), afirmam que, embora exista o risco de contaminação do solo com o uso de lodo de curtume, quando bem manejados, definidos e respeitados os critérios técnicos agronômicos sua utilização na agricultura torna-se uma alternativa de descarte, diante da composição variável do resíduo.

Este tipo de resíduo atua de duas maneiras após sua aplicação e incorporação no solo. Primeiramente como condicionante, melhorando as propriedades físicas, atuando sobre a agregação de suas partículas e consequentemente sobre a aeração.

A segunda maneira de contribuição da utilização destes compostos está relacionada com a presença da matéria orgânica (Nakatani et al., 2011; 2012) que em conjunto com partículas de argila no solo, formam complexos de adsorção, melhorando suas propriedades químicas por reter nutrientes que poderiam ser

perdidos pelo processo de lixiviação. Uma vantagem adicional é que esses nutrientes estão ligados à matéria orgânica e são disponibilizados lentamente para as plantas, à medida em que a matéria orgânica vai se decompondo, em um processo conhecido como mineralização.

Mesmo com todas estas vantagens de utilização de LCC em áreas agrícolas, faz-se necessário o conhecimento sobre sua composição, doses e tempos de utilização de modo a se respeitar os limites pré-estabelecidos pela legislação, pois a aplicação sucessiva aos solos causa aumento na concentração de metais nos ecossistemas (Joutey et al., 2015) gerando possíveis efeitos tóxicos na cadeia trófica (Sunitha et al., 2014) e contaminação ambiental.

## 2.2 Efeito do lodo de curtume nos atributos químicos e na estrutura da comunidade bacteriana do solo

Elevadas concentrações de cromo são encontradas em resíduos oriundos de indústrias curtumeiras, devido a forma como é processada a matéria prima para produção do couro.

Pesquisadores afirmam ser possível e principalmente necessário, promover a quebra das moléculas de Cr (VI) à Cr (III), anteriormente à sua utilização no solo, devido aos efeitos tóxicos do Cr (VI) (Ahemad, 2015) e a imobilidade do Cr (III), sendo considerado inclusive como um método de remediação de solos contaminados (Liao et al., 2015). Além disso, sua essencialidade como micronutriente o faz requerido para o crescimento de seres vivos como plantas (Santos et al., 2014) e microrganismos (Abdu et al., 2016).

Aumentos das concentrações de sódio (Na), cálcio (Ca), magnésio (Mg), potássio (K) no lodo de curtume, são observados após aplicações do resíduo ao solo (Rath et al., 2016; Sousa et al., 2017). A alta concentração de Na pode ser tóxica às plantas, bem como causar salinidade e sodicidade em solos adjacentes com sucessivas aplicações (Bareen; Tahira, 2011), assim como alterações de condutividade elétrica. De acordo com Possato et al., (2014), a elevação nos valores de CE correlaciona-se com os teores de sais dissolvidos em solução, principalmente os cátions $Ca^{2+}$, $Na^{+}$ e $Mg^{2+}$. Devido a isso, restrições de uso ao longo prazo, precisam ser consideradas, principalmente devido à presença de elementos traço, como o Cr (Santos et al., 2014; Oliveira; Araújo; Melo, 2015).

Técnicas de remediação em solos contaminados, incluem não somente

métodos químicos de redução, mas também, métodos biológicos (Liao et al., 2015) e, em alguns casos, considerados como mais eficientes (Wang et al, 2015). Métodos que envolvem a remediação microbiana são baseados no uso de microrganismos capazes de degradar moléculas químicas de difícil decomposição devido suas estruturas de ligações químicas, tais como metais pesados, resíduos de petróleo, hidrocarbonetos ((benzeno, tolueno, etilbenzeno e os três isômeros de posição do xileno) e pesticidas (Pereira e Freitas, 2012). Diversos grupos bacterianos têm sido encontrados no ambiente com efetivo potencial de redução do Cr (VI) (Zhu et al., 2013) e com adaptações ambientais à presença de metais pesados, como por exemplo, Acidobacteria, Actinobacteria, Firmicutes e Proteobacteria (Yin et al., 2015).

De maneira geral, os microrganismos são conhecidos como biosensores de distúrbios nos ecossistemas de solo. Isto se deve ao fato de serem mais sensíveis ao estresse ambiental (Zhang et al., 2016) quando comparados a outros organismos presentes sujeitos às mesmas condições.

Impactos causados pela presença de metais pesados têm sido demonstrados por pesquisadores ao longo do tempo, como efeitos adversos no microbioma do solo (Wang et al., 2015; Zhang et al., 2016), na biomassa microbiana (Breton et al., 2013; Silva et al., 2014; Deng et al., 2015; Araújo et al., 2016) e na estrutura da comunidade (Xu et al., 2009; Zhu et al., 2013; Ahemad et al., 2015).

Actinobacteria, Protebacteria e Firmicutes são considerados grupos importantes de microrganismos nativos capazes de estabelecer-se em ambientes com altas concentrações de material orgânico, inorgânico e metais pesados no solo, possuindo habilidade metabólica e versatilidade em ações de remediação ambiental (Polti et al., 2014), participando de importantes processos ecológicos e ciclagem de nutrientes, utilizando ácidos húmicos e matéria orgânica para seu crescimento. Além disso, apresentam capacidade de quebra de moléculas de difícil degradação (Kieser et al., 2000), habilidade de remoção de óleos, pesticidas, plásticos, e metais pesados, como por exemplo, o cromo (Polti et al., 2009, 2010, 2014). Apesar dos efeitos deletérios do cromo em bactérias (Viti and Guiovannete, 2001; Cervantes et al., 2001; Desai et al., 2009), mostram-se adaptáveis ao longo do tempo.

É importante ressaltar que a presença de composições orgânicas e inorgânicas nos resíduos, promove crescimento de microrganismos, devido a disponibilidade de nutrientes, refletindo diretamente na abundância e diversidade da comunidade. Fatores como mineralização do carbono (Fierer et al., 2007) e competição entre grupos de bactérias (Fierer et al., 2012), também podem influenciar a abundância de uma

comunidade.

Organismos pertencentes a Acidobacteria, Proteobacteria e Verrucomicrobia, são sensíveis à presença de matéria orgânica no ambiente, tendo sua população variável de acordo com a disponibilidade de nutrientes, como nitrogênio, carbono orgânico (Ward et al., 2009; Goldfarb et al., 2011), fósforo (Pedrinho et al., 2009) e ainda valores de pH do solo (Fierer et al., 2007; Lauber et al., 2009; Navarrete et al., 2013). Do mesmo modo, os Bacteroidetes que, apesar de menor abundância (Desai et al., 2009), são muito comuns nos solos e podem ser encontrados em frações orgânicas de resíduos sólidos (Calleia-Cervantes et al., 2015).

Efeitos adversos na qualidade do solo por estresse metálico além de refletirem na biomassa microbiana, inibem o desempenho de funções específicas dos grupos de microrganismos, ou de espécies chaves responsáveis por processos de mineralização, desnitrificação e fixação de nitrogênio, fundamentais para o equilíbrio ambiental (Desai et al., 2009). A diversidade de grupos funcionais e o número de indivíduos por grupos compõem a estrutura da comunidade e estão relacionadas aos fatores ambientais. O desempenho de funções ecológicas similares ou não, depende não somente de adaptações dos organismos, mas também das capacidades naturais de competição. Estresses severos podem afetar espécies raras e sensíveis (Gans et al., 2007) decrescendo suas habilidades de competição, resultando em aumento populacional de espécies resistentes e adaptadas, preenchendo nichos de estabilidade ecológica (Giller et al., 1998; Desai et al., 2009).

De forma geral, as características químicas do solo estão diretamente ligadas e interferem no comportamento dos microrganismos, tanto no que diz respeito a composição química, como ao tempo de exposição às condições geradas após uso do resíduo no solo (Pan et al., 2014; Abdu et al., 2016).

Estudos sobre biomas bacterianos tem mostrado que a estrutura das comunidades é dirigida pelo tipo de solo e por características químicas gerais, incluindo pH (Pan et al., 2014), como fator de influência na composição e na distribuição de filos em escalas locais (Berg e Smalla, 2009; Jones et al., 2009), bem como na disponibilidade de Ca, Mg e Mn (Navarrete et al., 2013) confirmando os efeitos diretos e indiretos da deposição do lodo de curtume compostado, com as modificações nas características do solo e as alterações de estrutura da comunidade bacteriana, respectivamente.

## 2.3 Ecologia microbiana e os Métodos Moleculares no estudo de microbiomas

A ecologia microbiana, estuda a diversidade dos microrganismos presentes nos ecossistemas e, principalmente, como esses eles interagem entre si e com o ambiente, além, disso, busca compreender como essa interação pode gerar e manter essa diversidade.

Os microrganismos são extremamente importantes para todos os ecossistemas do planeta, desempenhando papéis centrais nos diversos processos geoquímicos, no equilíbrio biológico, realizando funções essenciais para a manutenção do ecossistema, possuindo ainda, um papel essencial na fertilidade do solo e no fluxo de gases do efeito estufa, além de serem capazes de degradar uma variedade de compostos, incluindo poluentes de origem antrópica (Engelhardt, 2001; Joshi, 2014).

Dentre as composições ambientais existentes, o solo representa o ecossistema de maior composição e complexidade de diversidade microbiana, estimando-se que um grama de solo possa conter da ordem de 1 trilhão de células microbianas, podendo compreender mais de 10 mil diferentes genomas (Torsvik, 2002), sendo considerado uma grande fonte de produtos para aplicações biotecnológicas.

A variedade de funções ocorre devido à grande diversidade metabólica existente entre os grupos microbianos presentes no solo, que podem utilizar diferentes fontes de carbono e de energia e colonizar ambientes com as mais variadas características, como por exemplo, ambientes alcalinos, ácidos, neutros, com temperaturas variáveis e níveis nutricionais distintos, tornando os fatores químicos, físicos e biológicos do solo fortes influenciados quanto a presença e abundância de grupos microbianos. Contudo, as alterações desses fatores promovem variações de micro e macro habitats tornando possível a colonização e adaptação de microrganismos nos mais diferentes nichos (Gomes, 2010).

A importância da estrutura e diversidade microbiana do solo tem sido alvo de diferentes estudos em solos agrícolas, florestais e/ou antropizados (Fierer, 2012; Rodrigues, 2013; Mendes, 2014; Miranda, 2018). Ao longo do tempo, desafios nos estudos em ecologia microbiana do solo têm sido sanados no intuito de desenvolver métodos eficazes para descrever a diversidade e a função das comunidades microbianas presentes no solo (Mendes, 2014, 2015). O grande entrave para a compreensão completa do da distribuição das funções de cada grupo microbiano, se dá pela flutuação de comportamento de acordo com o tipo de solo, fatores climáticos, pluviométricos, presença ou ausência de vegetais, atividades antrópicas que por meio

de interações, causam constantes modificações no ambiente, influenciando diretamente no microbioma.

Apesar das ferramentas moleculares revelarem que a diversidade microbiana sobressai da diversidade dos outros organismos vivos, o conhecimento acerca dos padrões de distribuição no espaço e no tempo dos microrganismos e os fatores que governam essa distribuição ainda é considerado insuficiente, frente a infinidade de grupos a serem descobertos.

Os grupos de organismos exibem diferentes padrões espaciais, devido a reação individual com o solo em diversas formas. A heterogeneidade espacial tem sido observada em escala de milímetros a centenas de quilômetros (Rodrigues et al., 2013) correlacionando-se com propriedades do solo, tais como a agregação, textura, teor de oxigênio, variações de pH, umidade, teor de matéria orgânica, fornecimento de nutrientes, níveis de precipitação e presença e estágio sucessional da vegetação. Algumas dessas propriedades são importantes numa escala microscópica, enquanto outras são consideradas em distâncias maiores (Maron, 2011; Mendes e Tsai, 2018).

A avaliação da diversidade microbiana proveniente de amostras ambientais era realizada apenas por métodos tradicionais de isolamento seletivo e de cultivo para identificar as espécies ou os grupos funcionais, estimar a abundância relativa e determinar a estrutura da comunidade (Amann et al., 1995), porém com o advento das técnicas moleculares, foi possível ter alcance a análises mais detalhadas e completas das composições da microbiota, uma vez que esta técnica tem com base estruturas de DNA dos organismos. As técnicas anteriores, tornaram-se incipientes, apesar de importantes, ou necessitadas de mais detalhes comprobatórios das comunidades, pois a detecção e a identificação dos microrganismos eram baseadas somente nas suas necessidades nutricionais, nas fontes de carbono e energia específicas e nas condições de cultivo favoráveis ao seu crescimento, além da observação direta da morfologia em microscópio (Ritchie, 2000), sendo, em muitos casos, impossível a detecção completa da comunidade.

Importantes avanços na área da biologia molecular possibilitam analisar microbiomas, por meio de métodos independentes de cultivo, o que permitiu avanço considerável no estudo da sistemática e da ecologia microbiana (Leckie, 2005), não requerendo ensaios preliminares de cultivo e isolamento microbiano, e sim, utilizando estratégias moleculares para analisar DNA e/ou RNA extraídos diretamente de amostras ambientais (Mendes et al, 2017).

Através de análises dos ácidos nucléicos torna-se possível estimar as

diversidades taxonômica, genética e funcional, além de gerar informações a respeito da composição da comunidade, das relações filogenéticas e do estado metabólico dos microrganismos, pois podem detectar grande número de espécies ainda pouco conhecidas e/ou de difícil cultivo (Muyzer, 1999), assim com, contribui para a investigação e descoberta de inúmeras espécies ainda não identificadas pelos métodos tradicionais.

O método de análise do gene do rRNA 16S, ou fragmentos específicos dele, tornou-se o mais empregado no estudo da diversidade dos microrganismos, devido a estas sequências serem consideradas marcadores moleculares importantes por estarem presentes em todos os organismos, possuindo essencialidade na produção de proteínas, além de serem estruturalmente conservados, apresentando tamanho satisfatório, com cerca de 1500 nucleotídeos, suficientes para fazer inferências filogenéticas (Rajemdhran, 2011). Importantes informações sobre a ecologia dos microrganismos provenientes de ambientes naturais podem ser obtidas utilizando diferentes estratégias para analisar a estrutura da comunidade microbiana (Leckie, 2005).

Estudos de diversidade taxonômica, diversidade funcional e de prospecção de genes e enzimas de interesse, podem ser realizados graças à metagenômica acoplada aos métodos de sequenciamento de alto rendimento (*high throughput*). As novas tecnologias de sequenciamento de DNA e as ferramentas de bioinformática abriram inúmeras possibilidades de análises, mostrando milhões de organismos nos mais diversos ambientes. Essas plataformas têm alcançado um alto número de sequências, suficientes para revelar a diversidade de comunidades microbianas complexas e caracterizar a “rara biosfera” que compreende a presença de taxa de micro-organismos de baixa abundância em vários ambientes (Parameswaran, 2010).

A análise de diversidade taxonômica baseia-se na construção de bibliotecas de um gene marcador filogenético (rDNA 16S para bactérias e arqueias, região do ITS/5, 8S para eucariotos) utilizando como substrato o DNA metagenômico extraído de um dado ambiente (amostras). Para análise de metagenômica de DNA total (análises funcionais) são feitas construções de bibliotecas utilizando como substrato o DNA metagenômico fragmentado aleatoriamente (*shotgun*). Estes fragmentos obtidos são ligados a sequências adaptadoras comuns, e então é realizada a reação de sequenciamento, permitindo análises do potencial biotecnológico dos microrganismos, com possível identificação de novas biomoléculas.

O grande quantitativo de informações obtidos por meio do sequenciamento,

traz consigo, desafios que precisam ser superados, e o uso conjunto das técnicas da bioinformática para interpretação dos dados metagenômicos torna-se fundamental, uma vez que o metagenôma inclui o genoma de milhões de espécies de um dado ambiente, muitas das quais não são atualmente descritas, tornando-se uma técnica promissora no preenchimento de lacunas existentes sobre a diversidade genética e o potencial biotecnológico dos organismos sejam estes abundantes ou raros.

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# CAPITULO I - Responses of soil bacterial community after seventh yearly applications of composted tannery sludge


## Abstract

Composted tannery sludge (CTS) contains organic compounds and inorganic elements, mainly chromium (Cr), and its long-term application in soil can alter the bacterial structure and diversity. Thus, we used the next-generation sequencing to assess the structure and diversity of bacterial communities in soils after seven years of CTS application. CTS was applied at 0, 2.5, 5, 10, and 20 Mg $ha^{-1}$ and the soil samples were collected at 75 days after application in the seventh year. The most abundant phyla were Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi. The abundance of some specific groups increased with application of CTS, such as Anaerolinea S0208 and Firmicutes. Six bacterial genera differed between amended and unamended soil. The abundance of *Bacillus, Paenibacillus, Symbiobacterium, Clostridium, Microlunatus*, and *Actinomadura* increased after application of CTS. The Redundancy Analysis between the structure of the bacterial community and chemical variables in soil did not cluster all treatments clearly, but showed Cr, pH, and organic C as significant chemical variables that influenced the bacterial communities. Application of CTS in soil has a primary effect on the bacterial communities that, negatively, alter the bacterial diversity and community similarity, while that, positively, it permits to select specific bacterial groups able to resist and biodegrade contaminants.


**Keywords**: 16S rRNA; soil microbial diversity; organic waste

## Introduction

The high generation of solid wastes has led to environmental concerns worldwide since they are disposed in landfilling and, therefore, posing risk to the environment (Srivastava et al., 2016). Agricultural use of solid waste has increased recently, being one of the most promising alternatives for its disposal (Araujo et al., 2010). However, these wastes can contain pathogens and organic and inorganic elements, such as heavy metals, which could lead to soil pollution in the long-term use (Hargreaves et al., 2008). Specifically, tannery sludge (TS), a solid waste produced by tannery industries, contains organic compounds and inorganic elements, such as chromium (Cr), salts, carbonates, and hydroxides (Araujo et al., 2015) that can accumulate in soil and cause environmental pollution.

The composting can be an alternative biological process for recycling and decomposition of TS before its agricultural utilization (Santos et al., 2011). It is also important since organic compost has potential of improving soil properties (Yuksel, 2015). In fact, previous studies have shown that the use of composted tannery sludge (CTS) has improved the physical and chemical properties of soil (Araujo et al., 2013; Araujo et al., 2016).

On the other hand, a concern about using CTS in agricultural soils is related with the soil biological properties, mainly soil microorganisms. Soil microorganisms are the key organisms involved in important ecological processes, such as nutrient cycling, and are known as the largest reservoirs of biodiversity (De Mandal et al., 2015). Also, soil microorganisms present biotechnological potential (Sleator et al., 2008) and are recognized as sensitive indicators of soil health and anthropogenic disturbances (Nakatani et al., 2011). Several previous studies were already carried out to assess the effect of CTS application on soil microorganisms in both short- and long-term (Santos et al., 2011; Miranda et al., 2014; Araujo et al., 2015; Araujo et al., 2016). In these studies, the responses of soil microorganisms and microbial process were contradictory, i.e. soil microbial biomass and activity showed negative responses; nodulation and nitrogen fixation responded positively, while enzymatic activity showed no response.

It is unclear how the long-term use of CTS affects the structure and diversity of microbial community and what the chemical factors are driving these effects. Although some studies have evaluated the effect of untreated TS on microbial communities in the short-term (Nakatani et al., 2011; Giordarno et al., 2016), the responses of microbial structure and diversity to a long-term (years of) exposure due to successive applications

of CTS has not been assessed.

Nowadays, the sequencing of 16S rRNA genes recovered from the environment is the most frequently used molecular method for evaluating bacterial diversity (Mishra et al., 2014) and the next-generation high-throughput sequencing method presents high accuracy to elucidate the structure of bacterial communities. In the last years, this technique has been used to study soil bacterial structure and diversity from several environments (Neelakanta and Sultana, 2013; De Mandal et al., 2015; Araujo et al., 2017) but it has not yet been used to evaluate the effects of application of composted wastes to soil bacterial communities.

In this study, we hypothesize that (1) the long-term applications of CTS alter the bacterial structure and diversity; and (2) specific chemical parameters drive the response of bacterial communities to CTS application. To test these hypotheses, we used the next-generation high-throughput sequencing method on Illumina Miseq in order to assess the structure and diversity of bacterial communities in soils after seven years of CTS application.

## Material and Methods

### *Sampling site and experimental procedure*

The experimental site is located at the Long-Term Experimental Field belonging to the Agricultural Science Center, Federal University of Piauí, Brazil. The soil is classified as Fluvisol with the following composition at 0-20 cm depth: 10% clay, 28% silt, and 62% sand. CTS was produced by mixing TS with sugarcane straw and cattle manure (ratio 1:3:1; v:v:v) and the composting was performed using the aerated-pile method (USDA 1980) for 90 days. The physicochemical characteristics of CTS were evaluated at the end of composting process (Table 1). The water content was determined after oven drying the samples at 105°C for 24 h. The pH was directly measured by mixing CTS with water (1:2.5 v:v) using a pH-meter, and total solids were measured by drying the samples at 65°C (APHA, 2005). The total organic C content was evaluated by dichromate oxidation of the samples under external heating (Nelson and Sommers, 1996). The total N content was determined using the Kjeldahl method after sulphuric acid digestion of the samples (Bremner, 1996). The total Ca, Mg, K, P, S, Na, Zn, Cu, Cd, Pb, Ni, and Cr concentrations were determined by atomic absorption spectrophotometry after nitric acid digestion of the samples in a microwave oven (USEPA, 1996).

**Table 1**. Composition of composted tannery sludge.

| | Moisture | pH | TOC | N | P | K | Ca | Mg | Na | Cr |
|---|---|---|---|---|---|---|---|---|---|---|
| | % | | | | | g kg$^{-1}$ | | | | mg kg$^{-1}$ |
| | 68 | 7.5 | 201 | 15 | 4.9 | 2.9 | 121 | 7.2 | 49.1 | 1943 |
| MLP[1] | - | - | - | - | - | - | - | - | - | 150 |

MLP - Maximum Limit Permitted (CONAMA, 2009); TOC - Total organic carbon

CTS has been applied annually since 2009 at five rates: 0 (without CTS application), 2.5, 5, 10, and 20 Mg ha$^{-1}$ of CTS (dry basis). The experimental site is arranged in a completely randomized design with four replicates for each treatment. Plots are 20 m$^2$ each, with 12 m$^2$ of usable area for soil and plant sampling, and rows are spaced 1.0 m apart. In the seventh year (2015), CTS was applied 10 days before maize (*Zea mays* L.) sowing. At application, the CTS was spread on the soil surface and incorporated into the 20 cm layer with a harrow. Maize was grown at a density of 5 plants m$^{-1}$ (approximately 62,000 plants ha$^{-1}$) for 75 days. CTS was spread on the soil surface and incorporated into the 20 cm layer with a harrow. Soil samples were collected, in 2015, from each plot at 75 days after CTS application. For soil sampling, four samples were collected in each plot (0-20 cm), sieved (2-mm), and stored at 4°C prior to analysis.

The soil samples for chemical analysis were air dried and sieved (2-mm). Soil pH, exchangeable $Ca^{2+}$, $Mg^{2+}$, $K^+$ and $Na^+$, and the available P were estimated according to EMBRAPA (1997). Soil electric conductivity (EC) was evaluated in water (1:2 v:v) according to the method described by Richards (1954). Total organic C (TOC) was determined by wet combustion using a mixture of 5 mL of 0.167 mol L$^{-1}$ potassium dichromate and 7.5 mL of concentrated sulfuric acid under heating (170ºC for 30 min) (Yeomans and Bremner, 1988).

***DNA extraction and sequencing***

Soil DNA was extracted from 0.5 g (total humid weight) of soil using the Power Lyzer Power Soil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The DNA extraction was performed in triplicate for each soil sample. The quality and concentration of the extracted DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA).

The V4 region of the 16S rRNA gene was amplified with region-specific primers (515F/806R) (Caporaso et al., 2011). Each 25 µl PCR reaction contained the following: 12.25 µL of nuclease-free water (Certified Nuclease-free, Promega, Madison, WI, USA),

5.0 μL of buffer solution 5x ($MgCl_2$ 2Mm), 0,75 μL de solution of dNTP's (10 mM ), 0,75 μL of each *primer* (515 YF 40 μM e 806 R 10μM), 1.0 unit of Platinum Taq polymerase High Fidelity in concentration of 0,5 μL (Invitrogen, Carlsbad, CA, USA), and 2.0 μL of template DNA. Moreover, a control reaction was performed by adding water in place of DNA. The conditions for PCR were as follows: 95°C for 3 min to denature the DNA, with 35 cycles at 98°C for 20 s, 55 °C for 20 s, and 72°C for 30 s, with a final extension of 3 min at 72°C to ensure complete elongation.

After indexing, the PCR products were cleaned up using Agencourt AMPure XP – PCR purification beads (Beckman Coulter, Brea, CA, USA), according to the manufacturer's manual, and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Once quantified, equimolar concentrations of each library were pooled into a single tube. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 8.0 pM with a 20% PhiX (Illumina, San Diego, CA, USA) spike for loading into the Illumina MiSeq sequencing machine (Illumina, San Diego, CA, USA).

***Data processing***

Sequence data were processed using QIIME following the UPARSE standard pipeline according to Brazilian Microbiome Project ((http://www.brmicrobiome.org/#!16s-profiling-pipeline-illumina/czxl) (Pylro et al., 2014), to produce an OTU table and a set of representative sequences. Briefly, the reads were truncated at 240 bp and quality-filtered using a maximum expected error value of 0.5. Pre-filtered reads were dereplicated and singletons were removed and filtered for additional chimeras using the RDP_gold database using USEARCH 7.0. These sequences were clustered into OTUs at a 97% similarity cutoff following the UPARSE pipeline. After clustering, the sequences were aligned and taxonomically classified against the Greengenes database (version 13.8).

***Data analysis***

Redundancy Analysis (RDA) was used to determine the correlation between bacterial community structure and soil physicochemical properties (ter Braak and Šmilauer, 2002). The OTU table was initially analyzed using Detrended Correspondence Analysis (DCA) to evaluate the gradient size of the species distribution, which indicated

linearly distributed data (length of gradient < 3), revealing that the best-fit model for the data was RDA. Forward selection (FS) and the Monte Carlo permutation test were applied with 1000 random permutations to verify the significance of soil chemical properties upon a microbial community. We used permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) to test whether sample categories harbored significantly different bacterial community structure. SIMPER analysis was run to compare the similarity between and within treatments. RDA plots were generated using the software Canoco 4.5 (ter Braak and Šmilauer, 2002), and PERMANOVA, SIMPER and alpha diversity measurements were calculated with the software PAST (Hammer et al. 2001). To determine the differences in abundance of bacterial groups among soil samples, the Statistical Analysis of Metagenomic Profiles (STAMP) software package was used (Parks et al. 2014). The OTU table was used as input, and *P*-values were calculated using the two-sided Welch's t-test (Welch 1947), while confidence intervals were calculated using DP Welch's inverted and correction was made using Benjamini-Hochberg false discovery rate (Benjamini and Hochberg 1995). Spearman's rank correlation coefficients were calculated to explore the relationship between relative abundance of microbial groups and soil factors according to the different treatments using the 'multi test' package in R (R Development Core Team 2017) and the correction was made using Benjamini-Hochberg false discovery rate.

**Results**

The application of CTS during seven years has changed some of the chemical properties of the soil, with a significant increase of pH, EC, Cr, TOC, P, and Ca (Table 2). Markedly, Cr has increased strongly in all treatments after the seven years of CTS application. To compare the soil attributes among different treatments, we performed a principal component analysis (PCA). The biplot presented in the figure 1A reveals a clear segregation, clustering the samples according to the treatment. Samples from the T4 and T5 showed correlation with the high content of the all factors.

In order to visualize the differences in community structure among the treatments, the abundance matrix of taxonomy at genus level was used for redundancy analysis (Fig. 1B). Although the RDA did not show a clear clustering among all the treatments, the T1 and T5 was significantly clustered apart (PERMANOVA, $P < 0.05$), revealing a clear distinction of the bacterial community between the unamended and amended soils. Also, the analysis showed that the bacterial community structure of T4 and T5

treatments correlated strongly with the soil chemical factors. The results also pointed that CTS rate, Cr and pH significantly influenced the overall bacterial communities in this study. Interestingly, the Spearman's rank correlation analysis revealed that CTS rate and Cr were the factors that most correlated with microbial groups, presenting interaction with 79 and 76 genera, respectively (Table 3 and Supplementary Table 1). It's notable that most of these correlations (>90%) are negative interactions. These results reinforce the effect of CTS application on the soils chemical properties and, consequently, on the bacterial community structure.

**Table 2.** Chemical properties of the soil after 7 yearly applications of composted tannery sludge (CTS).

| CTS | pH | TOC | P | K | Ca | Mg | Na | EC | Cr |
|---|---|---|---|---|---|---|---|---|---|
| Mg $ha^{-1}$ | $CaCl_2$ | g $kg^{-1}$ | mg $kg^{-1}$ | ---------- $mmol_c$ $kg^{-1}$ ---------- | | | | dS $m^{-1}$ | mg $kg^{-1}$ |
| 0 | $6.5^{b}$ | $5.5^{b}$ | $6.0^{b}$ | $2.2^{a}$ | $13.3^{c}$ | $6.5^{a}$ | $4.6^{b}$ | $0.71^{c}$ | $4.4^{e}$ |
| | ±0.1 | ±0.8 | ±0.3 | ±0.2 | ±1.1 | ±0.1 | ±0.2 | ±0.03 | ±0.1 |
| 2.5 | $6.7^{b}$ | $7.0^{ab}$ | $6.8^{b}$ | $2.4^{a}$ | $20.0^{bc}$ | $6.8^{a}$ | $5.0^{b}$ | $0.83^{b}$ | $29.3^{d}$ |
| | ±0.3 | ±0.4 | ±0.5 | ±0.3 | ±1.7 | ±0.3 | ±0.1 | ±0.05 | ±2.7 |
| 5 | $6.9^{b}$ | $7.3^{ab}$ | $7.3^{b}$ | $2.3^{a}$ | $26.0^{a}$ | $7.0^{a}$ | $5.3^{a}$ | $0.85^{b}$ | $50.8^{c}$ |
| | ±0.2 | ±0.7 | ±0.8 | ±0.2 | ±1.9 | ±0.3 | ±0.1 | ±0.06 | ±6.2 |
| 10 | $7.5^{a}$ | $7.7^{ab}$ | $10.3^{a}$ | $2.0^{a}$ | $24.3^{ab}$ | $7.5^{a}$ | $5.4^{a}$ | $0.90^{a}$ | $102.2^{b}$ |
| | ±0.5 | ±0.6 | ±0.9 | ±0.5 | ±2.3 | ±0.5 | ±0.3 | ±0.07 | ±10.5 |
| 20 | $7.8^{a}$ | $8.5^{a}$ | $12.5^{a}$ | $2.1^{a}$ | $27.3^{a}$ | $7.8^{a}$ | $5.6^{a}$ | $0.94^{a}$ | $150.1^{a}$ |
| | ±0.5 | ±0.5 | ±1.1 | ±0.3 | ±2.1 | ±0.2 | ±0.3 | ±0.05 | ±12.4 |

Means ± standard error. Means followed by the same letter in the column did not differ from each other ($p < 0.05$) by the Duncan test

**Table 3.** Number of correlations between soil chemical parameters and bacterial groups as indicated by Spearman ($P<0.05$).

| **Parameter** | **Nº of Phyla** | **Nº of Genera** |
|---|---|---|
| CTS_rate | 12 | 79 |
| Cr | 12 | 76 |
| P | 12 | 58 |
| Ca | 11 | 62 |
| TOC | 11 | 54 |
| pH | 10 | 55 |
| EC | 8 | 24 |
| Mg | 7 | 10 |
| Microbial biomass C | 6 | 9 |
| Na | 5 | 9 |
| K | 3 | 4 |

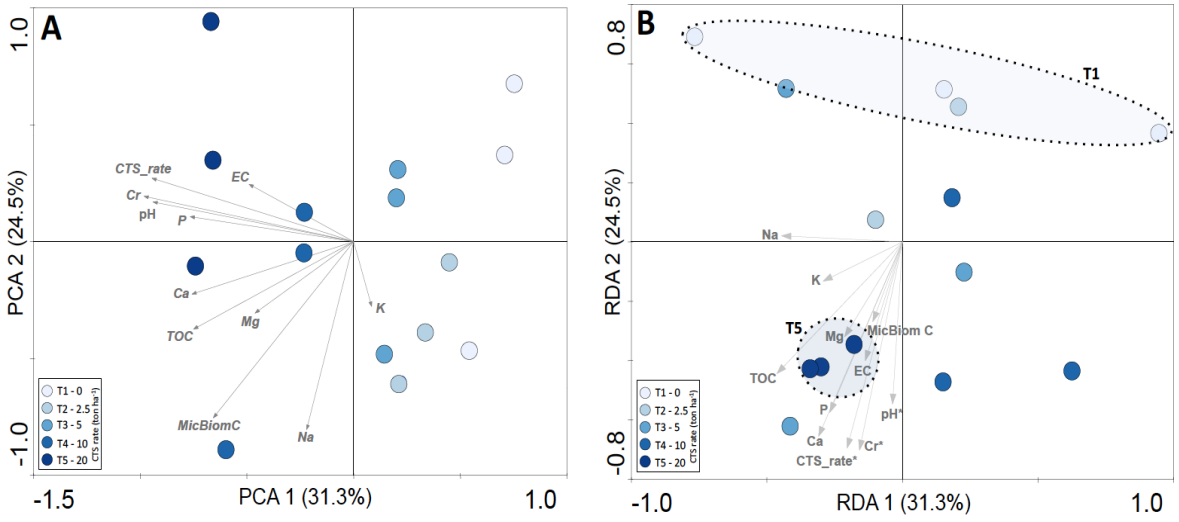


**Figure 1. (A)** Principal component analysis (PCA) biplot based on physicochemical soil parameters from different treatments. **(B)** Redundancy analysis (RDA) of the bacterial community structure (based on 16S rRNA) and chemical variables in soils after 7 yearly applications of CTS. T1 – 0 Mg $ha^{-1}$; T2 – 2.5 Mg $ha^{-1}$; T3 – 5 Mg $ha^{-1}$; T4 – 10 Mg $ha^{-1}$; T5 – 20 Mg $ha^{-1}$. The significance of these correlations was evaluated via the Monte Carlo permutation test and is indicated by asterisk ($P < 0.05$). The dashed lines in the graph indicate significant clusters (PERMANOVA, $P < 0.05$) between T1 and T5.

The analysis of the bacterial composition showed that the sequences were affiliated to 22 bacterial phyla, 113 families, and 99 genera within all evaluated sites. Ten phyla were considered most abundant with percentages of OTUs above 1% (Actinobacteria, Proteobacteria, Firmicutes, Chloroflexi, Planctomycetes, Acidobacteria, Gemmatimonadetes, Verrucomicrobia, Nitrospirae, and Bacteroidetes) (Fig. 2). Although the diversity measurements did not show significant differences between the treatments, they revealed a trend to decrease with the increased CTS application (Fig. 3A). Also, the effect of amendment on the bacterial community can be visualized by the SIMPER analysis (Fig. 3B), which shows a slightly decreased similarity between T1 and T5. On the other hand, the similarity within each community increased according to the CTS rate, indicating a homogenization of the community. In the treatment T5 the genus *Bacillus* contributed with 11.8% of this similarity.

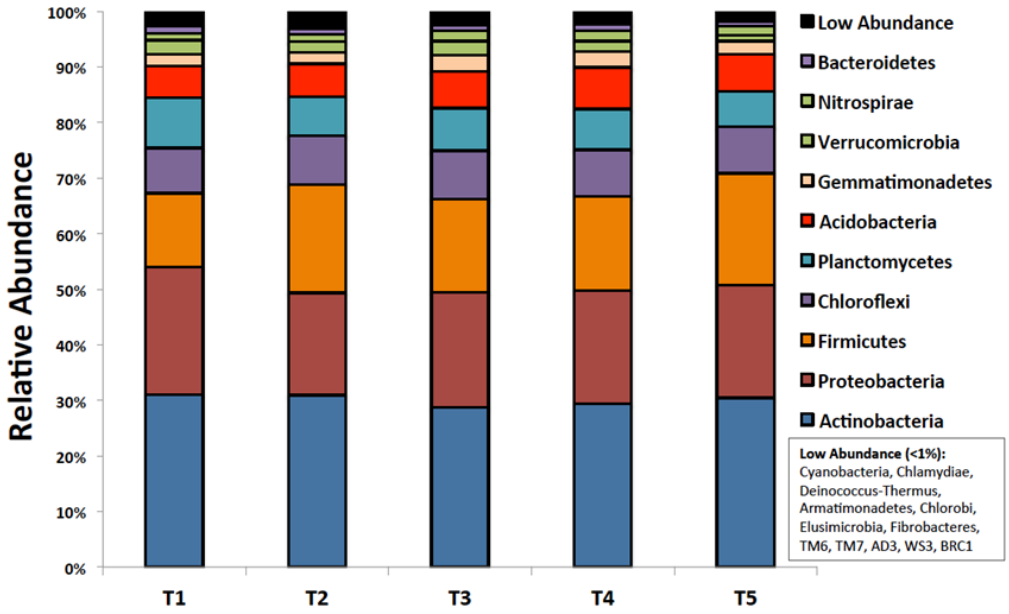


**Figure 2.** Taxonomic composition of the bacterial community (at phylum level) in soils treated with seven yearly different CTS rates. T1 – 0 Mg $ha^{-1}$; T2 – 2.5 Mg $ha^{-1}$; T3 – 5 Mg $ha^{-1}$; T4 – 10 Mg $ha^{-1}$; T5 – 20 Mg $ha^{-1}$.

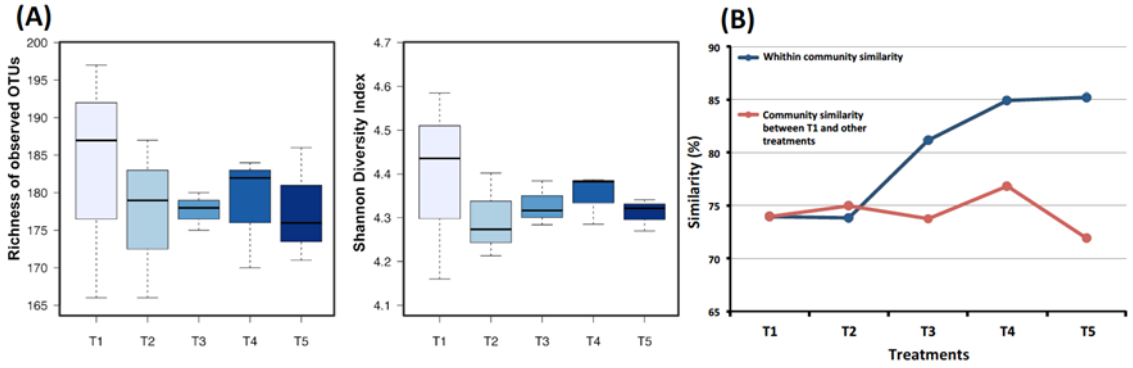


**Figure 3. (A)** Diversity measurements of bacterial communities in soils after seven yearly applications of CTS at different rates. Taxonomic diversity based on 16S rRNA OTUs classified at 97% os similarity. **(B)** Similarity percentage analysis (SIMPER) based on Bray-Curtis index of the bacterial community in soils with different rate of CTS amendement. T1 – 0 Mg $ha^{-1}$; T2 – 2.5 Mg $ha^{-1}$; T3 – 5 Mg $ha^{-1}$; T4 – 10 Mg $ha^{-1}$; T5 – 20 Mg $ha^{-1}$.

The relative abundance of some specific bacterial groups showed different patterns from unamended soil as compared with each CTS-treated soil (Fig. 4). More specifically, there was a shift in abundance of some phyla according to the treatments, such as decrease of Proteobacteria and an increase of Acidobacteria and Firmicutes (Fig. 4A-C). In a finer taxonomical level, the abundance of Anaerolinea S0208

(Chloroflexi) increased from T1 (unamended soil) to T2, T3, and T4 (2.5, 5, and 10 ton $ha^{-1}$, respectively). Acidobacteria Gp6 and S035 increased in T3 and T4, while Chloroflexi S085 increased in T3. The application of CTS at the highest rate (20 ton $ha^{-1}$) promoted a decrease in specific groups of Planctomycetes (Phycisphaera and Gemmatales) and Deltaproteobacteria (Myxococcales); while Firmicutes (Clostridiales) increased with the application of CTS at the highest rate (Fig. S1)

We found nine bacterial genera as most abundant, with percentages of OTUs above 1%, namely *Bacillus* (8%), *Rhodoplanes* (2%), *Paenibacillus* (1.5%), *Kaistobacter* (1.4%), *Streptomyces* (1.4%), *Ammoniphilus* (1.3%), *Alicyclobacillus* (1.3%), *Mycobacterium* (1.2%), *and Exiguobacterium* (1%). Comparing the sites with the application of CTS with the control, the relative abundance of *Bacillus, Paenibacillus, Symbiobacterium, Clostridium, Microlunatus*, and *Actinomadura* increased significantly with increasing CTS rates (Fig. 4D-I). Interestingly, these genera were influenced by CTS and Cr accumulation.

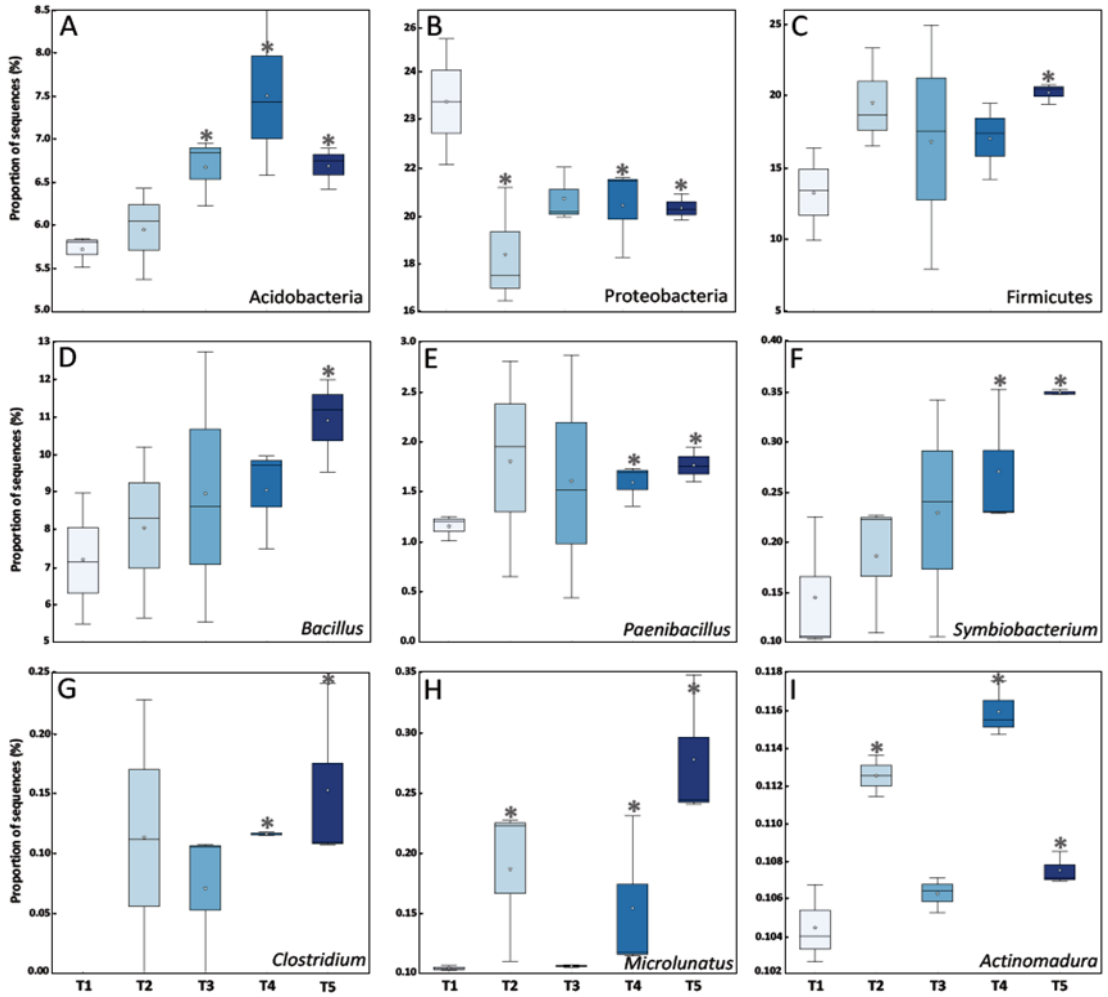


**Figure 4.** Distribution of the most differential bacterial phyla (**A** to **C**) and genera (**D** to **I**) in soils after 7 yearly applications of CTS. Boxes indicate IQR 75$^{th}$ to 25$^{th}$ of the data). The median value is shown as a

line within the box and outliers are represented by dots. Asterisks refer to significant differences compared to control, based on Tukey's test ($P < 0.05$). T1 – 0 Mg $ha^{-1}$; T2 – 2.5 Mg $ha^{-1}$; T3 – 5 Mg $ha^{-1}$; T4 – 10 Mg $ha^{-1}$; T5 – 20 Mg $ha^{-1}$.

## Discussion

As shown in Table 1, CTS has a high pH and high content of organic C, Ca, Na, and Cr. Therefore, successive yearly applications of CTS promoted shifts in some chemical variables of the soil as direct response of CTS composition. Indeed, the results of PCA and RDA showed that samples amended with CTS, mainly the highest rates, were correlated with all chemical variables and they influenced the structure and diversity of soil bacterial communities. The results showed that, significantly, CTS rates, Cr, and pH were the most important drivers of bacterial structure. Several previous studies have also found soil chemical variables, such as soil pH (Lauber et al., 2009), and chromium (Sheik et al., 2012) as drivers of microbial communities in soils. Fierer and Jackson (2006) have suggested pH as the main factor that drive the variation of microbial communities, mainly due to its integration with other physicochemical soil parameters. The pH has strong influence on abiotic factors, such as nutrient availability and metals solubility (Kemmitt et al., 2006). Also, the contamination by Cr can have adverse effects on soil bacterial communities, with effects on the diversity, structure and abundance (Li et al., 2017). Interestingly, soil pH and Cr were also the most significant variables influencing the microbial biomass and activity in soils amended with composted tannery sludge (Araujo et al., 2017). The result of RDA clearly separated the unamended soil from the highest CTS rate. It occurred because CTS rates drove the shifts in chemical variables from unamended to amended soil, mainly at the highest CTS rates, and consequently impacted the microbial communities.

Our study showed that the community variability within treatments decreased with increasing CTS rates and it could indicate an environmental filter (for example high Cr content). In this sense, soil pollution could have a repeatable influence on bacterial groups within the treatment and select more similar bacterial groups more as a result of soil disturbance. Although variability exists naturally in all population, soil disturbance can act increasing or decreasing the level of variability (Herren et al., 2016; Fraterigo and Rusak, 2008). Specifically, for disturbed soils, the disturbance decreases microbial variability through of selective pressure, such as metal accumulation, and separates communities more similar (Ranta et al. 1997). Thus, in our study, the repeated

application of CTS during 7 years could have selected a more similar bacterial community and, therefore, the variability of communities decreased in CTS-treated soils.

The results of richness and Shannon index showed that the application of CTS did not affected the diversity of bacterial community as whole (Figure 4). It means that CTS did not promote losses of biodiversity or simplification of soil bacterial community. In contrast, there was a shift in bacterial community from unamended soil to amended soil with 10 and 20 Mg $ha^{-1}$ CTS. These results can suggest that the applications of CTS, in long-term, selected bacterial groups which present characteristic to growth and colonize these soils.

The analysis of the sequences revealed Actinobacteria, Proteobacteria and Firmicutes as the most abundant phyla in CTS-treated soils. These findings are in contrast with several studies that found Acidobacteria, together with Actinobacteria and Proteobacteria, as the most abundant phyla across several ecosystems and environments (Kielak et al., 2016; Zeng et al., 2016; Araujo et al., 2017). Interestingly, Proteobacteria decreased and Acidobacteria and Firmicutes increased from unamended to amended soils. The increase in abundance of Acidobacteria and Firmicutes, with the increase in CTS rates, is probably a result of the highest tolerance of these groups to unfavorable conditions (Hartmann et al., 2014). Regarding to Firmicutes, this result may be explained due to the increase in Cr accumulation, which may have favored this phylum instead Proteobacteria. Firmicutes are endospore forming bacteria (de Hoon et al., 2010) that present the ability of living under environmental stresses (Barnard et al., 2013). Thus, the high Cr content found in soils may favor this group since they were found as the dominant bacterial group in metal-polluted soils (Ellis et al. 2003). Our finding agrees with Desai et al. (2009) that found a shift in bacterial communities from Proteobacteria to Firmicutes in a Cr-polluted industrial landfill. According to Janseen et al. (2006), usually the abundance of Firmicutes in uncontaminated soils is around 2% of the total bacterial community and increases in soils with high Cr content. Previous studies also found Firmicutes as abundant phylum in soils contaminated with Cr (Camargo et al., 2005; Wang et al., 2008; Chen et al., 2012; Stewart et al., 2010; Yao et al., 2010), being suggested as an effective bioindicator of Cr pollution (Desai et al., 2009). In addition, many of the isolates resistant to chromium are predominantly associated with the bacterial phyla Proteobacteria, Firmicutes and Actinobacteria (Kamaludeen et al., 2003), which were the most abundant in our amended soil.

Our results showed that other specific bacterial groups, usually found in metals-contaminated sites, were also influenced by CTS application. Chloroflexi also seems to increase in contaminated soils, e.g. with Cr. Katsaveli et al. (2012) assessed bacterial diversity in wastes containing Cr and found Chloroflexi as the most abundant phylum. According to Martins et al. (2010), members of Chloroflexi are recognized to be resistant to high content of Cr. As member of Chloroflexi, the class Anaerolinea can degrade recalcitrant compounds (Wu et al., 2001; Sekiguchi et al., 2003), which could explain its high abundance in soil with CTS. This finding is consistent with previous studies in metal-contaminated sites (Zhu et al., 2013; Yin et al., 2015), mainly in sludge wastewater treatment plants (Nielsen et al., 2009). According to Yin et al. (2015), the class Anaerolineae contains some specific genes related with metal resistance, which could explain their high abundance in metal-contaminated sites. The abundance of the class Acidobacteria GP6 also increased with the additions of CTS, which can be attributed to its ability to resist to metals. This class has been found in high abundance in soils contaminated with uranium as well (Barns et al., 2007; Gupta et al., 2012). On the other hand, the decrease in abundance of Planctomycetes may be associated to the increase in soil pH and, also, Cr concentration. According to Chronakova et al. (2015), the abundance of Planctomycetes decreases with increasing pH and organic matter content; while Firmicutes (both Clostridia and Bacilli), Chloroflexi, *Anaerolinea* all increases with pH and organic matter.

CTS rates and Cr were the drivers of abundance of specific genera, namely *Bacillus, Paenibacillus, Symbiobacterium, Clostridium, Microlunatus*, and *Actinomadura.* Specially, *Bacillus, Paenibacillus, Symbiobacterium,* and *Clostridium* increased their abundance at the highest CTS rates, and it occurred due to the characteristic of these bacteria in supporting high levels of pollution, mainly Cr contamination. Some studies have reported these bacteria, mostly belonging to phylum Firmicutes, to influence the fate and transport of toxic metals in soil (Ledin, 2000; Lovley and Lloyd, 2001) including Cr from polluted sites (Desai et al., 2009; *Poormina et al., 2010).* Also, some of these genera were found abundant in Cr-contaminated soils (Desai et al., 2009). For example, *Bacillus* was found as most dominant genus in soils exposed to high Cr content due to their characteristic to resist and also reduce Cr concentration (Desai et al., 2009).

This high tolerance of this specific group of bacteria to Cr may be related with their interactions with metals in the environment, i.e. by production of bacterial biofilms

that consist of extracellular polymeric substances (EPSs) (Davey and O'Toole, 2000). The composition of EPS is carbohydrates, protein, DNA, and sorbed abiotic constituents, and these characteristics protect individual cells from chemical attack (Sutherland, 2001). Previously, Teitzel and Parsek (2003) have suggested the mechanism of EPS for binding of contaminants and, thus, there would be an immobilization of metals. Our findings are important since further studies could isolate and investigate the potential of these bacteria for EPS production and their potential to bioremediation of Cr-polluted soils. Several studies have reported the utilization of bacteria for the bioremediation of Cr-polluted soils including *Clostridium, Paenibacillus,* and *Bacillus* (Inglett et al., 2011; Grady et al., 2016; Upadhyay et al., 2017).

In addition, it is important to highlight that despite CTS increased Cr content in soil, the application of this waste after 7 years contributed to the growth and yield of maize, mainly because of the improved soil fertility (Sousa, 2017). Probably, it occurred because *Bacillus, Paenibacillus*, and *Clostridium* were the most abundant bacterial genera found in CTS-treated soils and could help protect the plant against Cr toxicity. Previous studies have reported that some plant-promoting growth bacteria (PGPB), such as *Bacillus, Paenibacillus*, *Pseudomonas*, inhabit the rhizosphere of plants and can ameliorate the heavy metal effects and enhance the growth and biomass of plants (Braud et al., 2009; Kumar and Patra, 2013).

## Conclusion

In this work we showed that long term application of CTS in soil has a primary effect on the soil chemical properties and, consequently, on the structure and composition of bacterial communities. Negatively, this finding indicated an alteration in the bacterial diversity and community similarity. Positively, it permits to select specific bacterial groups able to resist and potentially biodegrade contaminants. These groups can be further used as biotechnological products for contaminated sites. Our study identified different bacterial groups more resistant to chromium concentration, such as *Bacillus, Paenibacillus*, and *Clostridium* (all belonging to Firmicutes phylum). Finally, this study showed that bacterial communities varied as effect of abiotic drivers, such as Cr, and pH, and it can suggest that these chemical properties could be important predictors of microbial communities composition in CTS-treated soils.

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## Supplementary Figures

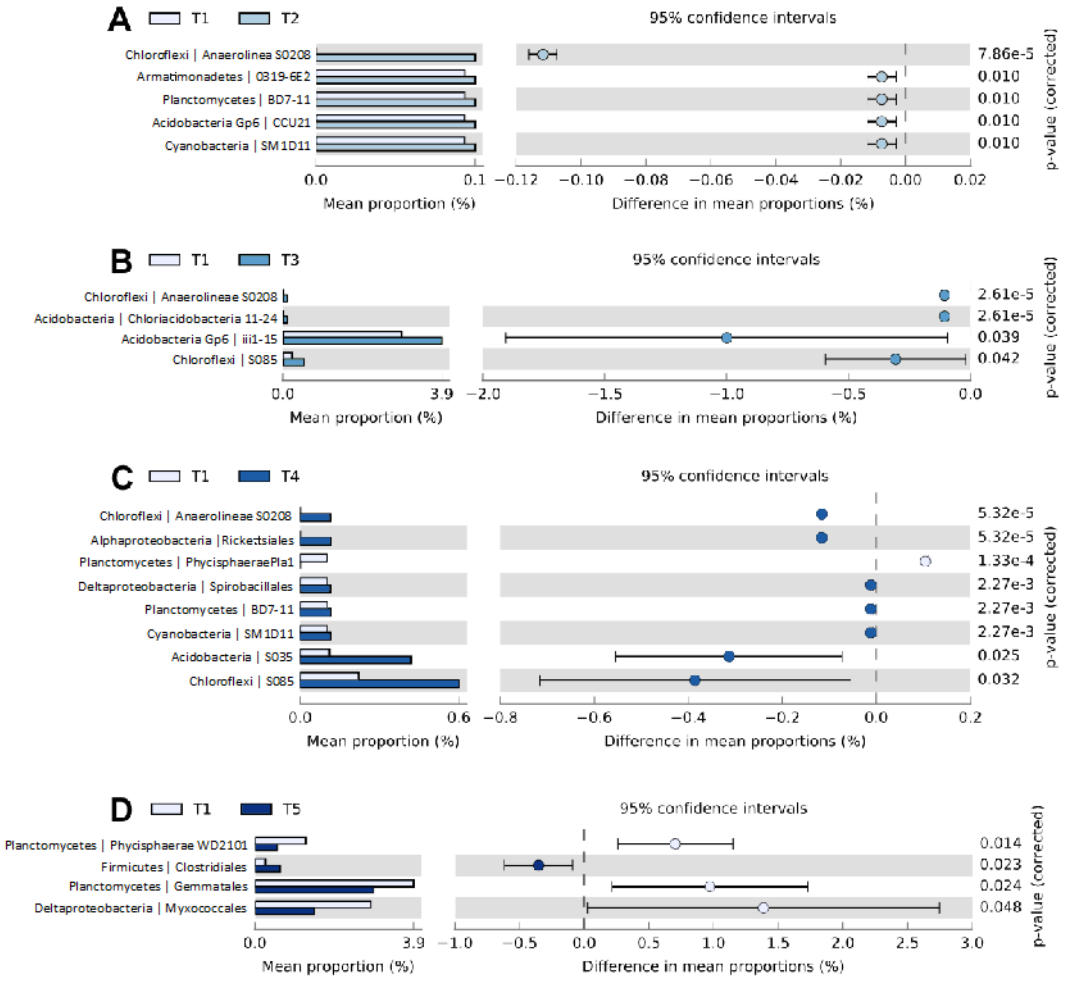


**Figure S1.** Differential abundance of OTUs, at the family level, in comparison between control and treatments, which consisted of 7 yearly applications of CTS. T1 – 0 Mg $ha^{-1}$; T2 – 2.5 Mg $ha^{-1}$; T3 – 5 Mg $ha^{-1}$; T4 – 10 Mg $ha^{-1}$; T5 – 20 Mg $ha^{-1}$

(Artigo Submetido à Revista Scientific Reports)

## CAPITULO II – Less abundant bacterial groups are more affected than those most abundant in composted tannery sludge treated soil


### Abstract

The application of composted tannery sludge (CTS) has promoted shifts in soil chemical properties and, therefore, could affect the soil bacterial community. This study assessed the effect of CTS on soil bacterial community over time. CTS was applied at five rates (0, 2.5, 5, 10 and 20 t/ha) and bacterial community was evaluated for 180 days. The principal curve response (PRC) analysis showed that the most abundant phyla were not influenced by CTS rates over time; while the analysis of bacterial community showed some of the less abundant phyla being influenced by the CTS rates. Similarly, the PRC analysis for bacterial classes showed a significant effect of CTS rates. The redundancy analyses for bacterial phyla and class showed the relationship between the significant chemical properties and bacterial community of soil after CTS amendment over time. Therefore, there was a shifting in the bacterial community over time with the application of composted tannery sludge. Our study has shown that the less abundant bacterial groups were more influenced by CTS than those most abundant bacterial groups, and that these bacterial groups were drove by soil chemical properties, mainly Cr and soil pH.


### Introduction

The generation of solid wastes is increasing worldwide and, therefore, it becomes necessary to find suitable methods for waste disposal in the environment. Unfortunately, in some regions, solid wastes are disposed in the environment without treatment and prevention. This practice has increased the accumulation of pollutants and thus promoted environmental pollution.

Specifically, tannery industries have released annually high amounts of tannery sludge (TS), a type of solid waste that contains a large amount of chromium (Cr), salts, carbonates, and hydroxides[1]. Although it is well-known that TS carries these chemical elements, this has not avoided its use as soil conditioner in agriculture[2,3]. Consequently, this practice has already promoted, in some regions, the accumulation of Cr and salinization of soils, affecting the soil microbial properties and plant growth[4].

Currently, new methods for detoxifying TS before its use have been proposed and composting has been reported as an efficient method[1]. The use of composted tannery sludge (CTS) has improved soil organic matter content and concentrations of plant nutrients, such as P, K, and Ca[1,5]. However, the permanent amendment of CTS

has promoted the accumulation of Cr and can cause soil pollution[5]. Therefore, the application of CTS may affect the soil properties, mainly the microbial communities that are driven by soil properties.

Indeed, previous studies have found significant influence of CTS on the chemical properties of soil[5] and these changes have affected soil microbial biomass and enzymes activities[1]. However, it is unclear how CTS affects the bacterial community over time, i.e. what the time-dependent effects of CTS are on the bacterial community and structure. Although previous studies have assessed the effect of TS on bacterial community[6,7], the effect of CTS on bacterial community remains unclear.

Bacterial community plays important roles in the soil ecosystem and is responsible for processes such as organic matter decomposition, mineralization, and plant growth promotion[8]. However, in the soil ecosystem, the bacterial community is influenced by several biotic and abiotic factors, such as chemical elements in soil. Therefore, the evaluation of the effect of CTS amendment, and consequently the changes in soil chemical properties, on soil bacterial community is important before its use. Nowadays, the main method for assessing soil bacterial community is the next-generation high-throughput sequencing[9] which is based on sequencing 16S rRNA genes recovered from the environment.

However, this method was not used previously for assessing the time-dependent effect of CTS on bacterial community. In order to fill this research gap, we have addressed two main hypotheses in this study: a) the dynamics of soil bacterial community will be affected by CTS in short-term; and b) the changes in chemical properties of soil will drive changes in the bacterial community.

## Results

### Chemical properties

Principal response curve (PRC) analysis of chemical properties showed that CTS rates explained 85% of the total variance and indicated that the values of most chemical variables increased in all CTS treatments (No observed effect concentrations; NOEC < 2.5 t/ha; Table S1). In contrast, the chemical properties did not change over time (Figure 2). Cr and soil pH were the main chemical variables influenced by CTS amendment showing weight ($b_k$) higher than 1.0, followed by Ca, P, electric conductivity (EC) and total organic C (TOC) with weight ($b_k$) higher than 0.25.

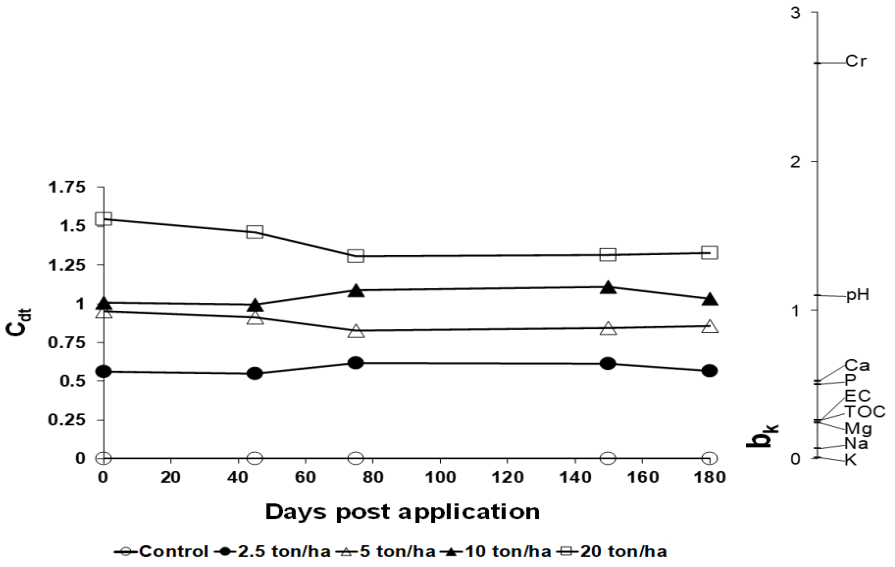


**Figure 2** Principal response curve diagram of physico-chemical data set indicating the effects of the composted tannery sludge (CTS) into the soil. The lines represent the course of the treatment levels in time. The weight ($b_k$) can be interpreted as the affinity of the variable with the Principal Response Curves ($c_{dt}$). Cr – chromium; pH – soil pH; Ca – calcium; P – phosphorus; EC –electric conductivity; TOC - total organic carbon; Mg – magnesium; Na – sodium; K – potassium.

## Effect on bacterial phyla

Our study found 16,494 bacterial operational taxonomic units (OTUs) affiliated to 35 phyla, 104 class, and 209 genera. The most abundant bacterial phyla (percentages of OTUs above 1%) were Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Nitrospirae, Proteobacteria, Planctomycetes and Verrumicrobia (Figure S1). However, these most abundant phyla were not influenced by CTS rates over time (Table S1). On contrary, the PRC analysis of bacterial community showed some of the less abundant phyla being influenced by the CTS rates (Figure 3). The analysis for bacterial phyla showed that 35% and 11% of all variance were explained by time and CTS rates, respectively. The canonical coefficient ($C_{dt}$) showed difference between amended and unamended soils over time, with greater difference at the highest CTS rate (NOEC = 2.5 t/ha; Table S1). Negatively, CTS rates affected phyla AD3, Tenericutes, and Verrumicrobia with weights ($b_k$) below -0.5. On the other hand, the phyla GAL15, NKB19, GN02, OP3, and WS3 were positively influenced by CTS rates with weights ($b_k$) above 1.5.

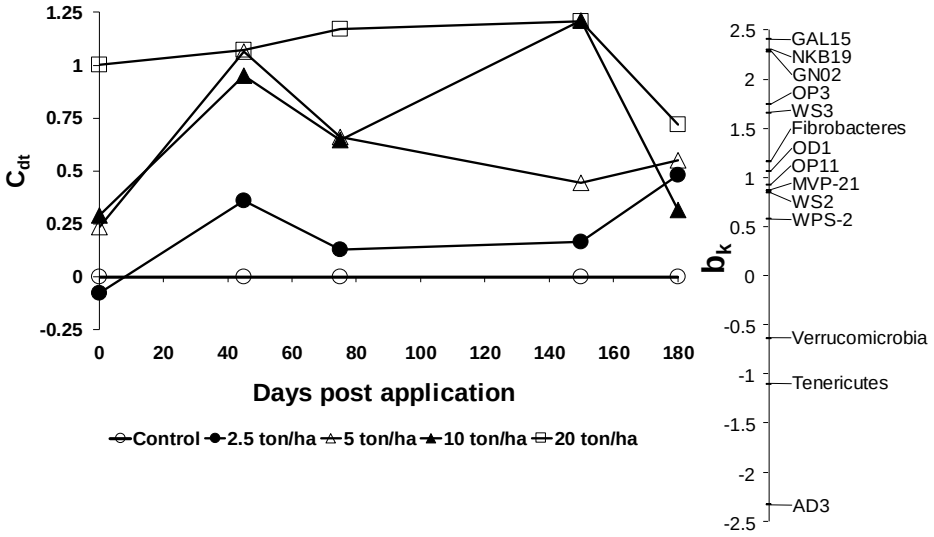


**Figure 3** Principal Response Curves between biological characteristics at Phyla level and treatments with tannery sludge (ton/ha$^{-1}$). The weight ($b_k$) can be interpreted as the affinity of the Phyla with the Principal Response Curves ($c_{dt}$).

## Effect on bacterial classes

Similarly, the PRC analysis for bacterial classes showed a significant effect of CTS rates (Fig. 4). The results showed different pattern between bacterial classes in CTS-treated soils and unamended soil, except for the lowest and highest CTS rates in different periods of sampling (NOEC = 2.5 t/ha at 45 and 150 days of sampling and NOEC > 20 t/ha at 75 and 180 days of sampling; Table S1). The classes were positively or negatively influenced by the CTS rates over time. In the analysis for classes, we only considered weight values ($b_k$) greater or lower than 1.0 and -1.0, respectively. Interestingly, the PRC for classes also showed an increase in the $C_{dt}$ at 45 and 150 days, while decreased at 75 and 180 days. For example, the class *Nitriliruptoria* (NOEC = 2.5 ton/ha based on an increase at 45 and 150 days of sampling, and NOEC = 10 ton/ha also based on an increase at 75 and 180 days of sampling) was positively influenced by CTS, while the class *JG37-AG-4* (NOEC = 5 ton/ha based on a decrease at 45 and 150 days of sampling and NOEC > 20 t/ha at 75 and 180 days of sampling) was negatively affected by CTS amendment over time.

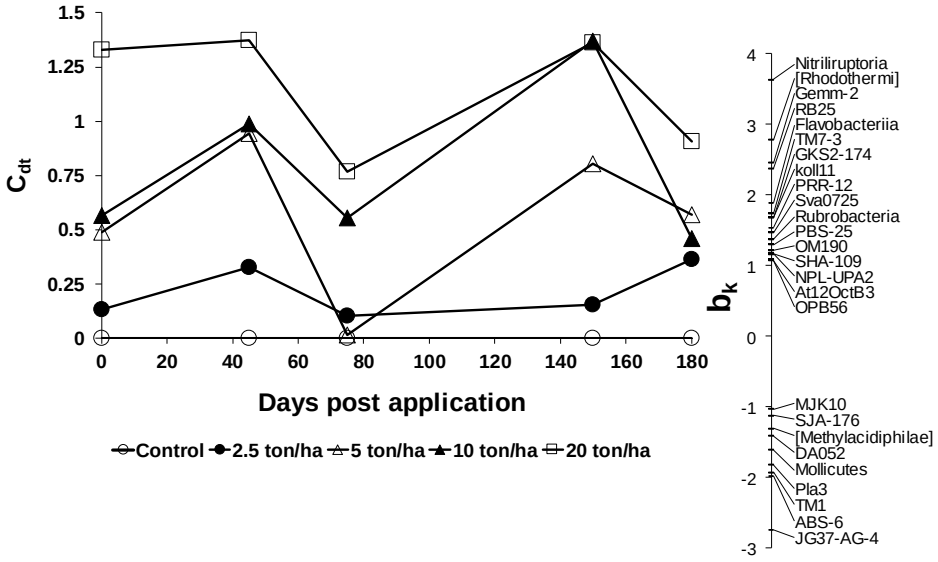


**Figure 4**. Principal Response Curve diagram of the OTU data at Class level and effects of treatments with CTS. The weight ($b_k$) can be interpreted as the affinity of the groups of Class with the Principal Response Curves ($c_{dt}$).

## Multivariate responses

The redundancy analyses (RDA) for bacterial phyla and class showed the relationship between the significant chemical properties and bacterial community of soil after CTS amendment over time (Fig. 5 and 6, respectively). These analyses showed that 27% and 25% of the variation for bacterial phyla and classes, respectively, were explained by chemical properties influenced by CTS amendment. For both phyla and classes, the results showed significant chemical variables, i.e. TOC, P, Ca, Cr and pH, clustered with the highest CTS rates (10 and 20 t/ha). For phyla, these chemical variables and the highest CTS rates clustered with Fibrobacteres, WS2, Gemmatimonadetes, GN02, NKB19, OP11, Nitrospira, GAL15, MVP-21, and OD1. In contrast, we found phyla Planctomicetes, Verrucromicrobia, Cyanobacteria, Tenericutes, TM7, [Thermi] and AD3 with a negative relationship with the chemical properties, thus clustered with the lowest CTS rate (2.5t/ha) and the unamended soil. For classes, these variables and the highest CTS rates clustered with *Nitriliruptoria, S085, Rubrobacteria, RB25, PRR12, Anaerolinea*, and *Gemm-2*.

The RDA also showed the pattern of bacterial community over time. From 0 day (without rhizospheric effect) to 180 days (rhizospheric effect of maize and cowpea) there was a shift in both phyla and classes. However, the bacterial communities associated with 45 days (flowering of maize) were separated from bacterial groups clustered with 75 days (senescence of maize). In contrast, the bacterial communities

associated with cowpea were more similar at 150 days (flowering of cowpea) and 180 days (senescence of cowpea).

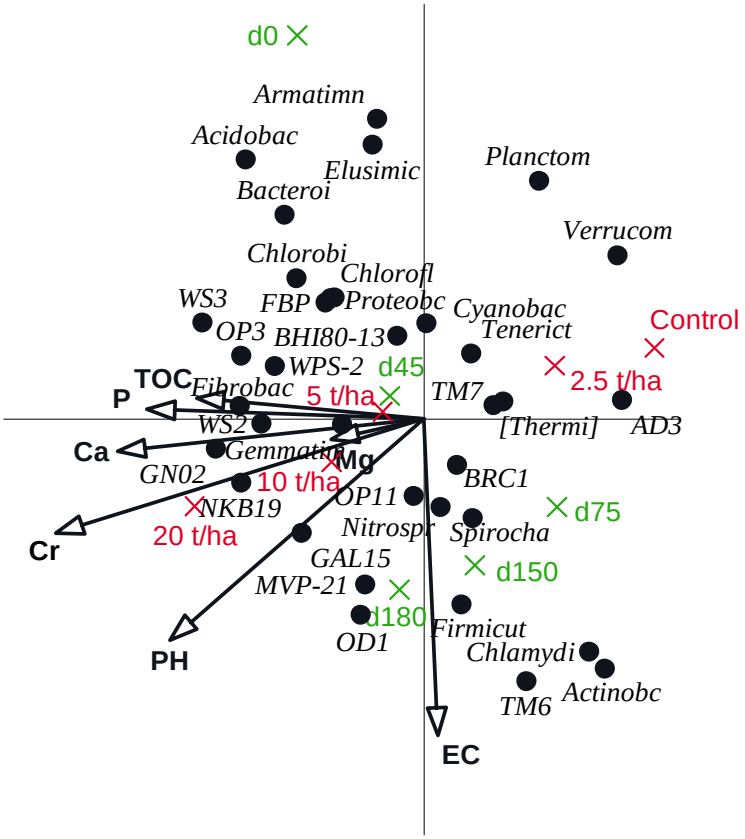


**Figure 5**. Redundancy analysis diagram (RDA) of correlations between significant physico-chemical proprieties and OUT's at the Phyla level. Physico-chemical properties were introduced as explanatory variables and explained 27% of the total variation of which 37% is displayed on the horizontal axis and another 30% on the vertical one. The variables treatment and sampling date were introduced as supplementary environmental variables. Rates of CTS (2.5, 5, 10 and 20 t/ha$^{-1}$); Time of sampling in days (0, 45, 75, 150 and 180); Total organic carbon – TOC; P – phosphorus; Ca – calcium; Cr – Chromium; pH – soil pH; EC –electric conductivity.

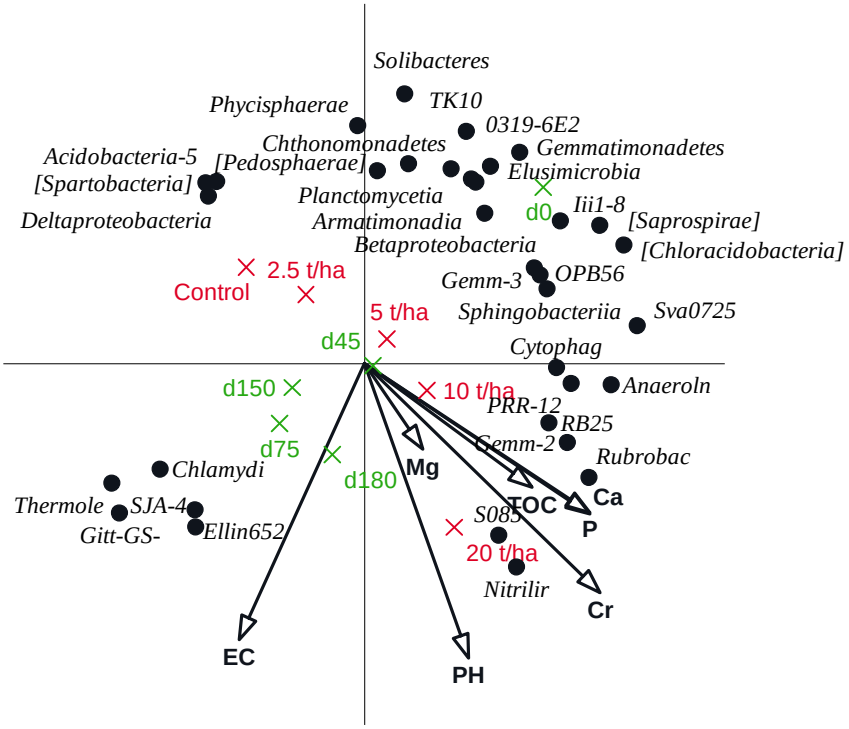


**Figure 6**. Redundancy analysis diagram (RDA) of correlations between significant physico-chemical proprieties and OUT's at the Class level. Physico-chemical properties were introduced as explanatory variables and explained 25% of the total variation of which 47% is displayed on the horizontal axis and another 24% on the vertical one. The variables treatment and sampling date were introduced as supplementary environmental variables. Rates of CTS (2.5, 5, 10 and 20 t/ha$^{-1}$); Time of sampling in days (0, 45, 75, 150 and 180); Total organic carbon – TOC; P – phosphorus; Ca – calcium; Cr – Chromium; pH – soil pH; EC –electric conductivity. For clarity only the 34 classes of which more than 30% of its variation is displayed on both axes are shown.

## Discussion

As expected, the application of CTS strongly increased some chemical variables, such as Cr, pH, Ca, P, EC, and TOC. This confirms that the chemical characteristics of CTS (Table 1) influenced the increase of these variables, mainly Cr and pH. However, there were no changes in the chemical properties over time, probably because the CTS has been applied only at the beginning of the experiment without additional amendment during 180 days.

**Table 1**. Chemical attributes of the CTS used in the experiment.

| pH | Moisture | TOC | N | P | K | Ca | Mg | Na | S | Cu | Ni | Cd | Cr | Pb |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $H_2O$ | % | ----------g kg$^{-1}$---------- | | | | | | | | ----------mg kg$^{-1}$---------- | | | | |
| 7.5 | 68 | 201 | 15 | 4.9 | 2.9 | 121 | 7.2 | 49.1 | 10 | 16 | 23 | 1.9 | 1,943 | 40 |
| MLP* | - | - | - | - | - | - | - | - | - | 200 | 70 | 3 | 150 | 180 |

* Maximum limit permitted by Brazilian regulation[44].

Cr was the main chemical element that increased more significantly and its increase with the application of CTS can be harmful to the soil and the plants[10,11]. Although the application of CTS has promoted increase of organic matter content in the soil[5] and could decrease Cr availability, the presence of this element can change the structure of the microbial community[7].

Acidobacteria and Actinobacteria were the most abundant phyla found in our study and it is in accordance with previous studies which found these phyla as most abundant in unpolluted soils[12] and also soil polluted with chromium[13]. Also, we found the phyla Bacteroidetes, Chloroflexi, and Firmicutes being more abundant than Proteobacteria, which could suggest that the application of CTS has influenced these groups to be more abundant than Proteobacteria, probably because Bacteroidetes, Chloroflexi, and Firmicutes are more resistant to the presence of metal and chemical modification in the soil.

However, these most abundant phyla were not influenced by both CTS rates and time. Interestingly, our results found the less abundant phyla being affected by CTS rates and time. Namely, phyla AD3, Tenericutes and Verrumicrobia were negatively influenced by CTS over time, which can be explained by the strict relationship between these organisms with pH, organic carbon, nitrogen and electric conductivity[14], showing greater abundance in sites with low concentrations of C and N and salinity, although AD3 showed higher abundance in soil with higher pH[15].

On the other hands, the phyla GAL15, NKB19, GN02, OP3, and WS3 were positively influenced by CTS, due positive correlation with salinity and high soil pH. This can be explained by the known adaptation of these organisms to fertile soils with low acidity, as well as their presence in residual treatment waters[14,16,17,18]. Previous studies have shown that bacterial community is influenced by the application of industrial wastes, such as tannery sludge, that change the chemical properties of soil, mainly soil pH and salinity[19], as well as the available Ca, Mg, and Mn[20]. Therefore, these changes in soil chemical properties could distribute bacterial phyla at local scales.

Although CTS is considered to be rich in chemical compounds, such as Na, Ca, Mg and K, these parameters presented in our study relative low weight within the main PRC. This can be explained by the absorption, adsorption, and leaching of the salts, promoting the reduction of the electrical conductivity in the soil[21]. Even given the

importance of the pH and temperature parameters for microbiota, salinity is considered to be a very important factor for the microbial environment, since it influences more directly the community structure than any other chemical factor in extreme values[22].

Several bacterial classes were influenced by application of CTS over time showing negative and positive effects. It confirms our first hypothesis (a), that bacterial community would change with the application of CTS over time. On the hand, the different CTS rates influenced differently the properties of soil, as discussed above, and consequently affected the bacterial groups.

On the other hands, the presence of different plant species cropped over time modified the soil environment, mainly rhizosphere that influenced the bacterial groups. Specifically, bacterial classes showed different pattern according to periods 45 and 150 days, and 75 and 180 days. It could be related to the presence of rhizosphere of different plants and their activities. The periods 45 and 150 days corresponded to flowering of maize and cowpea, while 75 and 180 days corresponded to senescence of both crops. These results showed a strong relationship between the flowering and senescence periods of these crops and the activity of the rhizosphere, and, consequently, on bacterial community.

Rhizosphere strongly influences the microbial communities directly, such as increased enzyme activity (amylase, urease, cellulase and protease), and indirectly, as an increase in soil chemical attributes[23]. Also, the rhizospheric effect varies according to plant species and stage of development[23,24]. Therefore, the pattern of increase in the abundance of bacterial classes during the flowering stages could be explained by a higher activity in the rhizosphere favouring the bacterial community[25]. In contrast, during the senescence, the plant decreases its absorption of nutrients and, thus, decrease the activity in the rhizosphere.

Although PRC diagram at bacterial class level does not show a significant difference between treatments (Figure 4), it is possible to observe a distribution of classes with a higher or lower sensitivity to CTS. As expected, some of these classes belong to those with higher or lower sensitivity phyla found in the PRC for phyla. Therefore, we found distinct bacterial groups in rhizosphere of maize during the flowering (Acidobacteria-5, [Spartobacteria], Deltaproteobacteria and Phycisphaerae) and plant senescence (Chlamydiia, Thermoleophilia, SJA-4, Giit-GS-136 and Ellin6529). On contrary, we did not find distinct bacterial groups during the flowering and senescence of cowpea. It suggests that cowpea can maintain the same bacterial

community while maize promotes a shift in bacterial groups. Therefore, different plant species present distinct rhizospheric effect and then affect the bacterial community[26]. Also, the quality and quantity of roots exudates can be determined by plant species[24] and the stages of growth that releases specific substrates and potentially antimicrobial compounds selecting specific microbial groups in the rhizosphere[23,27].

Chemical properties (pH, Cr, P, TOC, Ca, Mg and EC) showed a direct influence on the classes Nitriliruptoria and Rubrobacteria (Actinobacteria), Anaerolinea and S085 (Chloroflexi), RB25 (Acidobacteria), PRR12 (WS3) and Gemm-2 (Gemmatimonadetes), specifically, in the highest rates of CTS. It is known that these organisms, are sensitive to the presence of organic matter in the environment, presenting variable responses to the pH and availability of nutrients[12,20]. Also, Cr influenced strongly bacterial classes. Metal stress affects sensitive species[28] and decreases their competition ability, resulting in an increase in abundance of metal-resistant species capable of adapting to stress and filling up the empty niches in order to maintain ecological stability (functional redundancy)[29]. The chemical characteristics of the soil directly interfere with the behaviour and distribution of the microorganisms, both with respect to the chemical composition and the time of exposure to these factors[19,30].

In line with the second hypothesis (b), the bacterial community are influenced by change in chemical properties after application of CTS in the soil. Our study has shown that the application of CTS promoted increase in specific groups metabolically adapted to disturbed environment. It is important for selecting bacteria for bioremediation purposes. For example, Nitriliruptoria is a bacterium which is adapted to high pH values and produces enzymes able to metabolize nitriles and presenting great capability for environmental biotechnology[31]. Similarly, Rubrobacteria is found in polluted environment and could be useful for bioremediation[32]. Our study also found others important and potential groups which are observed in polluted environment. Thus, Chloroflexi were found in sites with application of the activated sludge from wastewater treatment industry[33], and Anaerolinea was found in sites contaminated with petroleum[34].

## Conclusion

There was a shifting in the bacterial community over time with the application of composted tannery sludge. Interestingly, our study has shown that the less abundant bacterial groups were more influenced by CTS than most abundant bacterial groups.

These bacterial groups were drove by soil chemical properties, mainly Cr and soil pH. Also, rhizosphere of plants presents different effects on bacterial community in soil with application of composted tannery sludge.

**Methods**

The experiment with CTS was carried out at the Agricultural Science Center from Federal University of Piauí, Brazil. The soil of the area is classified as a Fluvisol and the upper 20 cm layer contains 10% of clay, 28% of silt, and 62% of sand. The compost was produced by mixing tannery sludge with sugarcane straw and cattle manure (volume ratio 1:3:1) and the composting process was performed during 3 months. The properties of CTS were measured in the laboratory (Table 1). Water content was determined after oven drying the samples at 105 °C for 24 h, pH was directly read, and total solids were measured by drying the samples at 65 °C. The total organic C content was evaluated by dichromate oxidation of the samples under external heating. Total N content was determined using the Kjeldahl method after sulphuric acid digestion of the samples. The total Ca, Mg, K, P, S, Na, Zn, Cu, Cd, Pb, Ni, and Cr concentrations were determined by atomic absorption spectrophotometry after nitric acid digestion of the samples in a microwave oven[35].

We evaluated the application of CTS at five rates: 0 (control), 2.5, 5, 10, and 20 t/ha of CTS (dry basis). CTS treatments were applied to plots of 20 $m^2$ (four replicates) by spreading it on the soil surface and incorporating it into the 20-cm layer with a harrow. In this experiment, compost was applied 10 days before maize (*Zea mays* L.) AG 1051 sowing, and plants were grown at a density of 5 plants $m^{-1}$ (approximately 62,000 plants $ha^{-1}$) for 75 days. After this period, cowpea [*Vigna unguiculata* (L.) Walp.], cv. BRS Tumucumaque, was sowed at a density of 6 plants $m^{-1}$ (approximately 120,000 plants $ha^{-1}$) for 68 days. The plants were grown under rainfed conditions. The timeline of the experiment is shown in Figure 1.

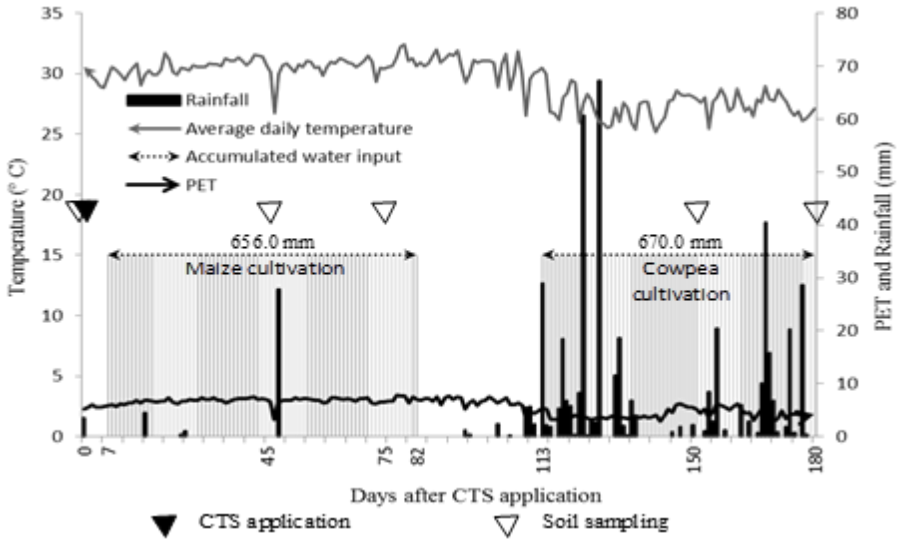


**Figure 1.** Timeline of the experiment (climatic data, CTS application, soil sampling, maize and cowpea cultivation, input of water). PET - potential evapotranspiration.

In order to measure the effect of CTS, over time, on the bacterial community, soil samples were collected from each plot at 0, 45, 75 (during maize growth), 150 and 180 (during cowpea growth) days after CTS application (from January to June 2015). Four soil samples were collected in each plot (0-20 cm), sieved (2 mm), and stored at -20 °C prior to analysis.

Soil DNA was extracted from 0.5 g (total humid weight) of the soil using the Power Lyzer Power Soil DNA Isolation Kit (MoBIO Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. The DNA extraction was performed in triplicate for each soil sample. The quality and concentration of the extracted DNA was determined using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA).

The V4 region of the 16S rRNA gene was amplified with region-specific primers (515F/806R)[36]. Each 25 µl Polymerase Chain Reaction (PCR) reaction volume contained the following: 12.25 µL of nuclease-free water (Certified Nuclease-free, Promega, Madison, WI, USA), 5.0 µL of buffer solution 5x (MgCl2 2Mm), 0.75 µL de solution of dNTP's (10 mM), 0.75 µL of each primer (515 YF 40 µM e 806 R 10 µM), 1.0 unit of *Platinum Taq polymerase* High Fidelity in concentration of 0.5 µL (Invitrogen, Carlsbad, CA, USA), and 2.0 µL of template DNA. Moreover, a control reaction was performed by adding water instead of DNA. The conditions for PCR were as follows: 95 °C for 3 min to denature the DNA, with 35 cycles at 98 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s, with a final extension of 3 min at 72 °C to ensure complete

elongation.

After indexing, the PCR products were cleaned up using Agencourt AMPure XP – PCR purification beads (Beckman Coulter, Brea, CA, USA), according to the manufacturer's manual, and quantified using the dsDNA BR assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Once quantified, equimolar concentration of each library was pooled into a single tube. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 8.0 pM with a 20% PhiX (Illumina, San Diego, CA, USA) spike for loading into the Illumina MiSeq sequencing machine (Illumina, San Diego, CA, USA).

Sequence data were processed using QIIME following the UPARSE standard pipeline according to Brazilian Microbiome Project (http://www.brmicrobiome.org/#!16s-profiling-pipeline-illumina/czxl)[37], producing an OTU table and a set of representative sequences. Briefly, the reads were truncated at 240 bp and quality-filtered using a maximum expected error value of 0.5. Pre-filtered reads were dereplicated and singletons were removed and filtered for additional chimeras using the RDP gold database using USEARCH 7.0. These sequences were clustered into OTUs at a 97% similarity cut-off following the UPARSE pipeline. After clustering, the sequences were aligned and taxonomically classified against the Greengenes database (version 13.8).

Prior to the statistical analyses, the physicochemical parameters (except pH) and the OTU's data sets were ln (Ax + 1) transformed, where x stands for the parameter value or abundance value and Ax makes 2 by taking the lowest value higher than zero for x. It was done in order to down-weigh high abundance values and approximates a normal distribution of the data (for rationale see[38]).

No observed effect concentrations (NOECs) were calculated for all physicochemical parameters and OTU's separately (Table S1). Effects were considered to be consistent when they showed statistically significant deviations pointing in the same direction for at least two consecutive sampling days. The NOEC calculations were performed by using the Williams test[39], which assumes a monotonic increasing effect with increasing exposure dose. The Williams tests were performed with the Community Analysis computer program, version 4.3.05[40], using a significance level of 0.05.

The physicochemical and OTUs data sets at level phyla and class were analysed by the principal response curve (PRC) method[41] to show and test temporal

changes in bacterial community and chemical properties of the soil caused by different rates of CTS as compared with those in the control (CTS-free soil) and also to quantify the contribution of each property to separate the treatments from the control. The PRC method is a multivariate technique which is based on the redundancy analysis ordination technique and was performed using the CANOCO Software package, version 5[43].

The PRC analysis results in a diagram showing time on the x-axis and the first principal component of the treatment effects on the y-axis, herewith showing the difference in the composition of the microbial and chemical properties of the treatments compared to the controls as they develop over time[41]. The overall significance of the tannery sludge treatment regime on the variation in species composition ($p \leq 0.05$) was tested by performing 999 Monte Carlo permutations[41]. The NOECs of the tannery sludge treatment per sampling date was calculated by applying the Williams test to the sample scores of the first principal component of each sampling date (for rationale see[43]).

Lastly to show the correlations between the treatment, time, physico-chemical parameters and the OTU's, an RDA was performed for the OTU's at the phyla and class level separately using the OTU's as response variables, physico-chemical parameters as explanatory variables and time and treatment level as supplementary environmental variables. Only physico-chemicals explaining a significant part of the variation in OTU's between the samples were included. This was tested by performing Monte Carlo permutation tests (999 permutations) including the single physico-chemical parameters as explanatory variable, sampling date as covariable and by permuting within covariables only.

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## Additional Information

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**Author contributions statement**
ARLM, JELA and WMB conducted the experiment(s) and analysed the data. ASFA and FFA designed the experiment. VMMM, ASFA and PJVB analysed the results, discussed, wrote and reviewed the manuscript.

List of figures

**Figure 1.** Timeline of the experiment (climatic data, CTS application, soil sampling, maize and cowpea cultivation, input of water). PET - potential evapotranspiration.

**Figure 2** Principal response curve diagram of physico-chemical data set indicating the effects of the composted tannery sludge (CTS) into the soil. The lines represent the course of the treatment levels in time. The weight ($b_k$) can be interpreted as the affinity of the variable with the Principal Response Curves ($c_{dt}$). Cr – chromium; pH – soil pH; Ca – calcium; P – phosphorus; EC –electric conductivity; TOC - total organic carbon; Mg – magnesium; Na – sodium; K – potassium.

**Figure 3** Principal Response Curves between biological characteristics at Phyla level and treatments with tannery sludge (ton/ha$^{-1}$). The weight ($b_k$) can be interpreted as the affinity of the Phyla with the Principal Response Curves ($c_{dt}$).

**Figure 4**. Principal Response Curve diagram of the OTU data at Class level and effects of treatments with CTS. The weight ($b_k$) can be interpreted as the affinity of the groups of Class with the Principal Response Curves ($c_{dt}$).

**Figure 5**. Redundancy analysis diagram (RDA) of correlations between significant physico-chemical proprieties and OUT's at the Phyla level. Physico-chemical properties were introduced as explanatory variables and explained 27% of the total variation of which 37% is displayed on the horizontal axis and another 30% on the vertical one. The variables treatment and sampling date were introduced as supplementary environmental variables. Rates of CTS (2.5, 5, 10 and 20 t/ha$^{-1}$); Time of sampling in days (0, 45, 75, 150 and 180); Total organic carbon – TOC; P – phosphorus; Ca – calcium; Cr – Chromium; pH – soil pH; EC –electric conductivity.

**Figure 6**. Redundancy analysis diagram (RDA) of correlations between significant physico-chemical proprieties and OUT's at the Class level. Physico-chemical properties were introduced as explanatory variables and explained 25% of the total variation of which 47% is displayed on the horizontal axis and another 24% on the vertical one. The variables treatment and sampling date were introduced as supplementary environmental variables. Rates of CTS (2.5, 5, 10 and 20 t/ha$^{-1}$); Time of sampling in days (0, 45, 75, 150 and 180); Total organic carbon – TOC; P – phosphorus; Ca – calcium; Cr – Chromium; pH – soil pH; EC –electric conductivity. For clarity only the 34 classes of which more than 30% of its variation is displayed on both axes are shown.

**Table S1**. OTU's and physico-chemical parameters showing consistent (two consecutive sampling dates) NOEC values. NOEC values are provided in ton/ha and the sign indicates the direction of the effect.

| **I2 data set** | | | | | |
|---|---|---|---|---|---|
| **Day** | **0** | **45** | **75** | **150** | **180** |
| All OTU's (PCA) | 10 | 2.5 | 2.5 | 5 | > 20 |
| Acidobacteria | > 20 | > 20 | 5+ | 2.5+ | > 20 |
| Chlamydiae | > 20 | > 20 | 10- | 10- | 10- |
| Fibrobacteres | > 20 | 2.5+ | 10+ | > 20 | 0+ |
| OP3 | > 20 | 0+ | 2.5+ | 5+ | > 20 |
| Planctomycetes | 5- | 5- | 0- | > 20 | 2.5- |
| Verrucomicrobia | 5- | > 20 | 10- | 2.5- | 0- |
| WS3 | 5+ | 2.5+ | 2.5+ | 5+ | > 20 |
| **I3 data set** | | | | | |
| **Day** | **0** | **45** | **75** | **150** | **180** |
| All OTU's (PCA) | 10 | 2.5 | > 20 | 2.5 | > 20 |
| *JG37-AG-4* | 0- | 5- | > 20 | 5- | > 20 |
| *Acidobacteria-5* | 5- | 5- | > 20 | 5- | 2.5- |
| *Acidobacteria-6* | 5+ | > 20 | 2.5+ | 2.5+ | > 20 |
| *RB25* | > 20 | > 20 | 0+ | 2.5+ | 0+ |
| *Solibacteres* | 10- | 5- | 2.5- | 10- | 5- |
| *Sva0725* | > 20 | 0+ | > 20 | 0+ | 0+ |
| *[Chloracidobacteria]* | > 20 | 2.5+ | > 20 | 5+ | 10+ |
| *iii1-8* | > 20 | > 20 | 5+ | 0+ | > 20 |
| *MB-A2-108* | > 20 | 2.5+ | 0+ | > 20 | > 20 |
| *Nitriliruptoria* | 2.5+ | 2.5+ | 10+ | 2.5+ | 10+ |
| *Rubrobacteria* | 5+ | 10+ | 2.5+ | 10+ | 2.5+ |
| *At12OctB3* | 0+ | 2.5+ | > 20 | > 20 | > 20 |
| *Cytophagia* | 10+ | 10+ | > 20 | 5+ | > 20 |
| *Chlamydiia* | > 20 | > 20 | 10- | 10- | 10- |
| *BSV26* | 10- | 10- | > 20 | > 20 | > 20 |
| *Anaerolineae* | 10+ | 10+ | > 20 | 5+ | 0+ |
| *C0119* | > 20 | 10- | > 20 | 10- | 5- |
| *Ktedonobacteria* | 10- | 2.5- | 2.5- | > 20 | > 20 |
| *S085* | 0+ | 2.5+ | 2.5+ | 2.5+ | > 20 |
| *TK17* | 10+ | 2.5+ | > 20 | 5+ | > 20 |
| *4C0d-2* | 5- | 5- | > 20 | > 20 | > 20 |
| *Fibrobacteria* | > 20 | 2.5+ | 10+ | > 20 | 0+ |
| *Clostridia* | > 20 | > 20 | 10+ | 5+ | 2.5+ |
| *Gemm-2* | 5+ | 0+ | > 20 | 10+ | 2.5+ |
| *Gemm-5* | 0+ | 2.5+ | > 20 | > 20 | > 20 |
| *koll11* | > 20 | 0+ | 5+ | > 20 | > 20 |
| *BD7-11* | > 20 | 10- | 10- | > 20 | 2.5- |
| *OM190* | 10+ | 2.5+ | > 20 | 0+ | > 20 |
| *Phycisphaerae* | 5- | 5- | 0- | 0- | 5- |
| *Planctomycetia* | > 20 | 5- | 10- | > 20 | > 20 |

| | | | | | |
|---|---|---|---|---|---|
| *Deltaproteobacteria* | 10- | 0- | 0- | > 20 | > 20 |
| *Gammaproteobacteria* | 10+ | > 20 | > 20 | 5+ | 0+ |
| *[Pedosphaerae]* | > 20 | 5- | 10- | 5- | 5- |
| *[Spartobacteria]* | 5- | > 20 | 10- | 2.5- | 0- |
| *PRR-12* | 5+ | 2.5+ | 2.5+ | 5+ | > 20 |
| **Physico-chemical data set** | | | | | |
| **Day** | **0** | **45** | **75** | **150** | **180** |
| PCA | 0 | 0 | 0 | 0 | 0 |
| pH | 0+ | 2.5+ | 2.5+ | 0+ | 0+ |
| EC | 10+ | 0+ | 10+ | > 20 | > 20 |
| TOC | 2.5+ | 2.5+ | 2.5+ | 10+ | 2.5+ |
| P | 5+ | 5+ | 10+ | 5+ | 2.5+ |
| Ca | 2.5+ | 0+ | 0+ | 2.5+ | 2.5+ |
| Mg | 2.5+ | 2.5+ | > 20 | 0+ | 2.5+ |
| Na | > 20 | 5+ | > 20 | 0+ | 0+ |
| Cr | 0+ | 0+ | 0+ | 0+ | 0+ |

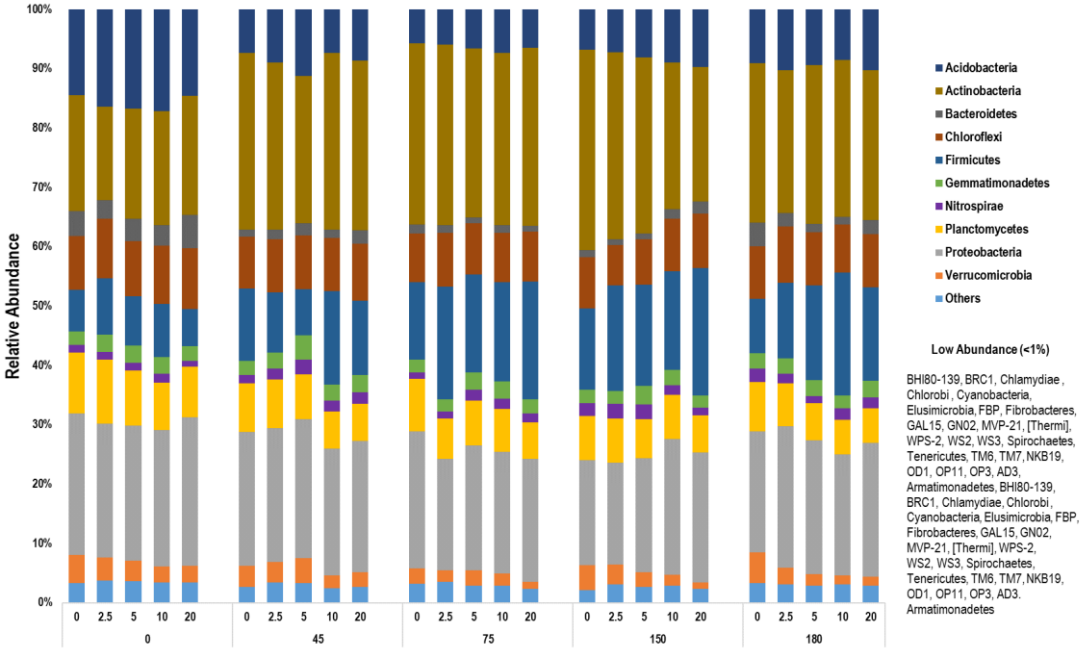


**Fig. S1.** Taxonomy at the Phyla level above 1% of relative abundance for all time and treatments after seventh years application of the composted tannery sludge (CTS). The numbers 0, 45, 75, 150 and 180 are time sampling (days) after application of the CTS and the numbers 0, 2.5, 5, 10 and 20 are rates of the CTS (Mg $ha^{-1}$).