JOSIANE FERREIRA PIRES

MICRORGANISMS SELECTION FOR PRODUCTION OF MIXED INOCULUM FOR DEPURATION OF WASTEWATER FROM COFFEE PROCESSING.

LAVRAS - MG

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Profa. Dra. Cristina Ferreira Silva e Batista

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Pires, Josiane Ferreira.
Microrganisms selection for production of mixedinoculum for depuration of wastewater from coffee processing / Josiane Ferreira Pires. - 2017.
136 p. : il.

Orientador(a): Cristina Ferreira Silva.

Tese (Doutorado) - Universidade Federal de Lavras, 2017.
Bibliografia.

1. Microbiologia. 2. Tratamento biológico. 3. Água residuária do processamento do café. I. Silva, Cristina Ferreira. II. Título.

JOSIANE FERREIRA PIRES

SELEÇÃO DE MICRORGANISMOS PARA ELABORAÇÃO DE INÓCULO MISTO PARA DEPURAÇÃO DA ÁGUA RESIDUÁRIA DO PROCESSAMENTO DO CAFÉ.

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APROVADA em 09 de março de 2017.
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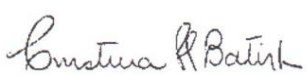

Dra. Cristina Ferreira Silva e Batista
Orientadora

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Aos meus pais Mariana e João pelo apoio e suporte as minhas escolhas, ao longo de todos os meus anos de formação acadêmica, mesmo que nem sempre entendessem ou concordassem. Meus exemplos de amor.
Dedico.

Agradecimentos

À Universidade Federal de Lavras, especialmente ao Departamento de Biologia e ao Programa de Pós-Graduação em Microbiologia Agrícola, pela oportunidade.

À CAPES, pela concessão da bolsa de doutorado. À FAPEMIG e ao CNPq.

À Fazenda Daterra, pela parceria e financiamento parcial da pesquisa.

À Fazenda Resfriado, por permitir a coleta de amostras de água residuária para minha pesquisa.

À professora Cristina Ferreira Silva e Batista, pelo suporte, dedicação e orientação no sentido mais amplo dessa palavra. Especialmente por acreditar nas ideias, no trabalho e em minha capacidade. Serei eternamente grata!

À professora Rosane Freitas Schwan, pela disposição para ajudar e contribuições ao trabalho.

Aos demais professores do programa de Pós-Graduação em Microbiologia Agrícola pelos ensinamentos.

Aos funcionários do DBI, especialmente Rose, Cidinha, Ivani, Jajá e Paulinho pelo auxílio e paciência.

A família NEMAI e aos colegas da microbiologia, pela amizade, auxílio nos momentos de necessidade, discussões científicas, companhia, conversas e bolos nos aniversários.

Ao Wesley e à Larissa, pelo auxílio no desenvolvimento do projeto e ao Gustavo Magno pelas consultas estatísticas.

À Gabriela e à Nara por todo auxílio em experimentos e disciplinas, pela hospedagem e amizade.

À Maísa e ao Gustavo Guimarães por todo suporte e ajuda nos experimentos.

À minha família, pelo apoio e carinho! Minha mãe Mariana e meu pai João, pelo amor incondicional e por suportarem minha ausência. A minha irmã Marinês, por ser exemplo de determinação e pelo companheirismo. Ao meu cunhado Jean, pelo incentivo, conversas e paciência. À Malu por ser meu alento.

Ao Marco Túlio pelas consultorias estatísticas e traduções, e principalmente por seu meu companheiro, pelo amor e apoio.

Muito obrigada!!

RESUMO

Técnicas de processamento com utilização de agua podem melhorar a qualidade do café, porém geram um grande volume de agua residuária, contendo elevada carga poluidora. Devido à presença de poluentes, é necessário que haja um tratamento adequado dessas aguas, antes de seu descarte no ambiente ou recirculação. O emprego do tratamento biológico com microrganismos é uma alternativa viável e eficiente para a melhoria das características do efluente. A bioaumentação de microrganismos nativos pode ser vantajosa uma vez que os microrganismos introduzidos têm capacidade de degradar compostos específicos, além de serem adaptadas as condições do ambiente. Nesse sentido, o objetivo deste trabalho foi isolar e caracterizar a microbiota presente nas aguas residuárias do processamento de grãos de café (ARC), a fim de selecionar microrganismos com capacidade de promover a redução da carga poluidora desses efluentes. Para isso, foram feitos o isolamento e a caracterização morfológica, bioquímica e do perfil proteico dos microrganismos. Observou-se maior densidade populacional de bactérias ocorrendo principalmente na presença de maior quantidade de oxigênio dissolvido ($9,9 \times 10^{11}$ UFC mL^{-1}). Foi selecionado um inóculo misto composto por *Serratia marcescens* CCMA 1010 e CCMA 1012, *Corynebacterium flavescens* CCMA 1006 *Acetobacter indonesiensis* CCMA 1002 baseando-se em análise de parâmetros biológicos de crescimento dos microrganismos, e físico químicos da ARC tratada em escala laboratorial. A depuração dos poluentes foi verificada na ARC proveniente da região de Cerrado e de Mata Atlântica. Além disso, o tratamento biológico promovido pelo inóculo misto selecionado foi avaliado na ARC em condições de campo, em um protótipo da Estação de Tratamento de Águas Residuárias (ETAR), em uma fazenda produtora de café, no município de Patrocínio, Minas Gerais. A inoculação de microrganismos apresentou redução de 85,46% na DBO e de 83,05% na DQO da ARC em laboratório e de 33% na BOD e 25% na DQO na ETAR piloto em condições de campo. Redução nos teores de açúcares e alguns ácidos, bem como na toxicidade também foram observadas tanto em laboratório quanto na ETAR piloto.

Palavras chave: Efluente. Isolamento. Tratamento biológico. Bioaumentação.

ABSTRACT

Processing techniques with water use can improve the quality of coffee, but generate a large volume of wastewater containing high pollutant load. Due to the presence of pollutants, it is necessary to have an adequate treatment of these waters, before their disposal in the environment or recirculation. The use of biological treatment with microorganisms is a viable and efficient alternative for the improvement of effluent characteristics. Bioremediation of native microorganisms may be advantageous since the introduced microorganisms have the ability to degrade specific compounds, in addition to the adaptation of environmental conditions. In this sense, the objective of this work was to isolate and characterize the microbiota present in wastewater from coffee bean processing (WP), in order to select microorganisms capable to promote the reduction of the pollutant load of these effluents. Thus, the isolation and characterization of morphological, biochemical and protein profile of the microorganisms were made. It was observed a higher population density of bacteria occurring mainly in the presence of greater amount of dissolved oxygen (9.9×10^{11} CFU mL^{-1}). A mixed inoculum composed of *Serratia marcescens* CCMA 1010 and CCMA 1012, *Corynebacterium flavescens* CCMA 1006 *Acetobacter indonesiensis* CCMA 1002 was selected based on analysis of biological parameters of growth of the microorganisms, and chemical physicists of the WP treated in laboratory scale. The clearance of the pollutants was verified in the WP from the Brazilian Cerrado and Atlantic Forest regions. In addition, the biological treatment promoted by the selected mixed inoculum was evaluated in the ARC under field conditions in a prototype of the Wastewater Treatment Plant (WTP) at a coffee producing farm in the municipality of Patrocínio, Minas Gerais, Brazil. The inoculation of microorganisms showed removal of 85.46% in the BOD and 83.05% in the COD of the WP in the laboratory and 33% in the BOD and 25% in the COD in the pilot WTP under field conditions. Reduction in sugars and some acids as well as in toxicity were also observed both in the laboratory and in the pilot WTP.

Key words: Effluent. Isolation. Biological treatment. Bioaugmentation.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Cerca de 60 países tropicais e subtropicais produzem café extensivamente, sendo para alguns deles, o principal produto de exportação agrícola (VIEIRA, 2008). O Brasil está entre os países onde o café é um dos produtos de notória importância e expressividade para a economia (FAO, 2014). A importância econômica do café deve-se principalmente à sua bebida, uma infusão preparada a partir dos grãos torrados e moídos (BERTRAND et al., 2003).

Diante da crescente exigência do mercado, a busca por qualidade tornou-se uma das maiores preocupações nos diversos segmentos produtivos e, em especial, no agronegócio cafeeiro (EMBRAPA, 2014). A qualidade do grão de café determina seu valor comercial e sua aceitação no mercado internacional (CAMPOS; PRADO; PEREIRA, 2010). Parte das características finais dos grãos são conferidas pela forma que ocorre a fermentação espontânea, após a colheita. A fermentação pode dar-se pelo processamento por via natural ou via úmida, ou uma combinação de ambos, o que é chamado de processamento semi-seco (ESQUIVEL e JIMÉNEZ, 2012; VILELA et al., 2010). Nos últimos anos, observa-se no Brasil uma tendência dos produtores por optarem pelo processamento semi-seco (BRUNO e OLIVEIRA, 2008), com maior utilização de água em relação a via natural.

A atividade de lavagem e despolpa de frutos do cafeeiro agrega valor ao produto, no entanto, é gerador de grandes volumes de águas residuárias, ricas em material orgânico em suspensão, constituintes orgânicos e inorgânicos em solução (MATOS e LO MONACO, 2003), tais como açucares, proteínas, pectinas, celulose, pequenas quantidades de corantes naturais e lipídeos (DIAS et al., 2014). Devido ao alto poder poluente que estas águas detêm, é necessário que haja, primeiramente, tratamento para o descarte ou sua reutilização adequada, atendendo às normas do órgão regularizador, responsável pelo meio ambiente, de modo a evitar danos à saúde humana e ao ambiente (MATOS, 2003).

As tecnologias convencionais de tratamento de efluentes comumente adotadas em países industrializados apresentam custos elevados para

construção, operação e manutenção (MAZUMDER e ROY, 2000). Além disso, para recuperar as águas residuarias, em conformidade com as normas ambientais, de forma a restaurar um ambiente seguro, tornou-se necessário encontrar tecnologias de tratamento inovadoras, que sejam menos onerosas e facilmente adaptáveis para esses efluentes (DEVI; SINGH; KUMAR, 2008).

Os microrganismos desempenham importante papel na degradação de matéria orgânica na natureza, exercendo participação fundamental na ciclagem de nutrientes. Devido a tais características, são os principais agentes dos chamados processos biológicos de tratamento de efluentes/resíduos de diferentes origens, incluindo efluentes agroindustriais, e asseguram a degradação de inúmeros poluentes (SANT'ANNA, 2013).

Dentre as diferentes técnicas que utilizam microrganismos para a depuração dos poluentes, o bioaumento tem sido aplicado com sucesso em uma variedade de ambientes, incluindo sistemas de tratamento de águas residuarias (BATHE et al., 2009; LORAH e VOYTEK, 2004; MA et al., 2009; IASUR-KRUH et al., 2011; ZHOU e GOUGH, 2016). Esta técnica consiste na introdução de microrganismos degradadores específicos para determinado ambiente contaminado, a fim de aumentar a taxa de degradação (MORIKAWA, 2006).

Diversos microrganismos são capazes de atuar em processos de biorremediação (GOMAA et al. 2011), no entanto, populações de microrganismos nativos, são certamente melhor adaptadas às condições climáticas, físico-químicas e de nutrientes (SEMPLE et al., 2007). Culturas puras ou consórcios microbianos podem ser introduzidos no sistema de tratamento e a seleção do inoculo deve considerar a natureza e a complexidade do contaminante a ser tratado (substrato) (SABRA et al., 2010). Normalmente, a diversidade microbiana é benéfica para o tratamento biológico (MILITON et al., 2015) e poluentes podem ser completamente degradados devido ao efeito sinérgico da composição da comunidade (Meng et al., 2015). Além disso, as bactérias despertam grande interesse, uma vez que formam o grupo mais abundante e atuante em sistemas de tratamento biológico (SANT'ANNA, 2013).

Apesar do grande volume de água residuária gerado durante o processamento de café (MATOS; CABANELLAS; BRASIL, 2006), e da

importância dos microrganismos para o seu tratamento biológico, pesquisas visando conhecer a microbiota associada ao efluente e o seu potencial para a depuração dos poluentes, são escassas. Estudos dessa natureza, além de inovadores, atuam na manutenção da sustentabilidade nos processos de produção em fazendas cafeeiras, uma vez que promovem benefícios econômicos, sociais e ambientais, que são os três pilares da sustentabilidade (IOC, 2006).

2 REFERENCIAL TEÓRICO

2.1 O café no Brasil e em Minas Gerais

O café chegou no Brasil e teve sua cultura iniciada em 1727, no Pará. Ao longo dos anos, a cultura foi disseminada pelo país, alcançando atualmente, praticamente todo o território nacional (SEDIYAM et al., 2001).

O Brasil é o maior produtor de café do mundo, sendo responsável por cerca de 35% da produção mundial (CONAB, 2015). Além disso, o café é um dos principais produtos agrícolas da pauta de exportações e comércio do país. A área total utilizada para a produção de café no país em 2016 foi 2,3 milhões de hectares (arabica e conilon) (CONAB, 2017).

O café conilon ocupa uma área de 450,84 mil hectares situadas principalmente nos estados do Espirito Santo, Bahia e Rondonia. A produção estimada entre 8,64 e 9,63 milhões de sacas em 2017 representa 20% da produção total de café do país.

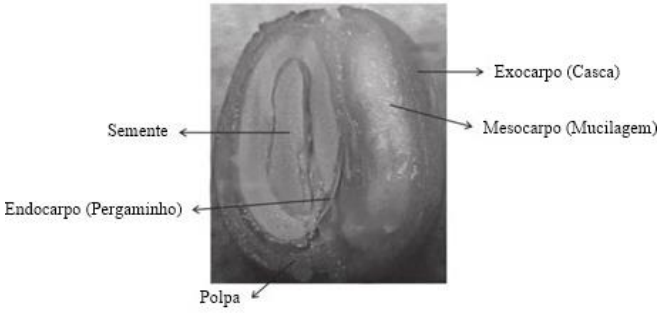
O café arabica é cultivado e produzido em maior extensão no Brasil, representando os outros 80% da produção total de café do país. Ocupa 1,78 milhões hectares, o que corresponde a 79,9% da área cultivada com lavouras de café. Para 2017 estima-se que sejam colhidas entre 35,01 e 37,88 milhões sacas (CONAB, 2017).

O cultivo de café arabica estende-se por todas as regiões do Brasil e Minas Gerais concentra a maior área plantada, com 1.190,6 mil hectares. A produção de café em Minas Gerais está estimada em entre 35,01 e 37,88 milhões sacas em 2017 (CONAB, 2017).

2.2 O Fruto do Café: Processamento

O fruto do café é composto por pericarpo e semente. O pericarpo é o conjunto de estruturas que envolvem a semente. No café, ele pode ser claramente diferenciado em exocarpo, mesocarpo e endocarpo (Figura 1) (SILVA, 2014).

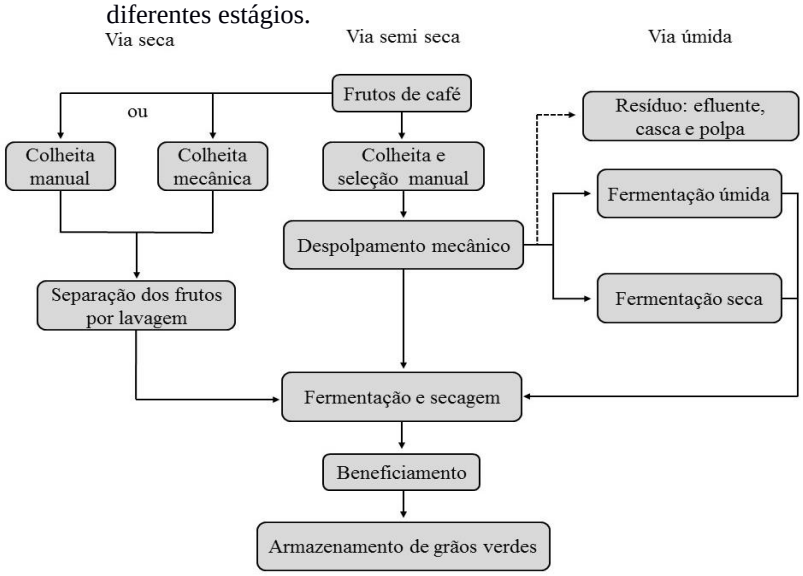
Figura 1 - Estrutura do fruto do cafeeiro *Coffea arabica L*.



Fonte: Adaptado de Silva (2014).

Frutos maduros de café podem ser processados por três métodos distintos (Figura 2). O processo mais simples e rústico é o processamento natural, no qual os frutos na sua forma natural são fermentados e secos ao sol, em terreiros. No processamento por via úmida, a polpa é removida mecanicamente, os grãos são fermentados em tanques com um grande volume de água para remoção da mucilagem e, posteriormente levados para secagem, geralmente em terreiro suspenso (TARZIA; SCHOLZ; PETKOWICZ, 2010). O processo semi-seco é uma variação do método por via úmida, em que os frutos de café são mecanicamente despolpados, porém são diretamente levados para plataforma de secagem onde ocorre a fermentação (VILELA et al., 2010).

Figura 2 - Diagrama de fluxo representando os três diferentes tipos de processamento dos grãos de café – seco, semi-seco e úmido – e seus diferentes estágios.



Fonte: Adaptado de Schwan et al. (2014).

2.3 O Processamento de Café por via semi seca

Na busca por qualidade dos grãos de café, é crescente a opção dos cafeicultores pelo processamento por via semi seca, que além de alcançar preço diferenciado no mercado, reduz o tempo de secagem e os riscos da ocorrência de fermentações indesejáveis (BRUNO e OLIVEIRA, 2008).

Durante o processamento, os frutos de café passam pelo lavador, onde os secos e "passas" flutuam e são separados - café boia - enquanto os verdes e cerejas são direcionados ao descascador, onde os frutos cereja são descascados e separados dos frutos verdes. O café cereja descascado pode então passar pelo desmucilador onde a mucilagem é retirada dos grãos (EMBRAPA, 2014).

Pela via semi seca, são retirados a casca e o mesocarpo externo dos frutos, que são encaminhados para tanques com água para remoção da mucilagem aderida ao pergaminho, e posteriormente para a secagem. Este processo leva à eliminação da parte externa do fruto, que representa 20% do café cereja e contém cerca de 60% da água, que não é aproveitado pelo cafeicultor (ARRUDA et al., 2012).

O grão de café despolpado tem a vantagem de proporcionar considerável diminuição da área do terreiro de secagem, que pode chegar a uma redução de 40%, além da redução do tempo (BARTHOLO e GUIMARÃES, 1997) e consumo energético na secagem, já que os grãos despolpados apresentam relativamente baixo teor de umidade, em torno de 50%, quando comparados com o fruto inteiro (BORÉM, 2008).

Além disso, as bebidas provenientes de cafés despolpados e lavados são mais suaves, o que normalmente é associado à remoção mecânica e lixiviação de alguns precursores de aroma (ARRUDA et al., 2012).

2.4 Resíduo líquido do processamento de café via semi seca

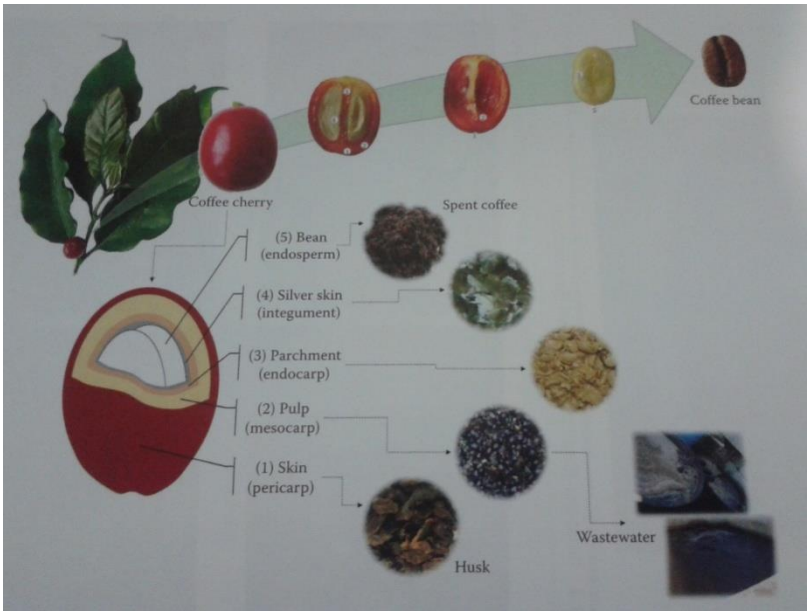
Grãos de café lavados e descascados/despolpados geram um produto com bebida diferenciada, que atinge melhores preços no mercado e, consequentemente, confere maior lucratividade aos produtores (FIA et al., 2010a). No entanto, o processamento utiliza grandes volumes de água limpa,

que é retornada ao ambiente com qualidade inferior, na forma de efluentes ricos em materiais orgânicos poluentes (MATOS et al., 2003).

As principais etapas geradoras do resíduo são a lavagem dos frutos e remoção da casca (descascamento) e da mucilagem que reveste os grãos (despolpa) (MATOS; CABANELLAS; BRASIL, 2006). Estes efluentes são referidos como as águas residuárias do processamento dos frutos do cafeeiro (ARC) (BRUNO e OLIVEIRA, 2008).

Aproximadamente 20% do café produzido no Brasil é processado com utilização de água (SOCCOL, 2002), e para cada litro de café processado são gastos quatro litros de água, que posteriormente originam os efluentes líquidos. Considerando a produção entre 43.650,1 e 47.509,8 mil sacas de 60 kg de café beneficiado (600 litros de café cereja), pode-se estimar que o volume de efluente gerado em 2017 (CONAB, 2017) será entre 14,1 e 15,4 bilhões de litros de ARC. No total, aproximadamente 200 milhões de toneladas de resíduos são gerados por ano durante o processamento dos grãos de café (DIAS et al., 2014). A Figura 3 apresenta um esquema demonstrativo das estruturas componentes do grão de café e os resíduos comumente gerados durante o processamento.

Figura 3 - Estrutura do grão de café e resíduos gerados durante o seu processamento.



Fonte: Dias et al. (2013)

As águas residuárias provenientes do descascamento possuem elevada carga orgânica em suspensão e compostos orgânicos e inorgânicos dissolvidos (MATOS e LO MONACO, 2003). Dentre os poluentes são encontrados cafeína, açúcares e compostos fenólicos, nitrogênio e potássio em altas concentrações (GONÇALVES et al., 2008).

O material sólido da água residuária encontra-se em suspensão ou dissolvido, sendo a maior parte volátil, que pode ser removido por tratamento biológico (BORÉM, 2008).

Devido à elevada carga orgânica, a água residuária não pode ser lançada em um corpo hídrico sem tratamento adequado. A degradação desses compostos na água pode causar decréscimo do teor de oxigênio dissolvido, comprometendo todo ecossistema aquático. O lançamento dessas águas no meio ambiente é uma fonte de contaminação para nascentes, rios, córregos e até mesmo ao solo. Isso faz com que seja necessário um tratamento prévio para descarte ou reutilização. Nesse sentido, vários sistemas de tratamento vêm sendo desenvolvidos (MATOS, 2008), de modo a atender às condições e padrões de lançamento de efluentes estabelecidos pela legislação.

Além disso, mais recentemente, devido à escassez e aos elevados custos, muitos produtores passaram a recircular a água no processo, como forma de diminuir o volume consumido. O gasto de água pode diminuir para aproximadamente um litro de agua por litro de fruto processado, com o reaproveitamento. Cerca de um quarto do que é consumido quando a recirculação não é feita (MATOS et al., 2007).

A qualidade da água em uso, no entanto, vai decaindo ao longo do tempo de recirculação no sistema, devido ao acúmulo de matéria orgânica, podendo, assim, comprometer a qualidade final do café processado. Outro agravante é que o aumento na concentração de poluentes nestas águas pode também dificultar seu tratamento, antes do lançamento no ambiente (FIA et al., 2010a).

São poucas as pesquisas sobre a melhor forma de tratamento dos efluentes provenientes da lavagem e descascamento/despolpa dos frutos de café, para sua reutilização no processamento. Dessa forma, a realização de estudos com intuito de disponibilizar técnicas de tratamento que possam ser

utilizadas para melhoria das características da água em recirculação passaram a ser necessários (FIA et al., 2010b).

Além disso, o desenvolvimento de técnicas visando o tratamento do efluente gerado é uma atividade nomeada “ecofriendly”, uma das melhores tentativas em direção a práticas de produção sustentáveis que atendam a uma série de necessidades ambientais, sociais e econômicas, vitais de quase um milhão de produtores de café (OIC, 2015).

2.5 Tratamento das águas residuárias do café

As tecnologias convencionais de tratamento de efluentes adotadas em países industrializados são bastante caras relativo à construção, operação e manutenção das Estações de Tratamento de águas Residuárias (ETARs) (MAZUMDER e ROY, 2000). Para manter a conformidade com as normas ambientais rigorosas e para a restauração de ambiente seguro, tornou-se imperativo encontrar tecnologias de tratamento menos onerosas e facilmente adaptáveis para o efluente (DEVI; SINGH e KUMAR, 2008).

Uma alternativa interessante é o desenvolvimento de métodos de tratamento biológico de águas residuárias, com o objetivo de gerir comunidades microbianas, promovendo a degradação de compostos orgânicos a transformação de substâncias tóxicas, e a remoção de nutrientes da água (WELLS et al., 2011), de forma a atender as exigências da legislação e de mercado.

As características da ARC são variáveis, dependendo da espécie de cafeeiro (MATOS et al., 2003); das características dos frutos processados, se verdes, maduros ou secos, dos tratos culturais utilizados na lavoura, entre eles a adubação e tratos fitossanitários; do tipo de processamento utilizado, e da recirculação ou não da água pelo sistema de processamento (FIA et al., 2010b). A ARC apresenta uma concentração elevada de matéria orgânica e nutrientes como nitrogênio e fosforo (FIA et al., 2010a). Além disso, a ARC apresenta valores de pH reduzidos, e sua correção favorece a aplicação de tratamento biológico. Quando se faz a recirculação da água no processamento, o volume gasto é reduzido (MATOS et al., 2006), no entanto, a ARC produzida apresenta maior concentração de matéria orgânica

e compostos recalcitrantes, dificultando o processo de tratamento (MATOS et al., 2007).

2.6 Tratamento biológico

O tratamento biológico utiliza organismos vivos, normalmente plantas ou microrganismos, para controlar a poluição e restaurar a qualidade ambiental por meio da degradação ou absorção de poluentes (SILVA e ESPOSITO, 2010). Este processo de remediação tem sido intensamente pesquisado, como alternativa viável para o tratamento de ambientes contaminados, tais como águas superficiais, águas subterrâneas e solos, além de resíduos industriais em aterros ou áreas de contenção (GAYLARDE; BELLINASO; MANFIO, 2005).

No tratamento biológico com microrganismos, podem ser empregados fungos, leveduras, bactérias, algas ou suas enzimas para remover compostos poluentes de forma que não ofereçam riscos de contaminação (GAYLARDE; BELLINASO; MANFIO, 2005). É uma metodologia atrativa e considerada muito eficiente, além de econômica, versátil e principalmente por causar menor perturbação ao ambiente (FORGACS; CSERHATI; OROS, 2004).

Considerado como um dos principais processos naturais de remoção das várias frações de poluentes do meio ambiente, o tratamento biológico utiliza a capacidade dos microrganismos em degradar substâncias orgânicas, produzindo CO_2 e água através da mineralização (HASSANSHAHIAN et al., 2014).

O tratamento biológico da água residuária do café com microrganismos já é relatado na literatura há algumas décadas (KIDA; IKBAL; SONODA, 1992). Embora a maioria dos trabalhos utilizem técnicas de tratamento anaeróbico (SELVAMURUGAN; DORAISAMY; MAHESWARI, 2010; SELVAMURUGAN et al., 2010; CAMPOS; PRADO; PEREIRA, 2014), bons resultados já foram descritos para remoção de matéria orgânica da água residuária do café utilizando processos microbianos aeróbios em reatores em batelada (Sequencing batch reactors (SBR)) (MAHESH; SRIKANTHA; LOBO, 2014; VILLA-MONTOYA;

FERRO; DE OLIVEIRA, 2016). Além disso, em seus trabalhos, Junior et al. (2014) e Matos, Júnior e De Matos (2015) demonstraram a eficiência de processos de aeração para auxiliar no tratamento da ARC, por facilitar a oxidação do material orgânico por micro-organismos aeróbios.

Uma das principais técnicas para tratamento biológico com microrganismos que pode ser usada no processo de depuração dos poluentes *in situ* (no local contaminado) é o bioaumento (HASSANSHAHIAN et al., 2014).

2.7 Bioaumento

O bioaumento é definido como uma técnica que promove a adição de uma cultura de microrganismos pré-crescidos, para realizar uma tarefa de remediação específica em um dado local no ambiente (MANCERA-LÓPEZ et al., 2007), ou seja, misturas específicas de microrganismos são introduzidas em um local contaminado ou em um biorreator para iniciar o tratamento biológico. Tem como objetivo adicionar microrganismos, pertencentes ou não à comunidade natural, crescidos em laboratório e com capacidade para degradar o contaminante e removê-lo (WIDADA et al., 2002).

A introdução de uma população de microrganismos isolados do local afetado pela contaminação é uma forma de otimizar os efeitos do bioestimulo (XIN et al., 2013), uma vez tais microrganismos que são melhor adaptados ao meio e mais resistentes a variações de condições ambientais locais, além de serem menos susceptíveis às variações genéticas causadas pelo stress no ambiente (CERQUEIRA et al. 2012), além de não causarem danos à comunidade microbiana local (concorrência) (TRIBEDI, 2013).

3 CONCLUSÃO

Durante o processamento de café por via semi-seca, uma grande quantidade de água residuária é gerada. Esta água é rica em matéria orgânica e outros compostos poluentes, que podem causar sérios problemas para o ambiente, se lançados sem o tratamento adequado.

O tratamento biológico, com microrganismos apresenta-se como uma alternativa viável e eficiente para a depuração das águas residuárias do processamento do café, visando a redução da carga poluidora a níveis aceitáveis ou sua completa remoção. Esta técnica considera a capacidade que os microrganismos têm de degradar poluentes, uma vez que são os principais degradadores de matéria orgânica na natureza.

Os microrganismos presentes nessas águas são fortes candidatos a agentes responsáveis pela depuração de águas residuárias e merecem atenção especial, já que são melhores adaptados às condições do ambiente. Portanto, estudos visando o isolamento, identificação e caracterização dos microrganismos em questão, bem como de suas capacidades de atuar de forma eficiente em processos de remoção dos poluentes são necessários.

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SEGUNDA PARTE – ARTIGOS

Artigo 1

Dynamics of microbiota found in coffee processing wastewater treatment plant

Elaborado de acordo com as normas do periódico Environmental Microbiology.

Artigo 2

Natural microbial consortium selected from wastewater coffee processing

Elaborado de acordo com as normas do periódico Bioresource Technology.

Artigo 3

Increasing efficiency in assisted depuration of coffee processing wastewater from mixed wild microbial selected inoculum

Elaborado de acordo com as normas do periódico Journal of Industrial Ecology.

ARTIGO 1: Dynamics of microbiota found in coffee processing wastewater treatment plant

Microbiota from coffee processing wastewater

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Summary

Cultivable microbiota presents in a coffee semi-dry processing wastewater treatment plant (WTP) were identified. Thirty-three operational taxonomic units (OTUs) were detected, these being 17 bacteria, 12 yeasts and 4 filamentous fungi. Bacteria dominated the microbial population (11.61 log CFU mL^{-1}), and presented the highest total diversity index when observed in the WTP aerobic stage (Shannon = 1.94 and Simpson = 0.81). The most frequent bacterial species were *Enterobacter asburiae*, *Sphingobacterium* sp., *Chryseobacterium bovis*, *Serratia marcescens*, *Corynebacterium flavescens*, *Acetobacter orientalis* and *Acetobacter indonesiensis*; these showed the largest total bacteria populations in the WTP, with approximately 10 log CFU mL^{-1}. Yeasts were present at 7 log CFU mL^{-1} of viable cells, with *Hanseniaspora uvarum*, *Wickerhamomyces anomalus*, *Torulaspora delbrueckii, Saturnispora* sp., and *Kazachstania gamospora* being the prevalent species. Filamentous fungi were found at 6 log CFU mL^{-1}, with *Fusarium oxysporum* the most populous species. The identified species have the potential to act as a biological treatment in the WTP, and the application of them for this purpose must be better studied.

Keywords: Bacteria, Yeast, Microbial Diversity, Effluent, Agroindustry

Introduction

Wet and semi-dry coffee processing are recognized as processes that produce higher quality coffees (Brando and Brando, 2014; Dias *et al.*, 2014; Silva, 2014). *Arabica* coffee is usually processed by these methods and accounts for approximately 62% of the world coffee market, which implies that most of the wastewater generated is from the production of quality coffees (ITC, 2011; Mussatto *et al.*, 2011). During semi-dry and wet processing, large amounts of wastewater are generated (from 20 to 45 kg per kg of coffee beans) (Dias *et al.*, 2014). It is estimated that 16.6 billion L of wastewater were generated in 2016, according to International Coffee Organization (ICO) (ICO, 2016).

Wastewater from semi-dry coffee processing (WRCP) is rich in organic matter (cellulose, hemicellulose, pectin, sucrose, monosaccharides, lipids, proteins, polyphenols and vitamins), which is released during coffee pulping and mucilage removal, thereby generating high levels (45 kg/ton of coffee beans) of chemical oxygen demand (COD) (3.4 to 50,000 mg L^{-1}), biochemical oxygen demand (BOD) (1.8 to 20,000 mg L^{-1}) and pH 4.0 in the final wastewater (Matos *et al.*, 2001; Bruno and Oliveira, 2008; Haddis and Devi, 2008; Campos *et al.*, 2010; Selvamurugan *et al.*, 2010; Oller *et al.*, 2011; Ferrell and Cockerill, 2012; Bonilla-Hermosa *et al.*, 2014; Rattan *et al.*, 2015). Coffee processing wastewater also presents high levels of ammoniacal nitrogen (40 to 60 mg/L), phosphorus (60 to 800mg L^{-1}), total nitrogen (180 to 250mg L^{-1}) (Matos *et al.*, 2001; Campos *et al.*, 2010; Rattan *et al.*, 2015), total solids (1,000 to 7,500mg L^{-1}) (Campos *et al.*, 2010; Villanueva-Rodríguez *et al.*, 2014) and residues of different fertilizers that usually contain potassium, nitrogen and phosphoric acid, used in agricultural practices (FAO, 2000). All of these characteristics classify coffee processing wastewater as highly pollutant.

Due to the physical chemical composition and large volume of residual waste from coffee processing (RWCP), it is necessary to have treatment for disposal in the environment or reuse, so as to comply with environmental legislation as CONAMA resolution 431/2011 (Matos and Lo Monaco, 2003). Some physico-chemical attempts for treatment of RWCP have been reported (Mahesh *et al.*, 2014; Villanueva-Rodríguez *et al.*, 2014); however, none of these reduced the pollutant effect completely. Therefore, a biological treatment could be an alternative for improving the recuperation of RWCP. Microbial communities naturally present in the effluent could promote the degradation of organic compounds, the removal of nutrients and the transformation of toxic substances from the residual water (Wells *et al.*, 2011). Biological treatment can be aerobic or anaerobic, with the use of activated sludge being one of the most used and efficient (Fredriksson *et al.*, 2012) because the microorganisms are already adapted to the environment.

The analysis of the microbial community can provide crucial information for wastewater biological treatment (Ma *et al.*, 2015). One challenge, however, is that the composition and structure of the community can vary at different stages of wastewater treatment (Ibarbalz *et al.*, 2013; Antwi *et al.*, 2017; Lin *et al.*, 2017; Xu *et al.*, 2017), and for many effluents are far from understood (Ma *et al.*, 2015). Researches on the microbiota associated with coffee wastewater and their potential for the purification of pollutants are scarce. Studies of this nature are innovative and aid to maintain sustainability in production processes in coffee farms (ICO, 2006). Our aim was to investigate the dynamics and dominance of microorganisms present in RWCP, isolate and identify these microorganisms, and evaluate their distribution, diversity and richness in the different stages of biological treatment at a wastewater treatment plant (WTP).

Results

Physicochemical composition of wastewater from coffee processing

The analysis of chemical physical parameters allowed the characterization of coffee wastewater and the identification and quantification of different compounds with nutrients and metals (Table 1).

High BOD (6,500 mg L^{-1}) and COD (13,232 mg L^{-1}) values were found, in addition to expressive amounts of dissolved and total solids (5,173 and 7,077 mg L^{-1}). Among the minerals, potassium (200 mg L^{-1}) showed the highest concentration, followed by calcium and cadmium (130 mg L^{-1} each one).

Table 1. Physicochemical parameters analyzed in wastewater from coffee processing.

Parameters	WP
BOD (mg L^{-1})	6,500
COD (mg L^{-1})	13,232
Color (mgPt L^{-1})	567
Turbidity (UT)	464
Total phosphorus (mg L^{-1})	1.97
Dissolved solids (mg L^{-1})	5,173
Total solids (mg L^{-1})	7,077
Total Nitrogen (mg L^{-1})	130
Ammoniacal nitrogen (mg L^{-1})	11.87
Electric conductivity (µs cm^{-1})	1,050
Total hardness (mg L^{-1})	3,600
Cadmium (mg L^{-1})	130
Zinc (mg kg^{-1})	1.5

Copper (mg kg^{-1})	0.7
Iron (mg kg^{-1})	56.2
Manganese (mg kg^{-1})	3.4
Magnesium (mg L^{-1})	10
Potassium (mg L^{-1})	200
Sulfur (mg L^{-1})	110
Calcium (mg L^{-1})	130

Isolation, purification and characterization of microorganisms present in the Wastewater Treatment Plant (WTP)

There were 4,514 colonies of bacteria, filamentous fungi and yeasts obtained from all samples. There were 1,851 yeast colonies, characterized in 12 different morphotypes (data not shown), represented by 116 isolates. There were 2,446 bacterial colonies obtained, characterized in 16 different morphotypes (data not shown) and represented by 127 purified isolates. There were 117 colonies of filamentous fungi (after 7 to 14 days of incubation); these were characterized in 3 different morphotypes (data not shown), and 25 isolates were purified for species identification.

Identification and frequency of occurrence of OTUs

Of the bacterial isolates, 10 different species were obtained. From the filamentous fungi isolates, 4 different species were identified, considering the score in MALDI-TOF analysis equal or superior to 1.8, which reflects the similarity between the sample and the reference spectrum (Table 2). Thirty-one yeast isolates were identified by the protein profile

in five different species (score in MALDI-TOF analysis equal to or greater than 1.7) (Table 2).

Forty-six unidentified isolates of bacteria and yeast were selected for identification by sequencing. In addition, 42 isolates already identified by MALDI-TOF were randomly selected to sequence and confirm their identification (Table 1). These results constituted the highest level of characterization, and determined the different OTUs that were quantified at each sampling point. Seventeen OTUs of bacteria, distributed in 13 genera, and 12 OTUs of yeasts, distributed in 10 genera were identified (Table 2).

Filamentous fungi were identified only by MALDI-TOF (Table 2), given that the generated spectra presented scores higher than 1.8, in relation to the database, and also there was high proximity between branches in the dendograms of each analysed morphotype. Four OTUs distributed in three genera of filamentous fungi were identified (Table 2).

The total viable microbial population present at each collection point ranged from 16.26 log CFU mL^{-1} (P1) to 23.61 log CFU mL^{-1} (P3). Bacteria formed the dominant group in all samples, with a minimum of 7.41 log CFU mL^{-1} and a maximum of 11.61 log CFU mL^{-1} (Table 2). *Sphingobacterium* sp., *Chryseobacterium bovis*, *Serratia marcescens*, *Corynebacterium flavescens*, *Acetobacter orientalis* and *Acetobacter indonesiensis* showed the largest total populations in WTP, with approximately 10 log CFU mL^{-1}.

Table 2. Microorganisms isolated from coffee wastewater samples. Score obtained for the isolates in the evaluation of MALDI-TOF, percentage of identification by sequencing and occurrence of each species in population of the sampling points.

	Population of OTUs (log UFC mL^{-1})	Isolates number	Score MALDI-TOF	Sequencing % ID*	Sequencing Access number	Sampling sites WO	P1	P2	P3	TW
Bacteria										
Bacteria population (log UFC/mL^{-1})						7.60	11.61	8.63	7.57	7.41
Bacillus cereus group	7.30	2	> 1.8	100	KM114617					
Sphingomonas sp.	7.70	1	-	> 99	AB696775					
Arthrobacter woluwensis	7.60	4	-	> 98	KT072630 , KM019881					
Sphingobacterium griseoflavum	10.11	1	-	> 97	KJ000806					
Enterobacter sp.	8.32	7	-	> 99	KR189400					
Pseudomonas lutea	7.00	1	-	> 98	AB495128					
Chryseobacterium bovis	10.77	25	-	> 99	HM217959, HM217955, HM217958, KM402106					
Enterobacter asburiae	9.10	9	> 1.9	100	HQ455820, CP007546					
Serratia marcescens	10.40	9	> 2.1	> 99	KR856196, JX103454, KT887950					
Staphylococcus xylosus	9.48	2	> 1.9	> 99	KJ958200					

Klebsiella oxytoca	7.60	1	> 1.8	> 99	AJ871858					
Corynebacterium callunae	9.61	5	> 1.8	> 99	KU922218					
Corynebacterium flavescens	10.32	13	> 1.8	> 99	JF496333					
Moxarela osloensis	9.78	4	> 1.9	> 99	AB643592, CP014234					
Acetobacter orientalis	10.40	2	-	> 98	LN884097					
Acetobacter indonesiensis	10.86	40	-	> 99	AJ419841, KU976968, JF793967, AB906398, EF681860					
Yeasts										
Yeast population (log UFC/mL^{-1})						5.83	6.39	6.28	5.38	5.48
Saturnispora gosingensis	7.09	12	-	> 97	KY105318					
Hanseniaspora. uvarum	6.83	6	> 1.8	> 99	KY816905					
Wickerhamomyces anomalus	7.43	25	> 1.8	> 99	KT175180, KY105896, KY105895, KY105887					
Torulaspora delbrueckii	7.60	25	-	> 99	KY203862, KY794753, KY105646, KM402069					
Kazachstania exigua	6.76	7	> 1.7	> 99	KY103637					
Cryptococcus albidus	4.30	1	> 1.9	> 98	JX174413					
Meyerozyma caribbica	6.56	11	-	> 99	KM402049, KU200440, KM676452,					
Cyberlindnera jadinii	6.57	2	> 1.8	> 99	KY103059					
Kazachstania gamospora	7.52	23	-	> 99	KY103643, KY103642					
Pichia fermentans	6.70	3	-	> 99	KM402060, KY816910					
Trichosporom domesticum	6.30	1	> 1.9	> 97	KT876717					

Filamentous fungi									
Fungi population (log UFC/mL^{-1})					4.48	5.61	4.08	3.31	5.65
Alternaria alternata	6.70	1	> 1.9	-	-				
Fusarium oxysporum	6.96	11	> 1.8	-	-				
Geotrichum silvicola	6.12	5	> 1.8	-	-				
Geotrichum candidum	6.06	4	> 1.8	-	-				
Total population (log UFC/mL^{-1})					17.90	23.61	18.98	16.26	18.54

Sampling sites: WO= Washer output; P1= Pond 1; P2= Pond 2; P3= Pond 3; TW= Wastewater after treatment. Percentage of occurrence in population: = < 1%; = 1 – 5%; = 5 – 10%; = 10 - 20%; = 30 – 50%; = > 50%.

*ID represents the identity with the sequences in the GenBank databases.

A. indonesienses, with a population of 10.86 log CFU mL^{-1}, stands out, and although it was not found in the washer output (WO), it represented 35.85%, 52.41%, 35.86% and 55.08% of the populations in P1, P2, P3 and TW, respectively. *Enterobacter* sp. and *Enterobacter asburiae*, together represented 77.55% of the WO total population (Table 2).

Saturnispora sp., *Toluraspora delbrueckii*, *Wickerhamomyces anomalus* and *Kazachstania gamospora* showed populations around 7 log CFU mL^{-1} (Table 1). Generally, yeasts were concentrated in the final stages of RWCP treatment (P3 and wastewater after treatment (TW), while the bacteria showed uniform distribution throughout the treatment. The species with the highest frequency of occurrence (f= 5) were the bacteria *E. asburiae* and the yeasts *Hanseniaspora uvarum*, *W. anomalus* and *T. delbrueckii*, found throughout the treatment system (Table 2).

Filamentous fungi presented a population of approximately 6 log CFU mL^{-1}. The OTUs of this group represented less than 5% of the species, with intermediate to low frequencies (f = 3 and f = 1) (Table 2).

The microorganisms deposited in the CCMA received accession numbers from CCMA 973 to CCMA 1056.

Diversity indexes of the microbial population

The P1 and P2 ponds presented the highest species richness, both with 22 OTUs, and 11 OTUs belonging to the bacteria found at all samples (Table 3). Simpson's (D) diversity indexes ranged from 0.46 to 0.84, from Shannon (H) 0.95 to 1.97, the equitability (J) ranged from 0.54 to 0.90. In the P2 pond, yeast presented the highest values for these three indices. The lowest H, J and D values were observed for bacteria

in the TW sample. However, considering the total microbial population, the P1 pond presented the highest diversity values of Simpson (0.81), diversity of Shannon (1.94) and Equitability (0.63).

Dynamics of the microbial community

The thirty-three OTUs isolated from the five different WTP points (WO, P1, P2, P3 and TW, FIG 1) were ordered by PCA, using information about abundance of microbial populations, in which the major components represented 78.87% of the total variance. The first major component accounted for 54.39%, and the second component accounted for 24.48% of the total variability. The generated dispersion diagram revealed the relationship among the studied samples, grouping the similar collection points into three groups (FIG 2). The collection points P3 and TW showed the closest composition of microbial species. This arrangement was mainly influenced by the absence of *Alternaria alternata*, *Cryptococcus albidus*, *Enterobacter* sp. and *Pseudomonas* sp. in both environments. The presence of *Fusarium oxysporum* in WO was the factor that approached the P3 and TW samples.

Acetobacter orientalis, *Sphingobacterium* sp. and *Trichosporom domesticum* were isolated only from P1, and were responsible for the differentiation of this collection point from the others. The differentiation of the P2 profile, in turn, occurred mainly due to the unique presence of the bacteria *Sphingomonas* sp, *Arthrobacter* sp, *Klebsiella oxytoca* and *Bacillus cereus* group. The other species influenced less significantly the differentiation of collection points.

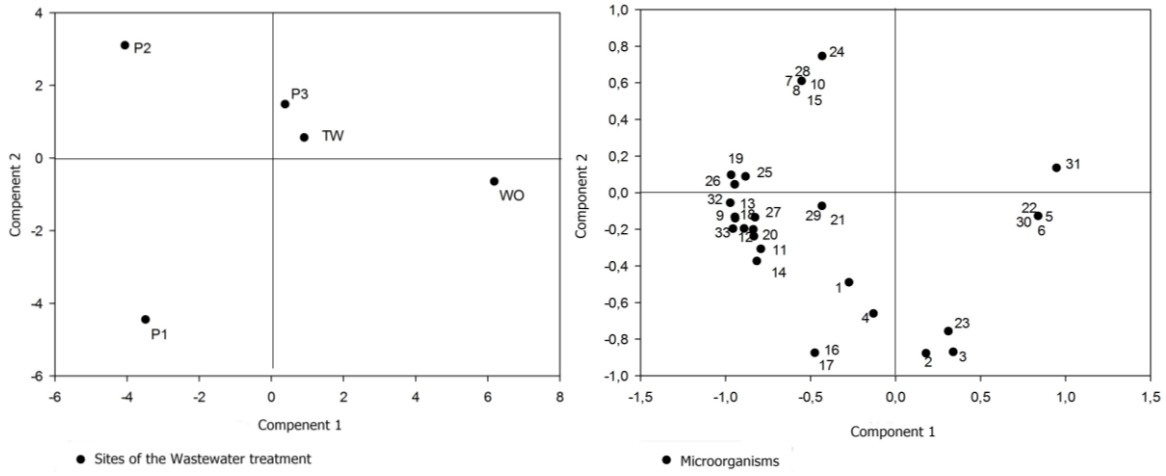

FIG 2. Principal components analysis (PCA) of species of bacteria, yeasts and filamentous fungi isolated from 14 wastewater samples from processing of coffee fruits in five different sites of wastewater treatment: WO= Washer output; P1= Pond 1; P2= Pond 2; P3= Pond 3; TW= Wastewater after treatment. Numbers of 1 to corresponded to OTUs, being: 1= *Serratia marcescens*, 2= *Pantoea aglomerans*, 3= *Chryseobacterium* sp., 4= *Enterobacter asburiae*, 5= *Enterobacter* sp., 6= *Pseudomonas* sp. 7= *Sphingomonas* sp., 8= *Arthrobacter* sp., 9= *Acetobacter indonesiensis*, 10= *Klebsiella oxytoca*, 11= *Corynebacterium callunae*, 12= *Corynebacterium flavescens*, 13= *Chryseobacterium bovis*, 14= *Moxarela osloensis*, 15= *Bacillus cereus* group sp., 16=*Acetobacter orientalis*, 17= *Sphingobacterium* sp., 18= *Hanseniaspora uvarum*, 19= *Wickerhamomyces anomalus* , 20= *Torulaspora delbrueckii*, 21= *Kazachstania exigua*, 22= *Cryptococcus albidus*, 23= *Meyerozyma caribbica*, 24= *Cyberlindnera jadinii*, 25= *Saturnispora* sp., 26= *Kazachstania gamospora*, 27= *Pichia fermentans*, 28= *Trichosporom domesticum*, 29= *Alternaria alternata*, 30= *Fusarium oxysporum*, 31= *Geotrichum silvícola*, 32= *Geotrichum candidum.*

Discussion

The microbiota naturally present in the RWCP samples collected at different points within the WTP showed differences in the composition and diversity of the population. The greater diversity and population density of microorganisms found in the Pound P1 certainly resulted from the effect of artificial aeration. Due to the turbulent

movement provided there was an incorporation of oxygen into the effluent (Eustáquio Júnior *et al.*, 2014) and the oxygen rate was maintained at a higher concentration, allowing the development of the aerobic microorganisms to be isolated during the experiment. The other lagoons did not receive artificial aeration and this may have contributed to a lower population of aerobic microorganisms and consequently a lower population density. Coffee wastewater contains considerable amounts of fermentable sugars and other nutrients (Mussatto *et al.*, 2011), which act as substrates for microbial growth (Bonilla-Hermosa *et al.*, 2014). In Pond P1, where the RWCP was directly after the coffee depulping, the higher load of these organic components may also have facilitated the increase in the microbial community. A higher bacteria diversity results, followed by yeasts and later by filamentous fungi, as agreed by (Sant'anna, 2013). The species of microorganisms identified according to protein and molecular profiles were very similar to those identified by molecular techniques during natural (Silva *et al.*, 2000, 2008), and also in semi-dry coffee fermentation as reported by Vilela et al. (2010) and Evangelista et al. (2015). The similarity of microbial species with the profile of microorganisms observed during coffee fermentation allowed them to infer that at least part of the microorganisms involved in the RWCP are from coffee cherries and naturally present during the processing.

The predominance of the bacteria *A. indonesiensis* over almost the entire RWCP treatment system can be justified by its ability to oxidize different types of sugars and alcohols (Huang *et al.*, 2014). Strains of the genus *Acetobacter* are referred to as decaying bacteria, responsible for the degradation of different substrates (Sokollek *et al.*, 1998; Bartowsky *et al.*, 2003; Huang *et al.*, 2014), besides being among the main

microorganisms responsible for acetic fermentation in vinegar (Yetiman and Kesmen, 2015).

Other predominant OTUs were *C. bovis*, *Enterobacter* and *S. marcescens* and *Corynebacterium*. *Chryseobacterium* species are commonly found in soil, associated with rhizospheres (Singh *et al.*, 2013; Nishioka *et al.*, 2016), and can metabolize nitrogen and ammonia (Ji *et al.*, 2016) and solubilize phosphate (Singh *et al.*, 2013). Bacteria of the genus *Enterobacter* are also involved in the degradation of hemicellulose-derived pentoses (Bi *et al.*, 2009) and in the removal of nitrogen and phosphorus nutrients as well as COD present in a synthetic effluent (Gonçalves *et al.*, 2016). Nitrogen, ammonia, nitrite and nitrate are relevant pollutants and must be removed by biological treatment (Sant'anna, 2013), so the presence of bacteria with this capacity is also fundamental in WTP. Most aerobic denitrification bacteria, including those mentioned above, are mesophilic, and nitrate or ammonia are common as a source of nitrogen to conduct biodegradation (He and Li, 2016).

Serratia marcescens strains from agroindustrial residues have been reported in the literature (Fulazzaky *et al.*, 2016). This bacterial specie has shown the ability to utilize effluents from cassava and corn processing for biomass growth (Montero-Rodríguez *et al.*, 2016). In addition, resistance to different metals has already been described for several species isolated from RWCP, including resistance to Ni, Cu and Zn by *Enterobacter* (Kang *et al.*, 2015; Paul and Mukherjee, 2016), Zn, Cu, Cd and Pb by *Corynebacterium* (Hussein *et al.*, 2013) and Ni, Co and Hg by *S. marcescens* (Kästner *et al.*, 1994; Marrero *et al.*, 2007; Thompson *et al.*, 2007; Giovanella *et al.*, 2015) reported that bacteria resistant to heavy metals may also grow in the presence of

122 persistent organic pollutants, as their occurrence is often concomitant in the
123 environment.

124 *Sphingomonas* sp., *Arthrobacter* sp., *K. oxytoca* and *Bacillus cereus* group are
125 described as denitrifying bacteria (Garrity *et al.*, 2004; Lin *et al.*, 2007; Song *et al.*,
126 2011). *Arthrobacter* and *Bacillus* are among the genera commonly found in microbial
127 communities that form flakes and biofilms in aerobic effluent treatment systems
128 (Sant'anna, 2013). *Arthrobacter* can capture and store sugars for later use. This
129 bacterium utilizes glucose rapidly and increases the substrate competition, thereby
130 reducing the diversity of the growing community (Mau *et al.*, 2014). *Bacillus* sp. is
131 normally able to synthesize a series of extracellular enzymes capable of degrading
132 complex substrates (Priest, 1977; Mala *et al.*, 2015; Siroosi *et al.*, 2016), which might
133 favour their growth in RWCP.

134 *Pichia anomala* and *Hanseniaspora uvarum* were the dominant yeasts
135 throughout Arabica coffee processing in East Africa (Masoud *et al.*, 2004). These yeasts
136 present high pectinolytic activity (Masoud and Jespersen, 2006), suggesting that they
137 act on the degradation of the mucilage (Masoud *et al.*, 2004) present in the RWCP after
138 the coffee wet processing.

139 *Pichia anomala*, *H. uvarum* and *T. delbruekii* are commonly found in
140 fermentation processes, such as beverage production, ethanol distillation and brewing
141 (Chniti *et al.*, 2014; Burgain *et al.*, 2015). The fermentative ability explained the
142 permanence of these microorganisms in WTP, regardless of aeration. Anaerobic
143 behavior for *P. anomala* (teleomorphic phase of *W. anomalus*) was reported in different
144 studies by Fredlund *et al.*, (2002, 2004). Bonilla-Hermosa *et al.* (2014) demonstrated
145 the ability of *H. uvarum* and *P. anomala* to grow on coffee residues as a substrate for

146 fermentation, for production of bioethanol and volatile compounds. Despite the ability
147 to survive and probably develop some degrading activity in WTP, yeasts generally are
148 not as prominent in aquatic systems as are bacteria (Sant'anna, 2013).

149 There are few fungi occurring in water, as they require specific features and
150 structures (Hageskal *et al.*, 2009). This is a plausible explanation for the low population,
151 richness and diversity of fungi found in the RWCP. The survival of *F. oxysporum* in
152 anaerobic submerged environments (Khallil and Abdel-Sater, 1992) was fundamental
153 for its presence in different stages of RWCP treatment, with and without aeration.

154 *Alternaria alternata* and *Fusarium oxysporum* appear to be resistant to adverse
155 environmental conditions, as previously found in aquatic environments contaminated
156 with effluent (Khallil and Abdel-Sater, 1992). *A. alternata* and *Fusarium* populations
157 also responded positively in soils irrigated with organic effluents, due to the large
158 amount of organic matter (Cwalina-Ambroziak and Bowszys, 2009; More *et al.*, 2010)
159 and the wide array of enzymes they secrete.

160

161 **Conclusion**

162 Bacteria are the predominant group of microorganisms in the RWCP, followed
163 by yeasts and filamentous fungi. The physico-chemical characteristics of each pond
164 allowed for observation of the prevailing species in each stage. Some species were
165 persistent throughout the treatment; among these were *A. indonesiensis*, *Enterobacter*
166 sp., *C. bovis*, *E. asburiae*, *S. marcescens* and C. *flavescens*. The metabolic functions,
167 already described in the literature for these predominant microorganisms in the RWCP,
168 allowed them to be associated with the degradation of organic compounds and nutrients
169 such as phosphorus and nitrogen. These characteristics confirmed the hypothesis that

some indigenous microorganisms, isolated from RWCP, can be selected as inoculants for acting in biological treatment, independent of the physicochemical composition present in WTP.

Experimental procedure

Culture media

Six different culture media were used to study the microbial community, according to the group of microorganisms. Culture media were prepared by mixing specified commercial components as follows: Nutrient Agar (NA, % w/v: 0.3 meat extract, 0.5 peptone and 1.5 agar), Dicloran Rose-Bengal Chloramphenicol Agar (DRBC, % w/v: 0.5 peptone, 1.0 dextrose, 0.1 monopotassium phosphate, 0.05 magnesium sulphate, 0.002 rose Bengal, 0.0002 dichloran and 1.5 agar), Yeast Extract Peptone Glucose Agar (YPG, % w/v: 1.0 yeast extract, 1.0 peptone, 2.0 glucose, 1.5 agar), Potato Dextrose Agar (PDA, % w/v: 2.0 dextrose, 4.0 potato infusion, 1.5 agar), Czapek Yeast Extract Agar (CYA, % w/v: 3.0 sucrose, 0.5 yeast extract, 0.1 dipotassium hydrogen phosphate, 0.03 sodium nitrate, 0.005 potassium chloride, 0.005 magnesium sulphate, 0.0001 ferrous sulphate, 0.0001 zinc sulphate, 0.00005 copper sulphate, 1.5 agar), Malt Extract Agar (MEA, % w/v: 3.0 malt extract, 0.5 mycological peptone, 1.5 agar) and Luria-Bertani Agar (LB, % w/v: 1.0 Bacto™ Tryptone, 5.0 Bacto™ Yeast Extract, 1.0 NaCl). The components of the media were dissolved in distilled water and sterilized in an autoclave (121ºC for 20 min).

Sampling

Samples of wastewater from the WTP were collected in sterilized glass bottles and immediately analysed. A total of 30 samples were gathered, with two samplings per day for three consecutive days at five different locations in the WTP on a coffee farm in southeast Minas Gerais (Brazil). The points at which the samples were collected were the washer output after the washing of the beans (FIG 1a), three water treatment ponds (FIG 1b, 1c, 1d), and in the effluent after spontaneous biological treatment (FIG 1e).

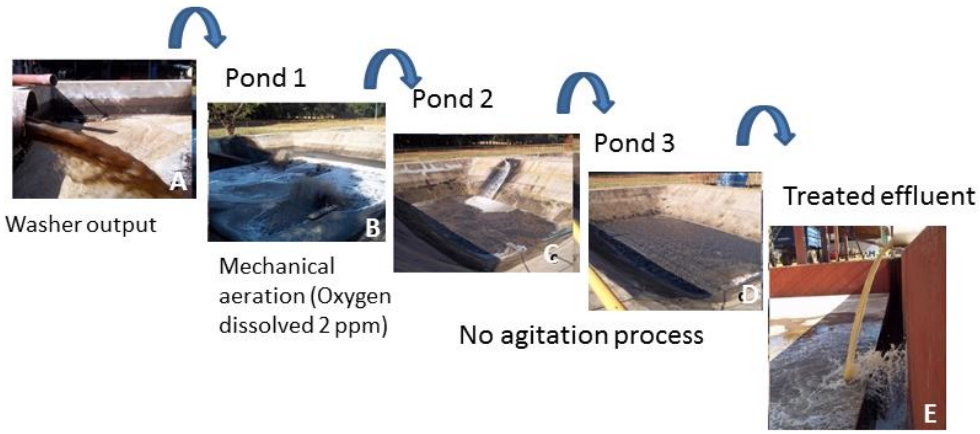

FIG 1. Sampling points of residual waste from coffee processing (RWCP) at the coffee producing farm wastewater treatment plant (WTP): washer output (a), pond 1 (b), pond 2 (c), pond 3 (d), and treated effluent (e).

Samples in ponds 1, 2 and 3 water were collected from the surface, 20 cm below the surface and from the bottom, through hoses responsible for water circulation between ponds. Each water sample from the surface was composed of 1 L of collected wastewater at four different points, to ensure the homogeneity and representativeness of the sample.

At the time of sampling, the spontaneous biological treatment of RWCP followed the procedures already established and standardized in the WTP. Pond 1 was aerated by shaking the water, and was the first to receive the water discharge from the processing of fruits (FIG 1b). In this first lagoon, the dissolved oxygen was maintained at approximately 2 ppm through mechanical aeration, and the pH value was adjusted to 7 with the addition of CaO. Ponds 2 and 3 were used for sedimentation of the solid compounds, storage of the water and water targeting for recirculation in the coffee processing system (FIG 1c and 1d). The total capacity of the lagoons is 300,000 L.

Isolation of microorganisms from wastewater treatment plants

The isolation of microorganisms from wastewater was carried out using a serial dilution technique. Aliquots of 100 µL of different dilutions were plated onto NA (bacteria) and DRCB (yeast and filamentous fungi) plates to ensure the growth of microorganisms. After at least 24 hours of incubation at 28ºC, the developed colonies were characterized morphologically, counted and randomly selected for isolation. Purified isolates were obtained by streaking colonies repeatedly of bacteria, filamentous fungi and yeast onto NA, PDA and YPG media, respectively, and were observed under light microscopy.

Morphological characterization

Bacteria, yeast and filamentous fungi colonies were characterized after growth in NA at 28ºC/24h, YPD at 28ºC/48h, YPD, CYA and MEA, at 25°C and 37°C/7 days, respectively.

Characterization of the protein profile in MALDI -TOF

All strains of bacteria, yeast and filamentous fungi were submitted to protein profile analysis by the MALDI-TOF mass spectrometry (MS) technique. For this analysis, bacteria were cultured on NA and yeast were grown on YPG for 24 hours at 28°C. Filamentous fungi were grown on PDA for 96 hours at 25°C. Small portions of the microbial biomass were transferred from the Petri dish to microtubes, to which were added, as specified by Miguel (Miguel *et al.*, 2017), 6μL of an aqueous solution of 47.5% acetronitrile and 2.5% trifluoroacetic acid (v/v) for bacteria or a solution of 70% formic acid in water (v/v) for yeasts. The preparation of the filamentous fungi samples using formic acid extraction followed the recommendations established by Bruker (Bruker, 2011). Immediately, 0.7 μL of each cell suspension was transferred to the MALDI flex plate and 1 uL of matrix solution (α-Cyano-4-hydroxycinnamic acid [HCCA]) was added and mixed gently.

An *Escherichia coli* K12 colony was obtained from the Public Portuguese Culture Collection of the Micoteca da Universidade do Minho (MUM, www. micoteca. deb. uminho. pt). These were used for in situ extraction of proteins, which in turn were used as the standard for the MALDI-TOF MS external calibration. Cells of *E. coli* BST were grown on LB agar at 30°C for 20 h. About 1 μg of cellular material from a single *E. coli* colony was processed and transferred to the MALDI flex plate as described above for the bacterial analysis. All sample plates were air dried at room temperature. Each sample was spotted in triplicate to test reproducibility. During the analyses, all solutions were prepared daily and stored at +5°C.

Spectrum acquisition was performed on a Microflex mass spectrometer (Bruker Daltonics). Each final spectrum was generated by the sum of 240 accumulated laser

pulses per profile. The resulting peak list was exported to the MALDI Biotyper 3.0 software package (version 3.0; Bruker Daltonics GmbH), which is a commercial Bruker Daltonics database (Bremen, Germany). In the database, a list of individual sample peaks was compared with reference spectra. Dendrograms of the spectral proximity among isolates were created.

Sequencing of 16S and ITS rDNA

Bacteria and yeasts were selected in the dendrograms by the proximity generated after analysis in MALDI-TOF MS. The proximity of the spectra presented in the dendrograms were considered for the selection of unidentified microorganisms, and those with a distance level higher than 0.4 were selected. Some microorganisms already identified by the proteomic profile were also selected to confirm the results by DNA sequencing.

Genomic DNA was extracted from the pure cultures using Instagene (Bio-Rad, Germany), following the manufacturer's instructions. Analysis was of the 16S rDNA region gene sequence in bacteria and the ITS region of rDNA in yeast. For the amplification of the 16S region the primers F27 (5'- AGRGTTTGATCMTGGCTCAG - 3') and R1512 (5′-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3′) were used (Felske *et al.*, 1997). For the ITS region the primers ITS1F (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS4r (5' – TCCTCCGCTTATTGATATGC – 3') were used (White *et al.*, 1990).

The PCR reaction was performed on a thermal cycler, using the components of the Top Taq Master Mix Kit (QUIAGEN®) and following the manufacturer's instructions. The PCR product was gel-loaded with 1.5% agarose (1.5% agarose diluted

in 50X TAE buffer), followed by 70 V electrophoresis for 30 minutes with 50X TAE running buffer. To each sample was added the SYBR Green dye, which after running on the electrophoresis gel allows the visualization of the formed bands by emission of fluorescence in ultraviolet light.

The amplified PCR products were sent for sequencing. The obtained sequences were compared for similarity with sequences from the same regions, deposited in the available GenBank database, using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990). An isolate will be assigned to the species with the highest corresponding identity sequence. Each species was considered an operational taxonomic unit (OTU).

The bacteria and yeasts that were identified were deposited at CCMA (Culture Collection of Agricultural Microbiology, UFLA, Lavras, Brazil) and the filamentous fungi at the Mycology Collection of Food Science Department, UFLA, Lavras, Brazil.

Ecological indices of species

The total population and species richness (S) were calculated by count colonies, considering for this the sample volume and the dilution plated. Closely related organisms formed by groups obtained from the molecular, proteomic and morphological characterization were represented as operational taxonomic units (OTUs), which were named at least to genera.

The species diversity of microorganisms isolated from the WTP was evaluated by the calculation of the total number of isolated individuals (n), equitability ($J = H'/Hmax$), Simpson's index ($1- (\Sigma (ni/n)^2)$) and Shannon's index ($H = -\Sigma(ni/n)\ln(ni/n)$,

where ni is the number of individuals of the taxon, and n is the total number of OTUs (Hammer *et al.*, 2001).

Software

The R software (version 2.15) was used to calculate the ecological indices of species. The PAST software (version 3.15) was used for principal component analysis (PCA) (Hammer *et al.*, 2001). Principal component analysis was performed using a correlation matrix, in which the distribution of microorganisms and the values of microbial populations were used to identify similarities between the samples that were collected at different sites in the WTP.

Wastewater physic-chemical analysis

Physicochemical parameters of coffee wastewater from the washer outlet were evaluated. The parameters color (2120 B), turbidity (2130 B), total nitrogen (Section 4500 A), ammoniacal nitrogen (4500 B), phosphorus (4500 B.5), COD (5220 B), BOD (5210 B), total solids (2540 B), electric conductivity (2510 B) and total hardness (2340 C) were determined according to recommended standard procedures in American Public Health Association (APHA 2012). Potassium, calcium, magnesium, manganese, zinc, copper, cadmium, sulfur, and iron were analyzed by atomic absorption spectrometry (Malavolta et al., 1997).

Acknowledgments

The authors are grateful to the CNPq, CAPES and FAPEMIG for their financial support and scholarship. We thank the Daterra farm, Patrocínio in the State of Minas

Gerais, for the opportunity to collect samples and partial financial support, and Marco Túlio Pacheco Coelho for helping with the diversity indexes in R.

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ARTIGO 2: Natural microbial consortium selected from wastewater coffee processing

Abstract

Native microbiota, previously isolated from semi-dry processed coffee wastewater (WP) was studied in relation to its ability to degrade pollutant compounds present in the wastewater. Physicochemical parameters were evaluated such as pH, biochemical oxygen demand, chemical oxygen demand, nitrogen and phosphorus, to aid the optimization of biological treatment. The selected bacteria inoculum was composed of *Serratia marcescens* CCMA 1010 and CCMA 1012, *Corynebacterium flavescens* CCMA 1006 and *Acetobacter indonesiensis* CCMA 1002 in WP with an initial pH value of 6. The mixed inoculum showed a highly viable and active population (11.18 log CFU mL^{-1}) with a reduction of 85% and 60% of BOD and COD values, respectively, and an 80% reduction of phosphorus and nitrogen. The ecotoxicity in *Triticum aestivum* (wheat) was low, representing a germination induction (Germination rate) higher than 80%, and a reduction in EC_{50} on *Daphnia similis* of up to 100%. The final pH of WP increased to 7.5, which is the recommended value for effluent disposal in water bodies. The microbial inoculum was tested in two WP treatments, with different physical and chemical characteristics, enhancing the efficiency of the selected strains according to the specific WP biological treatments.

Keywords: Agro-industrial effluent; Biological treatment; Mixed inoculum; Microbiota; Toxicity.

1 Introduction

Fresh water scarcity, exacerbated by the pollution of available water, is an issue that affects several countries worldwide (Paraskevas et al., 2002), and developing countries disproportionately (Wen et al., 2017). Wastewater discharge from cities and intensive livestock farms comprise the main organic pollutant load into water courses (Meybeck et al., 2003; Malaj et al., 2014). Wastewater from semi-dry processing coffee (WP) generates effluents in great volumes. In Brazil alone, in 2017, it is estimated that WP of between 14.1 and 15.4 billion L will be generated (CONAB, 2016).

The potential pollution in WP is due to the presence of high concentrations of diverse compounds, which is reflected in high values of chemical oxygen demand (COD) (50,000 mg L^{-1}) and biochemical oxygen demand (BOD) 1.8 to 20,000 mg L^{-1}), low pH (pH< 4.0) (Matos et al., 2001; Bruno and Oliveira, 2008; Haddis and Devi 2008; Campos et al., 2010; Selvamurugan et al., 2010; Oller et al., 2011; Ferrell and Cockerill 2012; Bonilla-Hermosa et al., 2014; Rattan et al. 2015), high ammoniacal nitrogen (40 to 60 mg L^{-1}), total nitrogen (180 to 250mg L^{-1}), phosphorus (60 to 800mg L^{-1}) (Matos et al., 2001; Campos et al., 2010; Rattan et al., 2015), and total solids (1,000 to 7,500mg L^{-1}) (Campos et al., 2010; Villanueva-Rodríguez et al., 2014). Wastewater may also contain components of fertilizers that normally contain potassium, nitrogen, and phosphoric acid that are required by coffee trees, and residue of fungicides and insecticides used in agricultural practices (FAO, 2000).

The treatment and reutilization of these effluents is important for the conservation of the hydric resources (Azizi et al., 2013; Paraskevas et al., 2002). In addition, the detoxification of domestic and industrial wastewater is of essential

617 importance to natural ecosystem protection, and to human health (Shchegolkova et al.,
618 2016).

619 Accelerated depuration processes by microbial action are effective because they
620 explore the degradable metabolic properties of microbes, by conversion of complex
621 organic compounds to most simple forms, besides effectively removing compounds of
622 low molecular weight (Singh et al., 2013). The aerobic treatment of wastewater offers a
623 more effective solution (Li, 2013) as compared to physical and chemical methods which
624 have a greater economic impact and are efficient just to remove of high molecular
625 weight compounds (Azizi et al. 2013).

626 Microorganisms are used to biodegrade several pollutants, due to their
627 biodiversity, versatility and great catabolic potential (Paisio et al., 2012). In particular,
628 natural microorganism populations, isolated from contaminated environments are
629 considered a valuable tool for treatment (Kamika and Momba, 2014), because they are
630 better adapted to climatic, physicochemical and nutrient conditions (Semple et al.,
631 2007).

632 In biological treatment systems, bacteria are the most abundant microorganisms,
633 and are more resistant to variations in environmental conditions such as temperature and
634 oxygen levels (Ding et al., 2016; Kekacs et al., 2015). Several studies use microbiota
635 and various substrates in biological treatments (Angelim et al., 2013; Banerjee et al.,
636 2014; 2016). For instance, a bacterial consortium isolated from activated sludge
637 promoted 98.62% of the decolorization and toxicity reduction of a highly toxic textile
638 effluent (Banerjee et al., 2014). Similarly, a hydrocarbonoclastic bacterial consortium
639 was used as a biological treatment strategy for oil-contaminated mangrove sediments
640 with positive results (Angelim et al., 2013). More recently, a bacterial consortium

isolated from activated sludge samples collected from a common effluent treatment plant was used to treat cosmetic effluents, with successful results (Banerjee et al., 2016).

Thus, in this study we aimed to select microorganisms previously isolated from the WP microbial inoculum, that had the potential for effluent depuration. The efficiency of the treatment for selection of the inoculum, was verified by the analysis of BOD, COD, chemical and physical composition, and acute and subchronic ecotoxicity of WP, and the biological activity of the microorganisms.

2 Materials and methods

2.1 Culture media.

Five different culture media were used in this work. Culture media were prepared by mixing specified commercial components as follows: Nutrient Agar (NA, % w/v: 0.3 meat extract, 0.5 peptone, 1.5 agar), Nutrient Broth (NB, % w/v: 0.3 meat extract, 0.5 peptone), Yeast Extract Peptone Glucose Agar (YPG, % w/v: 1.0 yeast extract, 1.0 peptone, 2.0 glucose, 1.5 agar), Yeast Extract Peptone Glucose Broth (YPGB, % w/v: 1.0 yeast extract, 1.0 peptone, 2.0 glucose), and Mineral Medium (MM % w/v%: 0.5 K_2HPO_4, 0.5 $(NH_4)_2SO_4$, 0.5 $MgSO_4.7H_2O$, 0.10 $FeCl_2.7H_2O$, 10 $CaCl_2$, 0.1 $MnCl_2$, 0.01 $ZnSO_4$). The components of the media were dissolved in distilled water, and sterilized in an autoclave (121ºC for 20 min).

2.2 Microorganisms

Microorganisms were previously isolated and identified by sequencing rDNA from cultivable microbiota present in a coffee semi-dry processing Wastewater

Treatment Plant (WTP) on a coffee farm in southeast Minas Gerais (Brazil, 18°45'18.4"S 46°58'16.1"W).

The selection of microorganisms called Wpc with the potential for biological treatment was carried out initially in wastewater from the Brazilian Cerrado. The first stage was based on a metabolic fingerprint created by analyzing the catabolic capacity of different nutrients, such as carbohydrates, caffeine, and nitrogen compounds, typically found as pollutants in wastewater. Thus 127 isolated bacteria and 116 isolated yeasts were tested (Table 1).

Bacteria. The initial characterization of each isolate comprised differential staining of gram, catalase and oxidase. The commercial Kit Bactray (Laborclin), in Systems I, II and III were used in the biochemical characterization of the gram-negative bacteria species. The gram-negative bacterial isolates with positive results in reaction to Malonate, Citrate, Maltose, L-Arginine, β-galactosidase, Lysine decarboxylation, H_2S production, Urea hydrolysis and Voges Proskauer, tests and all the gram- positive isolates were subjected to tests of pectin and caffeine assimilation, as a carbon and nitrogen source. The culture was made with MM increased of pectin and caffeine in 0.3%.

Yeasts. Yeasts were analyzed to investigate their assimilation capacity for carbon and nitrogen (Kurtzman & Fell 2011). Nutrient sources evaluated were: Glycose at 0.5% and 3.0%, Sucrose at 0.5% and 3.0%, Fructose at 0.5% and 3.0%, Pectin at 0.3% and Phenol at 0.3% and 1% as carbon source, Ammonium sulphate at 0.3% and 1%, and Urea at 0.3% and 1% as a nitrogen source.

687 **Table 1.** Bacteria and yeasts isolated from coffee wastewater and tested for the ability to
688 assimilate different carbon and nitrogen sources.

Microorganisms	**Isolates number**	**CCMA codes**
Bacteria	127	
Bacillus cereus group	2	CCMA 985, CCMA 1043
Sphingomonas sp.	1	CCMA 975
Arthrobacter woluwensis	4	CCMA 974, CCMA 980, CCMA 981
Sphingobacterium griseoflavum	1	CCMA 983
Enterobacter sp.	7	CCMA 986, CCMA 989
Pseudomonas lutea	1	CCMA 991
Chryseobacterium bovis	19	CCMA 987, CCMA 992 a CCMA 999, CCMA1054
Enterobacter asburiae	9	CCMA 988, CCMA 990
Serratia marcescens	9	CCMA 1010, CCMA 1012, CCMA 1013
Staphylococcus xylosus	2	CCMA 977
Klebsiella oxytoca	1	CCMA 973
Corynebacterium callunae	5	CCMA 1007
Corynebacterium flavescens	13	CCMA 1011, CCMA1044 a CCMA1047
Moraxella osloensis	4	CCMA 979, CCMA 1008
Acetobacter orientalis	2	CCMA 1006
Acetobacter indonesiensis	40	CCMA 976, CCMA 1009, CCMA 978, CCMA 982, CCMA 984, CCMA 997, CCMA 1000 a CCMA 1004, CCMA 1009
Yeast	116	
Saturnispora gosingensis	12	CCMA 1019, CCMA 1028, CCMA 1030 a CCMA 1032
Hanseniaspora uvarum	6	CCMA 1020
Wickerhamomyces anomalus	25	CCMA 1014, CCMA 1035 a CCMA 1037, CCMA 1041, CCMA1054 a CCMA1056
Torulaspora delbrueckii	25	CCMA 1018, CCMA 1023, CCMA 1029, CCMA 1034
Kazachstania exigua	7	CCMA 1021, CCMA 1024

Cryptococcus albidus	1	CCMA 1042
Meyerozyma caribbica	11	CCMA 1027, CCMA 1033, CCMA 1040, CCMA1048 a CCMA1053
Cyberlindnera jadinii	2	CCMA 1022
Kazachstania gamospora	23	CCMA 1016, CCMA 1017, CCMA 1025, CCMA 1026
Pichia fermentans	3	CCMA 1038, CCMA 1039
Trichosporon domesticum	1	CCMA 1015

2.3 Growth of isolates in coffee wastewater with different pH values

Yeast and bacteria pre-selected by metabolic fingerprinting were cultivated in 20 mL tubes, containing 10mL of WPc, sterilized in autoclave for 15 min, with pH values of 4.2 (wastewater pH), 6 and 7, adjusted with CaO. Bromothymol blue 0.04% was added as a pH indicator of the wastewater, to visually evaluate the alteration in pH values (acid production) (Sabnis, 2007).

Microorganisms at 10^7 CFU mL^{-1} were inoculated to a proportion of 10% of final volume of the WPc. Tubes were incubated in 25ºC, at 130rpm/6 d. Every 24 hours, tubes were assessed for cellular growth and acid production. At the end of incubation, viable cells were counted in a Petri dish containing NA medium to bacteria and in Neubauer chamber for yeasts.

Strains with superior values of cell concentration compared to the initial inoculum (10^7 CFU mL^{-1}), and pH values of WPc that favored the growing of these microorganisms were selected to the next stage.

2.4 Analysis of the ability to degrade pollutants in wastewater by microorganisms

Mixed inocula were compounded from selected strains, in order to optimize the degradation of the complex waste.

2.4.1 Evaluation of mixed inoculants using Plackett-Burman statistical design

Following the statistical design proposed by Plackett-Burman (PB) (1946), 24 assays were carried out, where 12 assays were composed of yeast isolates (L1 to L12) and other 12 assays were composed of bacteria (B1 to B12). Microorganisms and pH values were considered as variables (Tables S1 and S2). Microbial population and physicochemical parameters of WP were used as the response variable.

Experiments were conducted in bottles of 500mL capacity, containing 200 mL sterilized coffee wastewater at a temperature of 121 ºC/15 min. The inoculation, culture and counting of viable cells after incubation were conducted as described in section 2.4. Besides counting cells, the dry biomass of the microbial population was also determined. The wastewater used to microbial growing (considered here spent) was evaluated in physicochemical parameters of water (section 2.8).

2.4.2 Mixed inoculum selection

The PB design did not show a significant difference for the evaluated factors. Thus the selection considered the absolute values of growing and pH. Assays compounded by combinations of microorganisms and pH values that presented reduction in turbidity, BOD and COD were used as parameters for the selection of inoculuma. These combinations were evaluated in a completely randomized design (CRD).

In total, 12 treatments were analyzed in CRD (Table 2): Seven treatments (T1 to T7) were compounded only by bacteria isolate in different combinations; three treatments (T8 to T10) were compounded by combinations of different yeast isolates; one treatment (T11) comprised a mixture of bacteria and yeasts, compounded by the best combination of each group; one treatment was the control (T12), without inocula.

The WP pH value was adjusted to 6, and the assays were conducted in triplicate, as described in section 2.4.1. The evaluation of cellular viability, dry biomass and physicochemical parameters of wastewater (section 2.8.1) were done six days after beginning the inoculation.

The efficiency of selected mixed inoculum in WP depuration was verified using another sample of WP, from a distinct origin, located in the Brazilian Atlantic Forest (21°16'14.2"S 44°59'44.9"W) (WPaf), that presented a higher concentration of organic matter, color and turbidity (Table 2).

Analysis of biological activity, toxicity and composition of carbohydrates, acids and alcohols, described in sections 2.5, 2.6 and 2.7.2, respectively, were conducted as additional tests to evaluate the efficiency of treatments with WPc and WPaf spent and the mixed inoculum selected.

748

749 **Table 2.** Combination of microorganisms to composition of mixed inoculum inoculated in the coffee wastewaster completely randomized design
750 (CRD).

Treatments	Bacteria						Yeast						
	CCMA 1010	CCMA 1013	CCMA 1012	CCMA 1006	CCMA 1047	CCMA 1002	CCMA 1048	CCMA 1049	CCMA 1050	CCMA 1051	CCMA 1052	CCMA 1056	CCMA 1015
T1	+	-	+	+	-	+	-	-	-	-	-	-	-
T2	+	+	-	+	+	+	-	-	-	-	-	-	-
T3	-	+	-	+	+	-	-	-	-	-	-	-	-
T4	-	-	-	+	-	+	-	-	-	-	-	-	-
T5	+	+	-	-	-	-	-	-	-	-	-	-	-
T6	-	+	+	-	+	-	-	-	-	-	-	-	-
T7	+	+	+	-	-	-	-	-	-	-	-	-	-
T8	-	-	-	-	-	-	+	+	-	+	+	+	-
T9	-	-	-	-	-	-	+	-	-	-	+	-	-
T10	-	-	-	-	-	-	+	+	+	-	-	-	+
T11	+	-	+	+	-	+	+	+	+	-	-	-	+
T12	-	-	-	-	-	-	-	-	-	-	-	-	-

751 + indicates the presence and – indicates the absence of microorganism in treatment
752 CCMA 1010, CCMA 1013 e CCMA 1012: *Serratia marcescens*; CCMA 1011 e UFLA ARC 53: *Corynebacterium flavescens*; CCMA 1006: *Acetobacter orientalis;* UFLA
753 ARC 193, UFLA ARC 194, UFLA ARC 195, UFLA ARC 196, UFLA ARC 197: *Meyerozyma caribbica;* UFLA ARC 242: *Wickerhamomyces anomalus;* CCMA 1015:
754 *Trichosporon domesticum.*

2.5 Analysis of biological activity of the isolates using the biospeckle method

The quantification of the biological activity was performed for sets of 100 images with 320 × 240 pixels captured at 33.3 ms intervals. For each of these image sets, an intermediate matrix was constructed, known as the Time History of the Speckle Pattern (THSP), as proposed by Oulamara et al. (1989).

Numerical values were acquired for each THSP matrix. The operations to obtain a numeric value that represents the activity registered by the intensity variations of the selected pixels were performed using the Absolute Value of Differences (AVD) algorithm, proposed in Braga et al., (2011). This algorithm counts the changes in the value of intensity stored in the lines of a THSP matrix, assigns a greater weight to the more abrupt changes, and producing a dimensionless scalar value that, in this case, represents the level of biological activity registered in a set of images.

It was evaluated that the selected strains *Serratia marcescens* CCMA 1010 e CCMA 1013, *Corynebacterium flavescens* CCMA 1006 and *Acetobacter indonesiensis* CCMA 1002, were appropriate to be used, over the incubation time, after mixing the inocula in WPc and WPaf, and comparing the activity obtained in the two effluents. WP without inoculation of microorganisms was used as a negative control.

2.6 Toxicological analyses

Triticum aestivum and *Daphinia similis* were used to analyze WP toxicity.

2.6.1 Acute toxicity of WP

Tests of acute toxicity were conducted with *Daphinia similis*, using the WPc and WPaf spent in five concentrations (100, 75, 50, 25 e 12,5%), with four repetitions, and

779 with water of the organism in culture as the negative control. Five young organisms (6
780 to 24 hours), by repetition, were exposed to the effluent, and to the negative control in
781 10mL of solution, in 20 ± 2◦C, for photoperiod 12/12 hours. For each concentration,
782 immobility and/or mortality of the individuals was observed, after an exposition period
783 of 48h. The EC_{50} was calculated using the Sperman-Karber method to evaluate acute
784 toxicity by using the computer program "LC_{50} Programs JS Pear test" (Hamilton, 1977).
785 After the acquisition of numeric values of acute toxicity (EC_{50}), the
786 transformation in acute toxic unit (TU) (Equation (1)) (Karaouzas et al., 2010) and the
787 calculus of toxicity reduction percentage (%TR) (Equation (2)) (Isidori et al., 2003) was
788 calculated through the following formulas:
789
790 $$TU = 100/EC_{50} \quad (1)$$
791
792 $$\%\ TR = 1 - \left(EC_{50fresh}/EC_{50spent}\right) x\ 100 \quad (2)$$
793
794 **2.6.2 Subchronic toxicity of WP**
795 The phytotoxicity of WP was evaluated on wheat (*Triticum aestivum)* according
796 to the Sobrero method, with some modifications (Sobrero et al., 2004). Five
797 concentrations of fresh and spent WP (12.5, 25, 50, 75 and 100%) plus distilled water
798 (as control) were used. Three replications, containing fifteen seeds for each
799 concentration were analyzed. The relationship between WP and the control was used to
800 calculate the relative germination roots (RG) (Equation 3), root length (RL) (Equation
801 4), and the germination index (GI) (Equation 5), according to the following equations:

802

803 $$RG(\%) = \text{Number germinated seeds WP/Number germinated seeds control x 100} \quad (3)$$

804

805 $$RL(\%) = \text{Average root length WP/Average root length control x 100} \quad (4)$$

806

807 $$GI(\%) = ((RG\%) \times (RL\%))/100 \quad (5)$$

808

809 **2.7 Coffee processing wastewater composition**

810 Analysis of WP composition was important to evaluate microorganism action
811 during the biological treatment. Analyses were conducted before and after biological
812 microbial treatment.

813

814 **2.7.1 Physicochemical analysis**

815 Physicochemical parameters were evaluated during all processes of inocula
816 selection (items 2.4.1 and 2.4.2), and the results were decisive in choosing the better
817 group of microorganisms to be used in the WP treatment.

818 Characteristics were determined according to recommended standard procedures
819 suggested by the American Public Health Association (APHA 2012). The analyzed
820 parameters were: Color (2120 B), turbidity (2130 B), total nitrogen (Section 4500-N.C),
821 phosphorus (4500-P B), COD (5220 D) and BOD (5210 B).

822

823 **2.7.2 Determination of acids, carbohydrate and alcohol content**

824 Concentration of carbohydrates (sucrose, glucose and fructose), acids and
825 alcohols were determined by High Performance Liquid Chromatography (HPLC).

826 Analysis were conducted using a Shumadzu chromatograph (Shimadzu Corp., Japan),
827 equipped with a refraction index detector (RID-10A).

828 For quantification of carbohydrates, ethanol and glycerol were used in an
829 exclusion column of Shimadzu ions (Shim-pack SCR-101H, 7.9 mm x 30 cm). The
830 analysis was operated in 30º C, with ultrapure water, acidified with perchloric acid (pH
831 2.1) as the effluent, and a flow of 0.6 mL min^{-1}. The other identified acids in the
832 samples were quantified following the same procedure, however, the temperature was
833 adjusted to 50ºC.

834 Trigonelina, chlorogenic acid and caffeine were determined using a Shimadzu
835 column of the reverse phase (Shim-pack CLC-ODS (M)® C18, 100 mm of lenght x 0.3
836 mm DI) operated at 30ºC. The mobile phase was compounded using a proportion of
837 ultrapure water: methanol: acetic acid of 79:20:1, with flow of 0.6 mL min^{-1}.

838 Compound quantification was conducted using calibration curves, built with
839 different concentrations of standard compounds, injected in the same conditions as that
840 of the samples (Duarte et al., 2013, 2011). All the samples were analyzed in duplicate.

841

842 **3 Results**

843 **3.1 Physicochemical composition of wastewater from coffee processing**

844 Physicochemical parameters, such as BOD (6,500 and 10,000 mg L^{-1}), COD
845 (13,232 and 25,570 mg L^{-1}), total solids (7,077 and 21,865 mg L^{-1}), color (567 and
846 11,249 mgPt L^{-1}) and turbidity (467 and 9,100 UT), among others, showed greater
847 values in WPaf than WPc (Table 3). Parameters such as color, turbidity, BOD and COD
848 are directly related to the solids content in the effluent. These, in turn, can be influenced

849 by the variety of coffee processed, the stage of maturation of the beans, and the region
850 of cultivation.

851

852 **Table 3.** Physicochemical parameters of coffee processing wastewater from Brazilian
853 Cerrado (WPc) and Atlantic forest (WPaf).

Parameters	Values	
	WPc	WPaf
BOD (mg L^{-1})	6,500	10,000
COD (mg L^{-1})	13,232	25,570
Color (mgPt L^{-1})	567	11,249
Turbidity (UT)	464	9,100
Total phosphorus (mg L^{-1})	1.97	9.38
Dissolved solids (mg L^{-1})	5,173	16,710
Total solids (mg L^{-1})	7,077	21,865
Total Nitrogen (mg L^{-1})	130	629
Ammoniacal nitrogen (mg L^{-1})	11.87	67.00
Electric conductivity (µs cm^{-1})	1,050	3,130
Total hardness (mg L^{-1})	3,600	5,700

854

855 **3.2 Biochemical characterization and pre-selection of microorganisms**

856 Microorganism biochemical characterization (data not shown) was used in the
857 initial screening process. The ability to degrade different organic compounds whose
858 structure could resemble the structures of compounds present in the WP, was considered
859 in the selection process, thus, indicating which potential microorganisms were able to
860 remove pollutants from the WP.

861 Twelve bacteria were characterized as Gram-negatives (from 111 bacterial
862 isolates), and showed positive results to Malonate, Citrate, Maltose, L- Arginine, β-
863 galactosidase, Lisine decarboxylation, H_2S production, Urea hydrolysis and the Voges
864 Proskauer test. These bacteria and the 16 bacteria characterized as Gram-positive were
865 subjected to the tests of pectin and caffeine assimilation, as carbon and nitrogen sources.

Nineteen bacterial isolates (*Arthrobacter* sp. CCMA 974, *S. marcesens* CCMA 1010, CCMA 1012 and CCMA 1013, *S. xylosus* CCMA 977, *A. indonesiensis* CCMA 984, CCMA 997, CCMA 978 and CCMA 1002, *C. flavescens* CCMA 1011, UFLA ARC 44, UFLA ARC 47, UFLA ARC 48, UFLA ARC 49, and UFLA ARC 53, *C. bovis* CCMA 993, *Sphingobacterium* sp. CCMA 983, *B. cereus* group CCMA 985 and UFLA ARC 95) presented growth in pectin and caffeine.

All yeasts (116 isolates) could grow in glycose, fructose and sucrose as a carbon source, and ammonium and urea sulfate as sole nitrogen source. However, only 12 isolates were grown on pectin as a carbon source. Among these, 8 isolates (*M. caribbica* CCMA 1040, CCMA1048, CCMA1049, CCMA1050, CCMA1051, CCMA1052, CCMA1053, CCMA1056 e *T. domesticum* CCMA 1015) also grew in the presence of caffeine.

3.3 Analysis of the isolates growth in coffee wastewater with different pH values

Thirty bacteria and yeasts that presented good growth in different carbon and nitrogen sources were pre-selected for culturing in WPc (Table 4).

Growth in pH of value 4.2 was not considered to select bacteria growth in WPc, once only five (*S. marcescens* CCMA 1012, *A. indonesiensis* CCMA 978, *C. flavescens* CCMA1046, *B. cereus* group CCMA 985 and CCMA1043) of the nineteen tested bacteria presented viable cells.

Table 4. Viable population of microbial isolates after 6 days in wastewater from semi-dry coffee processing at different pH values.

Microorganisms	log CFU mL^{-1}		
	pH 4.2	pH 6	pH 7
Bacteria			
Arthrobacter sp. CCMA 974	-	8.23 Ca	8.38Ba
Serratia marcescens CCMA 1010	-	8.57 Aa	8.59 Aa
Serratia marcesens CCMA 1013	-	8.38 Ba	8.32 Ba
Staphylococcus xylosus CCMA 977	-	7.80 Da	7.88 Ca
Serratia marcescens CCMA 1012	-	7.85 Db	8.26 Ba
Acetobacter indonesiensis CCMA 978	-	5.04 Eb	8.61 Aa
Corynebacterium flavescens CCMA 1011	-	7.34 Da	7.04 Ea
Corynebacterium flavescens CCMA1044	-	6.78 Eb	7.63 Ca
Corynebacterium flavescens CCMA1046	-	5.85 Eb	8.83 Aa
Corynebacterium flavescens CCMA1045	-	6.08 Eb	8.18 Ba
Corynebacterium callunae CCMA 1007	-	8.23 Ca	7.68 Cb
Corynebacterium flavescens CCMA1047	-	7.25 Db	8.34 Ba
Chryseobacterium bovis CCMA 993	-	6.97 Db	6.04 Ea
Acetobacter indonesiensis CCMA 997	-	5.04 Ea	6.15 Ea
Sphingobacterium griseoflavum CCMA 983	-	6.30 Ea	7.28 Da
Acetobacter indonesiensis CCMA 1002	-	7.81 Ca	7.92 Ba
Corynebacterium flavescens CCMA 1006	-	8.56 Aa	8.56 Aa
Bacillus cereus group CCMA 985	-	6.64 Ea	7.36 Da
Bacillus cereus group CCMA1043	-	6.00 Ea	7.00 Ea
Yeasts			
Torulaspora delbrueckii CCMA 1029	6.04 Fb	6.30 Ca	6.28 Ca
Meyerozyma caribbica CCMA1048	7.30 Aa	6.61 Bb	6.08 Dc
Meyerozyma caribbica CCMA1049	6.77 Ca	6.53 Ab	6.30 Cc
Meyerozyma caribbica CCMA1050	6.30 Dc	6.69 Aa	6.49 Ab
Meyerozyma caribbica CCMA1051	6.28 Db	6.66 Aa	6.28 Cb
Meyerozyma caribbica CCMA1052	6.34 Eb	6.61 Aa	6.40 Bb
Wickerhamomyces anomalus CCMA1054	5.96 Fb	6.23 Ca	6.23 Ca
Wickerhamomyces anomalus CCMA1055	6.34 Ea	6.08 Db	6.00 Db
Wickerhamomyces anomalus CCMA1056	5.86 Fb	6.26 Ca	5.26 Eb
Trichosporon domesticum CCMA 1015	5.32 Fb	6.32 Ca	4.65 Eb
Meyerozyma caribbica CCMA 1040	7.04 Ba	6.28 Cc	6.35 Bb
Meyerozyma caribbica CCMA1053	6.69 Da	6.04 Db	5.51 Ec

*Means followed of same upper case in columns and the same letter lower case in lines, do not differ among them in each group of microorganisms, according to the Scott-Knott test, with 5% of significance.

All bacterial isolates grew at pH values of 6 and 7. Eight bacterial isolates presented difference of growth between pH values 6 and 7, and seven isolates had greater growth in pH 7 (Table 4). Isolates *S. marcescens* CCMA 1010 and *C. flavescens*

CCMA 1006 presented the greatest populations (8.57 and 8.56 log CFU mL^{-1}, respectively), which did no differ between them or other pH values. *S. marcenscens* CCMA 1013 presented the second greatest growth (8.38 log CFU mL^{-1}), and also did not show differences between the WP pH values 6 and 7. *S. marcescens* CCMA 1012, *C. flavescens* CCMA 1011 and UFLA ARC 53, *B. cereus* group CCMA 985, *A. indonesiensis* CCMA 1002 also presented expressive growth with 7.85, 7.34, 7.25, 6.64 and 7.85 log CFU mL^{-1}, respectively, in pH 7.

All yeasts grew in adjusted pH values (4.2, 6 and 7) to the WP (Table 3), however, showing different rates of growth in each one of pH values. The eight yeasts UFLA ARC 193 (7.30 log CFU mL^{-1}), UFLA ARC 194 (6.77 log CFU mL^{-1}), UFLA ARC 195 (6.69 log CFU mL^{-1}), UFLA ARC 196 (6.66 log CFU mL^{-1}), UFLA ARC 197 (6.61 log CFU mL^{-1}), UFLA ARC 242 (6.26 log CFU mL^{-1}), *T. domesticum* CCMA 1015 (6.32 log CFU mL^{-1}) and *M. caribbica* CCMA 1040 (7.04 log CFU mL^{-1}) presented high populations when cultivated in pH 6, or did not differ statistically between pH 6 and others pH values.

In general, yeasts presented better result in pH 6 and bacteria in pH 7, or there was no difference between pH 6 and 7. Therefore, two values of pH were evaluated in the preliminary tests (Plackett-Burman design), to verify if there is an influence of pH values in wastewater depuration.

3.4 Selection of mixed inoculum of microorganisms and pH value with better potential for WP clearance

It was not possible to detect the influence of isolates and pH in the wastewater depuration from the PB design. However, there was variation in absolute values to

922 variables BOD (91 to 1,224 mg L^{-1}), COD (224 to 2,227 mg L^{-1}) and turbidity (15.4 to
923 82.2 UNT) (Table S3). Thus, the mixed cultures that presented the lowest values for
924 these parameters, representing greater efficiency in biological treatment in WP were
925 analyzed again in DIC. Mixed cultures were evaluated in wastewater from the Brazilian
926 Cerrado (WPc) and the Atlantic Forest (WPaf).

927 The initial value of pH 6 was selected based on reduction of the amount of CaO
928 used to adjust the pH, reducing the disposal in the environment and the operational
929 costs. The WPc was altered after microbial treatment, achieving values between 7.7 and
930 8.5, when cultured with bacterial inoculum. In the WPaf, the final pH ranged from 5.4
931 to 7.6 after treatment with the yeast inoculum (Table 5).

932 The population observed in mixed bacterial culture was superior at 1 log, in WPc
933 (T4 to T6) or equal between the two effluents (T1 to T3). A similar, observation was
934 made in the biomass in yeast inoculum using WPc in all the treatments. Contrastingly,
935 when the inoculum was composed of bacteria and yeast, the produced biomass stayed
936 constant independent of the effluent origin (Table 5).

937 The mixed bacterial inoculum presented a population superior than the mixed
938 yeast inocula, in all evaluated observations. The maximum population was observed in
939 T7 (12.04 log CFU mL^{-1}) using WPaf, while in WPc the maximum population was in
940 10.18 log CFU mL^{-1} at T1. The lowest populations were detected in T9 in WPc and
941 WPaf, 5.61 and 5.11 log CFU mL^{-1}, respectively.

942 The T1 treatment presented the maximum efficiency obtained in the two tested
943 effluents which was 85.65 and 83.22% of the reduction of BOD in WPc and WPaf,
944 respectively. In actual values, after treatments (T1 to T12) BOD values presented great

945 variation, ranging from 312.22 to 2,121.33 mg L^{-1} and 475.32 to 3,389.33 mg L^{-1} in
946 WPaf and WPc, respectively.

947 COD values varied from 3,282 to 1,124 mg L^{-1} in WPc, with maximum
948 reduction of 65.74% in T2. The maximum reduction of COD in WPaf was 74.84% in
949 T11 and the medium values varied from 4,962 to 1,248 mg L^{-1} (Tables 5 and S4).
950 In general, treatments with bacteria as the inoculum were more efficient. BOD and
951 COD reduction by the action of mixed microbial cultures presented similar behavior in
952 wastewater of different origin, with better results achieved by T1 which promoted
953 greater rates of BOD and elevated rates compared to COD.

954 The mixed inoculum T1, compounded by *S. marcescens* CCMA 1010 and 1012,
955 *C. flavescens* CCMA 1006 and *A. indonesiensis* CCMA 1002 was considered the best
956 mixed inoculum for WP treatment and removal rates of BOD and COD were decisive
957 for the selection as these were two important parameters of pollution. The inoculum T11
958 presented similar efficiencies in meaurements of BOD and COD, however, the highest
959 number of involved isolates makes it disadvantageous, in relation to T1.

960 Besides the greatest efficiency in reduction of BOD and COD values, T1
961 promoted a greater percentage of nitrogen and phosphorus reduction (Table 5) using
962 both effluents. Reduction values above 50% in phosphorus content were observed when
963 in the presence of bacterial inoculum, except in T7, that differs from T1 by the presence
964 of *S. marcescens* CCMA 1013 and absence of *C. flavescens* CCMA 1006, and *A.*
965 *indonesiensis* CCMA 1002. Equally, there was a reduction of around 50% in nitrogen
966 content in WPc and WPaf.

967

968 **Table 5.** Mean values of microbial biomass, viable microbial cells and pH of the coffee wastewater from coffee producing farms in the Brazilian
969 Cerrado (WPc) and Atlantic Forest (WPaf).

Essays	Microrganisms - CCMA	Biomass (mg L^{-1})		log UFC mL^{-1}		pH		BOD		COD		Nitrogen		Phosphor	
		WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf
T1	1010, 1012, 1006, 1002	567.67 Ba	300.00 Da	10.18 Aa	11.18 Da	8.44 Aa	5.42 Cb	312 Cb	512 Da	1477 Da	1304 Da	0.09 Ca	0.01 Ca	0.93 Eb	1.37 Ca
T2	1010, 1013, 1006, 53, 1006	750.00 Ba	366.67 Da	10.08 Ab	11.75 Ba	8.22 Aa	5.51 Cb	569 Ca	677 Da	1124 Da	1323 Da	0.07 Db	0.12 Ca	0.90 Eb	1.27 Ca
T3	1013, 1006, ARC 53	850.00 Ba	966.67 Ca	9.81 Ab	11.61 Ca	7.98 Ba	5.40 Cb	644 Ca	631 Da	1962 Ca	2110 Ca	0.15 Ba	0.16 Ca	0.78 Ea	0.85 Ea
T4	1011, 1002	1033.30 Ba	166.67 Db	9.78 Ab	10.84 Da	8.46 Aa	5.65 Cb	453 Ca	520 Da	1940 Cb	2509 Ca	0.03 Db	0.14 Cb	0.52 Fa	0.57 Fa
T5	1010, 1013	766.67 Ba	51.67 Db	10.04 Ab	11.57 Ca	8.40 Aa	6.06 Bb	463 Ca	475 Da	2087 Ca	2077 Ca	0.07 Ca	0.09 Ca	1.01 Eb	1.31 Ca
T6	1013, 1012, ARC 53	916.67 Ba	53.33 Db	10.11 Aa	10.89 Ca	8.26 Aa	5.86 Bb	483 Ca	597 Da	1763 Ca	1385 Da	0.17 Ba	0.22 Ba	1.05 Ea	1.09 Da
T7	1010, 1013, 1012	666.67 Bb	1500.00 Ca	9.76 Ab	12.04 Aa	8.38 Aa	7.13 Ab	947 Bb	1118 Ca	2168 Cb	2463 Ca	0.32 Aa	0.35 Aa	1.38 Da	1.47 Ca
T8	193, 194, 196, 197, 242	1500.00 Ab	4866.67 Aa	5.18 Ba	5.88 Da	7.86 Ba	7.44 Aa	1118 Ba	994 Da	2844 Ba	2897 Ba	0.45 Aa	0.44 Aa	2.09 Ca	1.96 Ba
T9	ARC 193, ARC 197	1550.00 Ab	4433.33 Aa	5.11 Ba	5.61 Da	7.68 Ba	7.60 Aa	1199 Bb	1446 Ba	2572 Bb	3098 Ba	0.38 Aa	0.41 Aa	2.28 Bb	2.58 Aa
T10	193, 194, 195, 1015	1466.67 Ab	3916.67 Ba	5.30 Ba	5.71 Da	8.10 Ba	7.52 Ab	1017 Ba	1063 Ca	3131 Aa	3334 Ba	0.24 Ba	0.24 Ba	2.80 Aa	2.14 Bb
T11	1010, 1012, 1006, 1002, 193, 194, 195, 1015	733.33 Ba	266.67 Da	9.36 Ab	11.59 Ca	8.17 Ba	5.47 Cb	325 Ca	529 Da	1499 Da	1248 Da	0.10 Ca	0.15 Ca	0.52 Fb	0.90 Ea
T12*	-	*	*	*	*	6.61 Ca	5.46 Cb	2230 Ab	3389 Aa	3545 Ab	4962 Aa	0.40 Aa	0.44 Aa	2.94 Aa	2.55 Ab

970 Note: Means followed by the same capital letter in the columns, and the same lowercase letter in the lines, do not differ according to the Scott-Knott test, with 5% significance, within
971 each variable.
972 * There are no data on biomass and microbial population because it is the uninoculated control.
973

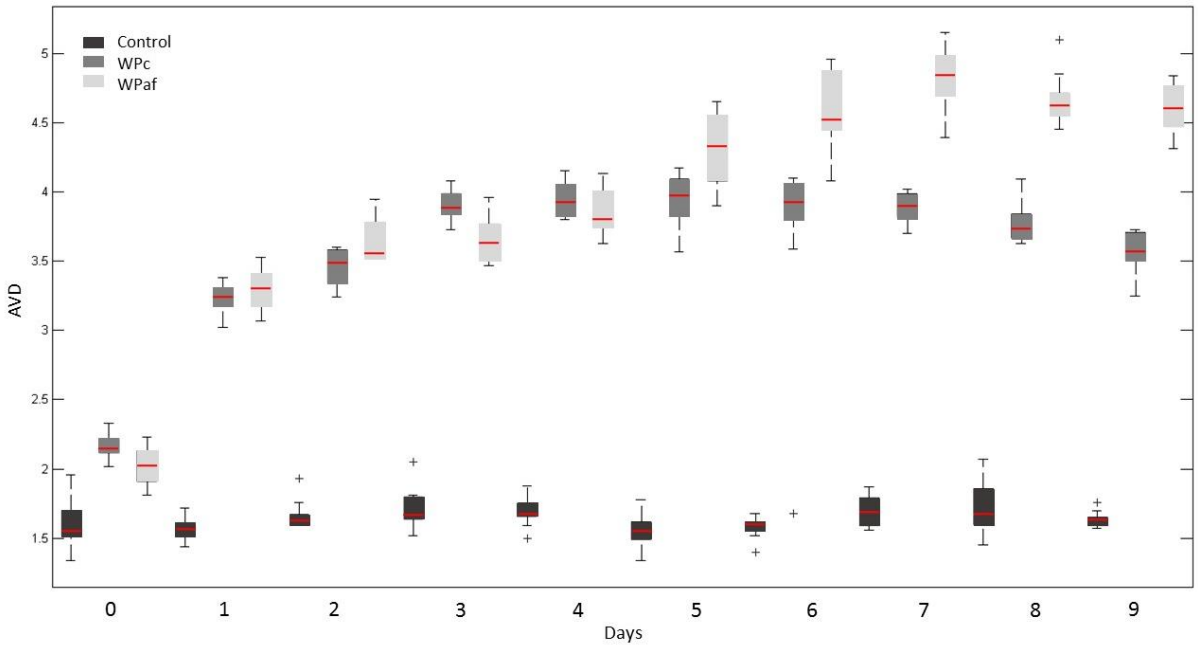
974 Color and turbidity were not reduced by microorganisms in WP. Color was greater in WPaf, and values varied from 890.67 (T12) to 2290
975 mg L^{-1} PtCo (T10); in WPc values varied from 738 (T12) to 2027 mg L^{-1} PtCo (T10). Turbidity varied from 0.143 (T12) to 2.800 uT (T0) in
976 WPc and from 0.153 (T12) to 2.577 uT (T9).

977

978 **3.4 Analysis of the biological activity of the isolates by the biospeckle method**

979 Biological activity in WPc and WPaf after inoculation with selected bacterial
980 inoculum was monitored for nine consecutive days. Activities observed in WP samples
981 were compared between them, and to the activity in the middle of BN without inoculum
982 (Figure 1).

983



984

985 **Figure 1.** Samples activity detected by the Speckle Temporal Spatial (STS) over nine days:
986 average values obtained with 16 replications of each treatment group. Control= WP
987 without inoculum; WPc= Wastewater from semi-dry coffee processing from a farm in the
988 Cerrado region inoculated with *S. marcescens* CCMA 1010, *S. marcescens* 1012, *C.*
989 *flavescens* CCMA 1006, *A. indonesiensis* CCMA 1002; WPaf – Wastewater from semi-
990 dry coffee processing from a farm in the Atlantic forest region inoculated with CCMA
991 1010, CCMA 1012, CCMA 1011, CCMA 1002.

992

993 Biological activity in WPc and Wpaf did not differ until the 4th day, it was
994 increased of 2, after inoculation, until approximately 4. Although a statistical difference
995 was still not observed between WP, after the 4th day, it is noted that the activity in WPc
996 stabilized, while in WPaf it continued its upward behavior until the 7th day, achieving a

997 value close to 5. A statistical difference was observed to values from day 7, when a
998 decline in biological activity was observed in both WPs.

999 The biological activity in the Control group was constant and inferior to the
1000 activity in samples inoculated with the WP.

1001

1002 **3.5 Toxicological analyses**

1003 **3.5.1 Acute toxicity of WP**

1004 The EC_{50} of the WP in *D. similis* was greater to fresh WP in both wastewater
1005 treatments. Maximum toxicity was observed to fresh WPaf, with EC_{50} of just 17.68%,
1006 equal to 5.66 TU (Table 6).

1007

1008 **Table 6.** Acute toxicity of coffee wastewater from the Cerrado (WPc) and Atlantic forest
1009 (WPaf) before and after biological treatment with mixed bacterial inoculum.

WP	EC_{50} (%)	TU	%TR
WPaf in natura	43.30	5.66	
WPaf treated	17.68	2.30	59.17
WPc in natura	90.86	1.10	
WPc treated	--	--	100.00

1010

1011 Biological treatments with mixed bacterial inocula reduced the acute toxicity by
1012 59.17% in WPaf and 100% in WPc, representing the absence of negative effects in
1013 motility and mortality of *D. similis.*

1014

1015 **3.5.2 Subchronic toxicity of WP**

1016 The phytotoxicity of spent and fresh WP was evaluated in *Triticum aestivium*,
1017 considering the percentage of relative germination (RG), rate of root length (RL) and the
1018 germination index (GI) (Figures 2A, 2B e 2C).

There was germination of seeds in all the analyzed samples, and low sensibility of *T. aestivium* was observed in WP. A greater phytotoxicity was observed in seeds exposed to fresh WPaf. RG, RL and GI presented an inverse correlation to the used concentration.

The relative germination presented between 31 and 82% in fresh WPaf, and values superior to 80% in spent WPaf and WPc, independent of treatment. The growth rate of roots presented values between 14 and 62% in fresh WPaf, and between 27 and 89% in othersamples of WPaf.

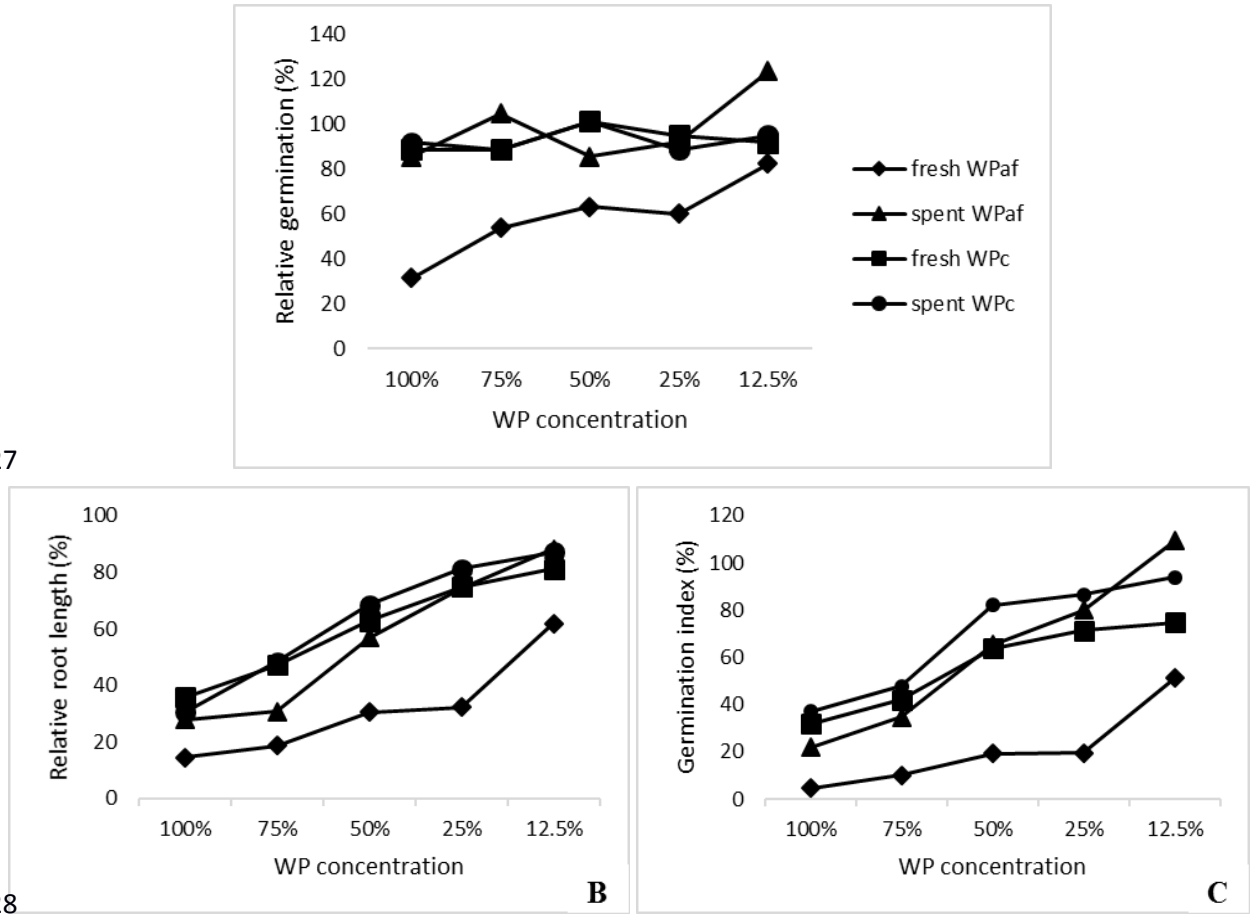


Figure 2. Subchronic toxicity of wastewater from coffee processing evaluated in seeds of *Triticum aestivium*. Relative Germination (A); Relative root length (B); Germination index (C).

fresh WPaf= wastewater from coffee from an untreated Atlantic forest area; spent WPaf= wastewater from coffee from Atlantic forest after biological treatment; fresh WPc= wastewater from coffee from the cerrado region without treatment; spent WPc= wastewater from coffee from cerrado region after biological treatment.

The germination index varied from 4 to 51% in seeds exposed to fresh WPaf, and from 21 to 109% in spent WPaf; this is the greatest GI observed to seeds of *T. aestivum*. GI values for treated WPc and in nature varied from 31 to 75% and from 37 to 94%, respectively. Although toxicity was observed, the two spent WPs presented inductor effects of germination (GI >80%) of wheat seeds, that were below 25% in WPaf and 50% in WPc.

3.6 Coffee processing wastewater composition: Determination of acids, carbohydrates and alcohol content

Possible alteration in WPc and WPaf composition resultant from aerobic biological treatments with selected inocula related to composition of carbohydrate, alcohols and acids was conducted by HPLC (Table 7).

Fructose was detected in higher concentration, with 1,445.89 mg L^{-1} in WPc and 1,426.80 mg L^{-1} in WPaf. Glycose and sucrose presented initial concentrations of approximately 33 and 8 mg L^{-1}, respectively, and a final concentration equal to 0. Only fructose was detected after the treatment, however, in concentrations approximately 10 and 20 times lower in relation to fresh WPaf and WPc, respectively. Thus, it may be inferred that the analyzed carbohydrate was consumed by microorganisms during the treatment.

Alcohols were detected in both fresh wastewater, with values of around 200 mg L^{-1} of ethanol and of around 1400 mg L^{-1} of glycerol; however, after treatment alcohol was not detected in WPc, and just glycerol was detected in WPaf, with a final concentration of 40.79 mg L^{-1}, which represents a value that is 35 times lower that observed in the beginning of treatment.

Table 7. Mean concentrations of different compounds in coffee wastewater from Brazilian Cerrado (WPC) and Atlantic forest (WPaf) quantified by HPLC.

Origin	Compound	Concentration (mg L^{-1})			
		Fresh WPc	Spent WPc	Fresh WPaf	Spent WPaf
	Carbohydrates				
CP	Sucrose	10.90 ± 0.17	nd	5.82 ± 0.97	nd
CP	Glucose	33.54 ± 0.65	nd	33.47 ± 0.91	nd
CP	Fructose	1465.89 ± 7.78	68.75 ± 8.07	1426.80 ± 6.36	196.32 ± 0.31
	Total carbohydrates	1510.33	68.75	1466.09	196.32
	Alcohols				
MA	Ethanol	237.97 ± 3.17	nd	195.29 ± 1.01	nd
MA	Glycerol	1408.64 ± 4.44	nd	1393.85 ± 6.81	40.79 ± 5.35
	Total alcohols	1646.61	nd	1589.14	40.79
	Acids				
CP	Chlorogenic acid	0.63 ± 0.01	2.46 ± 0.09	0.56 ± 0.07	0.74 ± 0.01
MA	Citric acid	78.33 ± 4.14	nd	74.94 ± 1.25	nd
MA	Tartaric acid	16.16 ± 1.83	355.57 ± 7.05	19.91 ± 0.81	56.65 ± 8.51
MA	Malic acid	0.81 ± 0.02	3.21 ± 0.92	0.71 ± 0.10	28.20 ± 7.75
MA	Succinic acid	nd	275.42 ± 2.71	nd	134.56 ± 8.25
MA	Lactic acid	2277.93 ± 137.69	nd	2152.66 ± 4.76	nd
MA	Acetic acid	1019.77 ± 0.51	nd	1008.87 ± 0.55	nd
MA	Propionic acid	111.28 ± 0.47	546.23 ± 1.35	105.06 ± 0.68	763.93 ± 62.61
MA	Butyl acid	nd	33.17 ± 0.57	nd	27.61 ± 1.41
MA	Isovaleric acid	nd	9.99 ± 0.30	nd	18.97 ± 3.39
	Total acids				
	Others				
CP	Trigonellina	108.25 ± 3.26	6.36 ± 0.46	94.29 ± 17.07	20.25 ± 7.91
CP	Caffeine	32.05 ± 0.89	33.15 ± 0.39	111.92 ± 11.99	31.93 ± 0.43
MA	1,2-propanediol	17.03 ± 0.42	nd	19.02 ± 0.36	nd
	Total others	157.33	39.51	225.23	52.18

CP= Coffee processing; MA= Microbial activity.

Among the acids, lactic acid presented the greatest initial concentrations in WPc and WPaf (2,277.93 and 2,152.66 mg L^{-1}, respectively); however, it was not detected after treatment in both WPs. The same observations were made with respect to citric and acetic acids, and with 1,2- propanediol. In contrast, butyric, isobutylic and isovaleric acids were detected just after the biological treatment. The others had their concentrations increased by the microbial activity. Trigonelline concentration was reduced in both WPs after treatment with the selected mixed inoculum.

4 Discussion

Pre selection of inoculum was based on evaluation of the degradation of WP by a mixed culture of microorganisms once it is established that the microbial diversity is beneficial for biological treatment (Bathe et al. 2009; Jia et al. 2016; Lorah & Voytek 2004; Militon et al. 2015; Zhang et al. 2016; Zhou & Gough 2016; Zhu et al. 2017). Degradation of many pollutant substances found in effluents is faster and more effective in relation to microbial communities if formed by diverse species, with different relationships to various substances (Ding et al. 2016; Xie et al. 2014), such as nitrates, ammonium, carbohydrates and caffeine. BOD and COD are global indicators of organic matter, and diverse studies focus on reduction of these parameters using a microbial consortium as biological treatment of food waste, petrochemical wastewater, synthetic textile effluent, industrial wastewater and landfill leachate (Cydzik-Kwiatkowska & Zielinska 2016; Ding et al. 2016; Fong & Tan 2000; Hu et al. 2015; Karunya et al. 2014; Kekacs et al. 2015; Xie et al. 2014).

BOD reduction after biological treatment indicates an active contribution of organisms (Zaveri et al., 2015). A greater percentage of BOD and COD in WP by the action of mixed cultures with bacteria was expected (Ding et al. 2016; Karunya et al. 2014; Kekacs et al. 2015; Wang et al. 2013). The advantage of using a consortium with bacteria seen by the greater number of viable cells in WP, demonstrating its resistance to culture conditions (Cydzik-Kwiatkowska and Zielinska, 2016).

The rates of BOD (85.65%) and COD (83.05%) due to the microbial consortium action in WPc, the final values of 312.33 and 1477.00 mg L^{-1}, respectively, were high, but not enough to reach the recommended values by WHO (WHO 1995) and CONAMA 430 (BRASIL., 2005). However, according to CONAMA standards, the effluent could be released into the environment after this treatment, once the BOD reduction was greater

than 60% in relation to the value initially found. Thus the discharge of the effluent depends on rigorous studies on the capacity of self-purification of the receiving body, demonstrating that the minimum dissolved oxygen (DO) concentrations will not be disobeyed.

The final pH values between 7.5 and 8.5 observed in all WPc and WPaf treatments using yeasts, were in conformity with the recommendation of the WHO (6.5 to 8.5) (WHO 1995). This interval of 7.5 to 8.5, observed after biological treatment, is still beneficial because it results in nitrification, besides organic matter removal (Tchobanoglous, 1991).

On the other hand, final pH values of around 5.5 with WPaf treatment and microbial consortia stayed over of the recommended values by law (WHO, 1995). It can thus be inferred that this result is attributed to the WPaf composition, and consequently the alteration in available nutrients, used metabolic routes and generated subproducts. Acid production by microbial activity, such as succinic, butylic and isovaleric acids, observed in the biological treatment with T1 are responsible for reduction in pH values. If just WP pH values must meet legal limits, it is possible to infer that yeasts are more useful than bacteria in WPaf treatments.

The observed differences between WPc and WPaf in viable populations of each treatment and in biological treatments over the time is important; for instance, T1 can be associated with higher concentrations of biodegradable organic matter (BOD) and with elements such as phosphorus and nitrogen found in WPaf, which can be used as nutrients by microorganisms (Truper and Schleifer, 2006).

Besides the differences observed in microbiota in WPs composition, biological treatments achieved similar efficiencies in reduction of pollutants of biodegradable compounds, including carbohydrates, alcohols and some acids. The availability of

nutrients present and bioavailability of compounds that are degradable, as well as the distribution and microbial activity are decisive elements that depend on the effectiveness of the bioremediation process (Diplock et al., 2009).

Diverse characteristics and properties presented by bacteria in the mixed microbial inoculum could explain the action in the treatment of organic waste and the hardiness observed. *Acetobacter indonesiensis*, which has already been isolated from vinegar (Wu et al. 2012; Yetiman & Kesmen 2015), whey (Lima et al., 2016), brewery effluent (Olorode and Fagade, 2012), fermented rice flour (Tanasupawat et al., 2011) and fruits (Kommanee et al., 2012) is capable of oxidizing different sugars and alcohols, and participating in the degradation process of extracts such as that of beer, apple, pear, and pineapple (Huang et al., 2014).

Corynebacterium flavescens has already been described in association to rice roots (Bacilio-jim et al., 2003), and colonizing cheese (Brennan et al. 2002; Masoud & Jakobsen 2003). It has also been isolated from grey water treatment systems (Keely et al., 2015) and from cooking wastewater treatment systems (Joshi et al., 2016), and is involved in phosphate accumulation (Tarayre et al., 2017) and in metabolization of nitrogen compounds and different carbohydrates (Bacilio-jim et al. 2003; Gtari et al. 2012).

Serratia marcescens isolated from agroindustrial wastewater has been reported by Fulazzaky et al. (2016) as able to grow in effluents rich in organic matter, from cassava and corn processing (Montero-Rodríguez et al., 2016). These bacteria presented good activity of pectinolytic enzymes, responsible for the degradation of pectin, one of the main components of the coffee mucilage (Silva 2014).

Besides reduction of the measured compounds, biological treatment with mixed inocula was responsible for WP toxicity reduction. WP toxicity reduction may be verified

by germination induction in seeds of *T. aestivum*, and increasing values of EC_{50} in *D. similis*; moreover, using WPc in biological treatments seems been more efficient, resulting in a reduction of toxic effects in *D. similis* by 100%.

D. similis. is considerable sensitive, and has been the most efficient species in detecting toxicity of effluents generated by a veterinary pharmaceutical company (Maselli et al., 2015), therefore, the reduction in TU of *D. similis* in spent WP enhances the efficiency of the biological treatment. Ethanol removal can be influenced by a reduction of acute toxicity in *D. similis*, once the organisms of this genera are sensitive to the presence of ethanol (Hu et al. 2015; Silva et al. 2016).

In contrast, alteration in organic acid concentration is one of the factors related to phytotoxicity reduction; once these can have inductor or inhibitor effect in seeds germination according to the concentration that they are (Smith et al. 2003; Tunes et al. 2012). A reduction in the concentration of phenolic compounds in particular polyphenolics, as a result of the increase in pH after biological treatments (Chen et al., 2008), may also have contributed to the reduction of toxicity on the seeds of *T. aestivum*, because compounds like catechin, ferulic acid, and others are substantially phytotoxic (Li et al., 2010; Al Harun et al., 2015).

The fundamentals that no species is sensitive to all toxic substances, and the sensitivity to them varies with the toxic substance concerned, and with environmental conditions justifies the importance of evaluating more than one species for toxicological testing (Zagato and Bertoletti, 2006). Therefore, a reduction in toxic effects after biological treatment with mixed inoculum resulted in a removal of the most toxic compounds in WP.

5 Conclusion

A mixed bacterial inoculum compounded by using *S. marcescens, C. flavescens* e *A. indonesiensis*, with the capacity to reduce the polluting load of was selected. The growth, biological activity and efficiency of treatments in WPaf are indicators of the strength of the selected mixed bacterial inoculum for WP biological treatment, independent of the region where they were isolated. Organic compound concentration and WP toxicity were reduced after treatment. Although BOD and COD contents are below the official legal recommendation, the treatment needs to be improved; a reduction of more than 60% of BOD in relation to initial values allowed for the effluent be disposed.

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Supplementary material

Table S1. Composition of the assays using Plackett-Burman statistical design for selection of mixed yeast inoculum and pH value for the analysis of the pollutants present in the coffee wastewater.

Essay	Factors								
	Yeast								pH
	CCMA 1048	CCMA 1049	CCMA 1050	CCMA 1051	CCMA 1052	CCMA 1056	CCMA 1015	CCMA 1015	
L1	+	-	+	+	+	-	-	-	7
L2	-	-	+	-	+	+	-	+	6
L3	-	-	-	-	-	-	-	-	6
L4	+	-	+	+	-	+	+	+	6
L5	+	+	-	+	+	+	-	-	6
L6	-	+	-	+	+	-	+	+	7
L7	-	+	+	+	-	-	-	+	6
L8	-	-	-	+	-	+	+	-	7
L9	+	+	-	-	-	+	-	+	7
L10	+	-	-	-	+	-	+	+	6
L11	-	+	+	-	+	+	+	-	6
L12	+	+	+	-	-	-	+	-	7

*L1 to L12= Assays composed of the yeast combination and pH value.

Table S2. Composition of the assays using a Plackett-Burman statistical design for selection of mixed bacteria inoculum and pH value for the purification of the pollutants present in the coffee wastewater.

Essay	Factors							
	Bacteria							pH
	CCMA 1010	CCMA 1013	CCMA 1012	CCMA 1006	CCMA 1047	CCMA 1002	CCMA 1010	
B1	+	-	+	+	+	-	-	6
B2	-	-	+	-	+	+	-	7
B3	-	-	-	-	-	-	-	6
B4	+	-	+	+	-	+	+	7
B5	+	+	-	+	-	+	-	6
B6	-	+	-	+	+	-	+	7
B7	-	+	+	+	-	-	-	7
B8	-	-	-	+	-	+	+	6
B9	+	+	-	-	-	+	-	7
B10	+	-	-	-	+	-	+	7
B11	-	+	+	-	+	+	+	6
B12	+	+	+	-	-	-	+	6

*B1 a B12= Assays composed of the bacteria combination and pH value.

Table S3. Biological parameters of the biomass and physical chemical of the coffee wastewater evaluated after mixed culture of bacteria and mixed culture of yeasts, according to the treatments in a Plackett-burman design.

Essay*	Parameters					
	Biomass (mg L^{-1})	pH	CFU mL^{-1}	BOD (mg L^{-1})	COD (mg L^{-1})	Turbidity (UNT)
Bacteria						
B1	228.5	8.16	6.8×10^{9}	499	948	27.0
B2	194.1	8.35	1.2×10^{10}	1043	1877	25.7
B3	80.4	7.60	0	997	1499	24.0
B4	192.2	8.29	1.0×10^{10}	272	661	18.9
B5	230.6	8.17	2.5×10^{10}	136	264	15.4
B6	269.6	8.25	2.9×10^{10}	317	436	19.7
B7	172.8	8.24	1.4×10^{10}	725	1341	62.8
B8	212.3	8.13	1.0×10^{10}	272	308	88.2
B9	193.9	8.28	2.8×10^{10}	181	463	17.1
B10	224.6	8.24	1.3×10^{9}	680	1156	29.8
B11	203.9	8.31	2.1×10^{10}	227	639	21.0
B12	212.1	8.24	2.6×10^{10}	453	771	41.0
Yeast						
L1	477.0	8.02	9.9×10^{6}	1224.0	2227.38	25.6
L2	551.0	8.01	5.5×10^{6}	725.0	1279.09	19.2
L3	062.0	4.18	0	907.0	3660.85	30.3
L4	489.0	7.69	2.8×10^{6}	544.0	1808.37	46.0
L5	495.0	7.80	3.9×10^{6}	635.0	926.24	48.9
L6	661.0	8.06	5.9×10^{6}	731.0	815.97	53.9
L7	447.0	7.68	5.8×10^{6}	1133.0	1852.48	38.8
L8	591.0	7.95	5.3×10^{6}	725.0	1940.69	39.0
L9	566.0	8.15	4.1×10^{6}	725.0	1234.98	28.0
L10	566.0	8.00	3.3×10^{6}	544.0	1050.3	40.1
L11	589.0	7.97	5.8×10^{6}	907.0	1995.4	40.2
L12	672.0	7.74	7.2×10^{6}	91.0	727.96	18.9

*B1 to B12: Assays composed of different combinations of 7 bacteria cultured at pH 6 or 7; L1 to L12: Assays composed of different combinations of 8 yeasts grown at pH 6 or 7.

Table S4. Mean values of BOD, COD, nitrogen, phosphorus, color and turbidity of the coffee wastewater from a cerrado farm (WPc), and another farm in a region of Mata Atlântica (WPaf), measured after biological treatment with different combinations of microorganisms.

Assay	BOD (mg L^{-1})		COD (mg L^{-1})		Nitrogen (mg L^{-1})		Phosphor (mg L^{-1})		Color* (mg L^{-1} PtCo)		Turbidity* (uT)	
	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf	WPc	WPaf
T1	312 Cb	512 Da	1477 Da	1304 Da	0.033 Ca	0.093 Ca	0.522 Fb	0.573 Fa	1039.00 Cb	1833.00 Ba	0.933 D	1.367 E
T2	569 Ca	677 Da	1124 Da	1323 Da	0.067 Db	0.123 Ca	0.896 Eb	1.266 Ca	852.00 Db	1077.33 Ea	0.897 D	1.267 E
T3	644 Ca	631 Da	1962 Ca	2110 Ca	0.150 Ba	0.160 Ca	0.784 Ea	0.849 Ea	931.33 Db	1163.67 Ea	0.783 D	0.850 G
T4	453 Ca	520 Da	1940 Cb	2509 Ca	0.087 Db	0.137 Ca	0.933 Ea	1.367 Ca	788.33 Db	1261.00 Da	0.523 E	0.573 H
T5	463 Ca	475 Da	2087 Ca	2077 Ca	0.073 Ca	0.097 Ca	1.008 Eb	1.307 Ca	817.00 Db	1175.00 Ea	1.010 D	1.307 E
T6	483 Ca	597 Da	1763 Ca	1385 Da	0.173 Ba	0.220 Ba	1.045 Ea	1.092 Da	954.67 Db	1236.67 Da	1.047 D	1.093 F
T7	947 Bb	1118 Ca	2168 Cb	2463 Ca	0.323 Aa	0.353 Aa	1.381 Da	1.470 Ca	1160.33 Ca	1248.33 Da	1.383 C	1.470 D
T8	1118 Ba	994 Da	2844 Ba	2897 Ba	0.450 Aa	0.436 Aa	2.091 Ca	1.965 Ba	1312.00 Ba	1441.67 Ca	2.093 B	1.963 C
T9	1199 Bb	1446 Ba	2572 Bb	3098 Ba	0.380 Aa	0.407 Aa	2.277 Bb	2.577 Aa	1390.33 Ba	1469.67 Ca	2.277 B	2.577 A
T10	1017 Ba	1063 Ca	3131 Aa	3334 Ba	0.237 Ba	0.243 Ba	2.800 Aa	2.14E Bb	2027.00 Ab	2290.00 Aa	2.800 A	2.147 B
T11	325 Ca	529 Da	1499 Da	1248 Da	0.100 Ca	0.147 Ca	0.523 Fb	0.902 Ea	882.00 Db	1093.00 Ea	0.523 E	0.903 G
T12	2121 Ab	3389 Aa	3282 Ab	4962 Aa	0.400 Aa	0.443 Aa	2.943 Aa	2.553 Ab	738.99 Da	890.67 Fa	0.143 F	0.153 I

*The color and turbidity parameters were not reduced by the biological treatment with mixed inocula. **The values for turbidity were not influenced by WP, so it was compared only among the treatments.

Note: Averages followed by the same capital letter do not differ from each other in the columns and averages followed by the same lower case do not differ from each other in the lines, for each parameter, according to the Scott-Knott test, with 5% significance.

ARTIGO 3: Increasing efficiency in assisted depuration of coffee processing wastewater from mixed wild microbial selected inoculum

Abstract

This work evaluated the efficiency of bacterial bioaugmentation to the biological treatment of wastewater from coffee processing (WP) in a pilot wastewater treatment plant (WTP). BOD and COD values were the base of the treatment efficiency. *Serratia marcescens* CCMA 1010 and CCMA 1013, *Corynebacterium flavescens* CCMA 1006, and *Acetobacter indonesiensis* CCMA 1002 were previously selected. The microbial cocktail was inoculated and persisted in WP during all treatments. The wild species richness suffered minimal alteration, and up to nine species were found in each sampled time. The microbiota composition presented variation of a total of 13 species, despite the inoculation of the microbial inoculum. The greatest reduction of BOD (~33%) and COD (~25%) were observed between 72 h and 8 days of the biological treatment. The WP physico-chemical composition was influenced by the community composition and microbial activity. The WP toxicity in *Allium cepa* seeds was up to 60% lower, and the germination index (GI) was beyond 100% in the treated WP. The results of the WP biological treatment by bioaugmentation from native microorganisms in the pilot WTP indicated the greatest efficiency related to the spontaneous biological treatment of ARC. The effluent could be release in the environment without toxic effects to the plants.

Key words: Bacteria, Bioaugmentation, Indigenous microorganism, Biological treatment, Toxicity

Introduction

Biological treatment with microorganisms uses competent microbial communities for promoting the organic compound degradation, toxic substance transformation, and nutrient removal (Wells et al. 2011).

Among the different techniques that use microorganisms for the depuration of pollutants, bioaugmentation has been the most successfully applied in various environments, including wastewater systems (Bathe et al. 2009; Lorah and Voytek 2004; Ma et al. 2009; Iasur-Kruh et al. 2011; Zhou and Gough 2016).

Bioaugmentation is used as an *in situ* (Alexander 1999) bioremediation technique, and it is considered a green technology (Okoh and Trejo-Hernandez 2006) that uses specific degrader microorganisms to treat contaminated environment, in order to increase the degradation tax (Morikawa 2006). The utilization of microorganisms native to the contaminated location can be favorable (Xin et al. 2013), owing to the greater adaptability of contamination and resistance to local environmental variations, as well as the lower susceptibility to genetic variations caused by stress in the environment (Cerqueira et al. 2012). Pure strain or microbial consortium can be introduced in the treatment system, and the inoculum selection must consider the nature and complexity of the contaminant (substrate) that will be treated (Sabra et al. 2010). Usually, the microbial diversity benefits from biological treatment (Militon et al. 2015). This has led to mixed cultures of microorganisms being frequently studied (Weathers et al. 2016; Zhu et al. 2017; Bengtsson et al. 2017). As a result, bacteria are presented positively, as they are the most abundant and active group in systems of biological treatment (Sant'anna 2013).

The implementation of bioaugmentation in a pilot-scale wastewater treatment plant (WTP), simulating the working and environmental conditions of a real WTP, can

provide more reliable results during effluent treatment, since the environmental conditions in the field vary (Bengtsson et al. 2017; Huang et al. 2015).

Wastewater from semi-dry coffee processing (WP) is among the effluents generated in large quantities by agro-industrial activities, whose production in 2017 is estimated between 14.1 and 15.4 billion L in Brazil (CONAB 2016). In addition, the residual waste from coffee processing is rich in pollutant components, such as ammoniacal nitrogen (40 to 60 mg L^{-1}), total nitrogen (180 to 250mg L^{-1}), phosphorus (60 to 800mg L^{-1}) (Matos et al. 2001; Campos et al. 2010; Rattan et al. 2015), total solids (1,000 to 7,500mg L^{-1}) (Campos et al. 2010; Villanueva-Rodríguez et al. 2014), and residues of different fertilizers that usually contain potassium, nitrogen, and phosphoric acid, used in agricultural practices (FAO 2000). WP has high levels of chemical oxygen demand (COD) (3.4 to 50,000 mg L^{-1}) and biochemical oxygen demand (BOD) (1.8 to 20,000 mg L^{-1}) and pH 4.0 (Haddis and Devi 2008; Selvamurugan et al. 2010; Oller et al. 2011; Ferrell and Cockerill 2012; Rattan et al. 2015; Matos et al. 2001; Bruno and Oliveira 2008; Campos et al. 2010; Bonilla-Hermosa et al. 2014). It is necessary to treat WP before disposal in the environment or reuse (Matos and Lo Monaco 2003).

Some species within the genera *Acetobacter* sp., *Corynebacterium* sp., and *Serratia* sp. are described as being capable of destroying many substrates, and the occurrence of these microorganisms has already been reported in different types of agro-industrial waste (Denis and Irlinger 2008; Fulazzaky et al. 2016; Bartowsky et al. 2003; Sokollek et al. 1998; Huang et al. 2014; Montero-Rodríguez et al. 2016; Olorode and Fagade 2012; Suárez-Estrella et al. 2013). The aim of this work was therefore to provide the biological treatment for wastewater of the coffee bean processed through the bioaugmentation technique in a pilot WTP, using *Acetobacter indonesiensis*,

Corynebacterium flavescens, and *Serratia marcescens*, previously isolated from this effluent. The efficiency of the treatment was verified by the evaluating the phytotoxicity, BOD, COD, and the impact of inoculation of the native microbiota of WTP.

Materials and methods

Inoculum preparation

Cultures of *Serratia marcescens* CCMA 1010 and CCMA 1013, *Corynebacterium flavescens* CCMA 1006, and *Acetobacter indonesiensis* CCMA 1002 were previously isolated from WTP, and deposited at Culture Collection of the Agricultural Microbiology (CCMA). Each isolate was reactivated in nutrient broth (NB, % w/v: 0.3 meat extract, 0.5 peptone) and was individually cultivated to reach 10^7cells mL^{-1}.

Assembly of the pilot-scale effluent treatment station

The WTP prototype was built to simulate the working conditions of a real-scale WTP, which has a volume of operation in the farm of around 300,000 L. The prototype was compounded from three tanks with capacity of 50 L arranged sequentially, of which the first was used for aeration and the others for sedimentation of the solid material (Figure 1). Two duplicate systems were built.

In comparison to the effect of the inoculation in the treatment, all analyses described for the pilot-scale WTP were also made for the real-scale WTP samples.

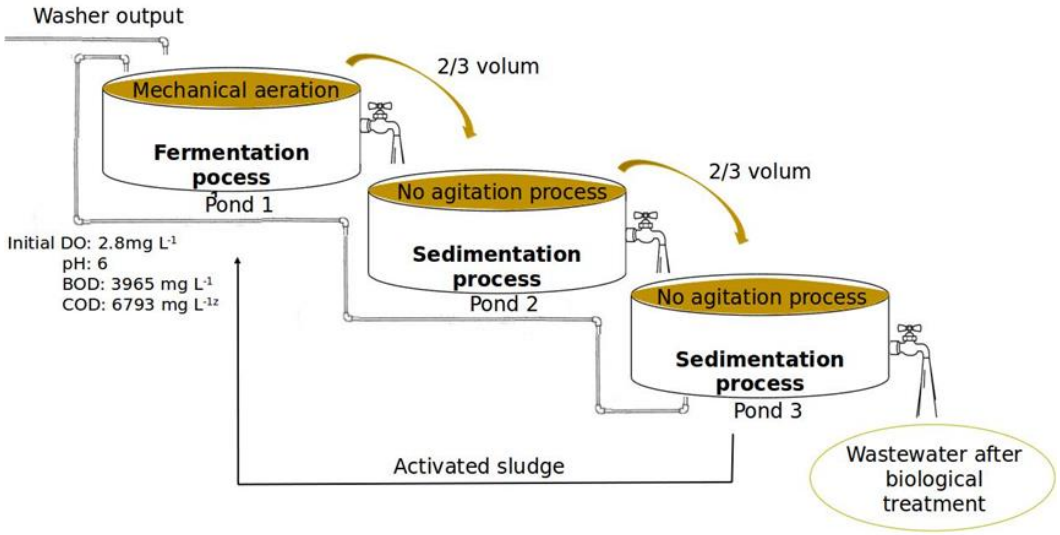

Figure 1. Schematic representation of the Wastewater Treatment Plant in pilot scale located at Fazenda Daterra, in Patrocínio, Minas Gerais, Brazil, to simulate the treatment conditions of wastewater from coffee in the field.

Bioaugmentation and operation of the pilot-scale effluent treatment station

The WP was filtered before being added to the WTP by using a system of hydrodynamic static sieves (pattern procedure in the farm), and it was immediately placed in Tank 1 of the prototype WTP. It was placed in 30 L of the effluent in each tank, the pH was adjusted to 6, and the aeration was supported mechanically.

The four studied bacteria (10^7 cells mL^{-1} concentration) were simultaneously inoculated in Tank 1 containing the WP, in proportion to 10% (v/v). After the microorganism inoculation, the tank stayed in constant aeration to maintain dissolved oxygen (DO) in 2 mg L^{-1} tax, measured periodically with a Dissolved Oxygen Measurer, Hanna HI9146. The first tank was initially operated in batches throughout seven days to allow the inoculum adaptation. From the 8^{th} day, operation started in a supplied batch system of the effluent, with a volume of 90 L of wastewater in each

prototype. The utilized effluent for feeding the pilot-scale WTP was collected daily after the filtering process already described and immediately placed in Tank 1.

In the subsequent stages, the water was transferred to the two sedimentation tanks, allowing the separation of the microbial flakes and further sedimentation. Every 24 h, 2/3 of wastewater was transferred to Tank 1 and from there to Tank 2, and 2/3 of the wastewater from Tank 2 was transferred to Tank 3. The sediment sludge was re-circulated to the aeration tank in order to maintain the microbial population (Sant'anna 2013).

The prototype WTP operated for a period of 10 days continuously, during which samples were removed to evaluate physico-chemical parameter, in order to monitor the permanency of the inoculums over the biological treatment of the coffee wastewater and to analyze the efficiency of the treatment process, as described in the following sections. The samples were removed within 0, 3, 5, 21, 24, 29, 45, 48, 53, 68, 72, 77, and 96 h, and 7, 8, 9, and 10 days of treatment. The temperature, pH value, DO, DOB, and COD inside of the prototype were monitored.

Monitoring of the bacterial population in the prototype WTP by DGGE

The persistence of the mixed inoculum and the impact of the inoculation in the bacterial diversity in the WTP during the treatment were verified by denaturing gradient gel electrophoresis (DGGE) (Pires et al. 2016).

Total DNA from the WP samples was extracted using the Power Soil® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. Genomic DNA was used as template for the amplification of microbial DNA in the target ribosome regions. Primer pairs 338 fGC (50-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG

CAG-30) (the GC clamp is underlined) and 518r (50-ATT ACC GCG GCT GCT GG-30) were used for bacteria.

Reaction and band observations were performed following the method reported by Souza et al. (2014) with modifications. To evaluate the DGGE profiles, data were clustered using STATISTICA® 8.0 software (StatSoft South America - Development Agency). The Euclidean distances were used for calculating the metric distances and cluster in the samples.

Analysis of sub chronic toxicity of WP

The phytotoxicity of WP was evaluated in onions (*Allium cepa*) according to the method reported by Sobrero and Ronco, with minor modifications.(Sobrero et al. 2004) Five concentrations of fresh and spent WP (12.5%, 25%, 50%, 75%, and 100%) as well as distilled water (as control) were used. Three replications, containing fifteen seeds for each concentration, were analyzed. The relationship between the spent vinasse and the control was used to calculate the relative germination (RG) of the roots (Equation (1)), root length (RL) (Equation (2)), and the germination index (GI) (Equation (3)), as follows:

$$RG(\%)=\text{Number germinated seeds WP}/\text{Number germinated seeds control} \times 100 \quad (1)$$

$$RL(\%)=\text{Average root length WP}/\text{Average root length control} \times 100 \quad (2)$$

$$GI(\%)=(((RG)x(RL)))/100 \quad (3)$$

Wastewater physico-chemical analysis before and after treatment

BOD and COD determination. The chemical oxygen demand (COD) and biochemical oxygen demand (BOD) parameters were determined according to the pattern recommended procedures in American Public Health Association (5220 D and 5210 B respectively) (APHA 2012).

Content determination of acids, carbohydrate, and alcohols. The analysis of the physico-chemical composition of WP is important for evaluating the microorganism activity during the biological treatment. Carbohydrates, caffeine, trigonelina, chlorogenic acid, and other acids are released in the water from the coffee fruits during the grinding of the beans, and they can be used (degraded) by the microorganisms for supply of nutrients. Acids and alcohols are normally results of the microbial metabolism during the metabolic process. The concentration of carbohydrates (sucrose, glucose and fructose), acids, and alcohols were determined by liquid chromatography of high efficiency (LCHE). The analysis was carried out using a Shimadzu chromatograph (Shimadzu Corp., Japan) equipped with detector of refractive index (RID-10A).

For quantification of carbohydrates, ethanol, and glycerol, an exclusion column of ions (Shimadzu -pack SCR-101H, 7.9 mm x 30 cm) was utilized. The analysis was operated at a temperature of 30°C by ultrapure acidified water with perchloric acid (pH 2.1) as effluent in a flux of 0.6 mL min^{-1}. The remaining identified acids in the samples were quantified following the same procedures, but at a temperature of 50°C (Bonilla-Hermosa et al. 2014).

Trigonelina, chlorogenic acid, and caffeine were determined using an O column of reverse phase Shimadzu (Shim-pack CLC-ODS (M)® C18, 100 mm of length x 0.3 mm DI) operated at 30°C. The moving phase was composed of ultrapure water, methanol, and acetic acid (79:20:1), with a flux of 0.6 mL min^{-1}.

The compound quantification was realized using the calibration curves built from different concentrations of standard compounds, injected under the same conditions of the samples (Duarte et al. 2011, 2013). All samples were analyzed in triplicate.

Results

Bioaugmentation and operation of the pilot-scale effluent treatment station

The temperature, pH, DO, and capacity of organic compound degradation by mixed inoculum was evaluated in the pilot-scale WTP (Figure 2).

Figure 2. Temperature variation and percentage of dissolved oxygen in the coffee wastewater over time, in a pilot scale wastewater treatment plant, under field conditions, after inoculation with mixed culture of the bacteria by *Serratia marcescens* CCMA 1010, *Serratia marcescens* CCMA 1013, *Corynebacterium flavescens* CCMA 1011, and *Acetobacter indonesiensis* CCMA 984.

The temperature varied during the treatment, exhibiting a minimum of 16.75°C and maximum of 25°C. The DO content increased over the time, varying from 1.7 mg L^{-1} (53 h) to 6.5 mg L^{-1} (9 days) and median value of 3.6 mg L^{-1}. The increase in DO

tax in the final half of the treatment coincided with the period where the pilot-scale WTP was operated in the feed batch system.

Monitoring of the bacterial population in the prototype WTP by DGGE

The permanency of inoculated microorganisms and the variation in the bacterial community in the pilot-scale WTP were evaluated by DGGE (Table 1). The profile of the bacterial community in the WP after treatment in the real-scale WTP was also compared.

The four strains of inoculated bacteria remained in the pilot-scale WTP until the 10^{th} day, with *S. marcescens, A. indonesiensis* and *C. flavescens* referred to as band A, band B, and band C, respectively. Ten other different bands were observed in the WP.

For the richness of the species in each sample, represented by the number of bands, there was a small change over the 10 days, with a variation between 7 and 9. The smallest number was found in the last day of the treatment. The composition of species, however, differed from the analyzed samples, and it was possible to observe a succession of species over time (Table 1). Band D, for example, was observed between 48 h and 8 days, bands G and I were observed in the initial stages (0 and 3 h), and bands F and H appeared at the end (9 and 10 days and 8 and 10 days, respectively).

Overall, the composition of the bacterial community in the WP in the real-scale WTP differed from all of the evaluated times in the pilot-scale WTP, although the richness of species was similar.

Table 1. Succession of bacteria in coffee wastewater during 10 days' biological treatment by bioaugmentation, in a pilot scale WTP.

Bands[1]	Isolates				Samples									
	CCMA 1010	CCMA 1013	CCMA 1006	CCMA 1002	0h	3h	24h	48h	72h	96h	8d	9d	10d	WTP
A	+	+	-	-	+	+	+	+	+	+	+	+	+	-
B	-	-	-	+	+	+	+	+	+	+	+	+	+	+
C	-	-	+	-	+	+	+	+	+	+	+	+	+	+
D	-	-	-	-	-	-	-	+	+	+	+	-	-	+
E	-	-	-	-	+	+	+	+	+	+	+	+	-	+
F	-	-	-	-	-	-	-	-	-	-	-	+	+	+
G	-	-	-	-	+	+	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	+	+	+	-
I	-	-	-	-	+	+	-	-	-	-	-	-	-	+
J	-	-	-	-	-	-	+	+	+	+	+	+	+	+
K	-	-	-	-	+	+	+	+	+	+	-	-	-	-
L	-	-	-	-	+	+	+	+	+	+	+	+	+	+
M	-	-	-	-	+	+	+	+	+	+	+	-	-	-

[1]Letters A, B and C correspond with the inoculated bacteria; Letters D to M correspond with the other bands detected on DGGE gel. + = detected and - = non-detected

The succession of species was also represented in a grouping of Euclidian distances that considered the similarity between the samples (Figure 3). The grouping formed two large groups, where the first was composed of samples from 0 h to 8 days of the experiment and the second was composed of samples from 9 and 10 days of the experiment and from the real-scale WTP.

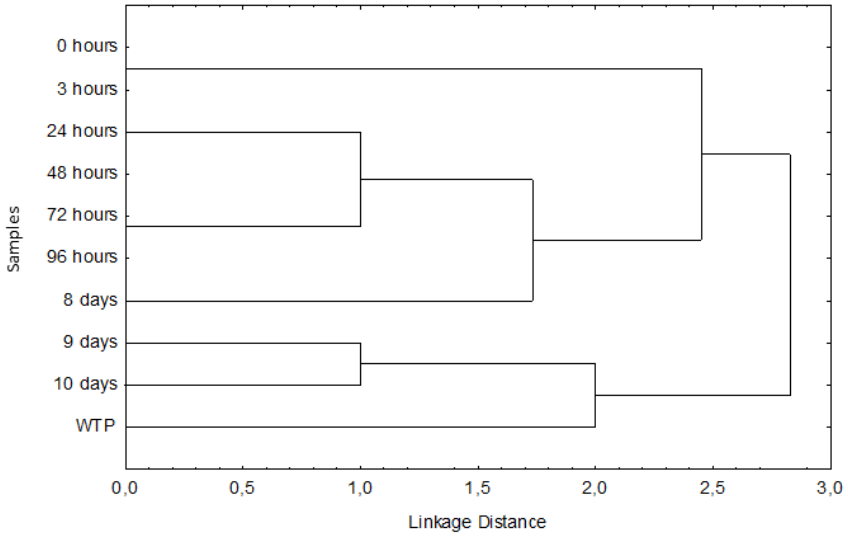

Figure 3. Cluster analysis of WP samples by Euclidean distances, based on the similarities of the microbial band profiles found in DGGE.

The grouping of the same profile of species for 0 and 3 h of the experiment was observed. This profile changed after 24 h and remained unchanged until 96 h after the bioaugmentation. After 8 days, there was a minimum alteration that decreased the similarity of the band profile with respect to the initial stages. Samples of 9 and 10 days were grouped in a separate branch because they presented larger alterations with respect to the species of all other samples. The species profile founded in the real-scale WTP was similar to that in the final stages in the pilot-scale WTP.

Physico-chemical analyses of the wastewater from the coffee processing

BOD and COD analyses. The removal of BOD and COD was more efficient between the interval of 72 h and 8 days of treatment, around 33% and 25%, respectively. This period embraced the beginning of the effluent entrance in the pilot-scale WTP in the batch system. Then, there was a small reduction in the values of BOD (7.86%) and COD (5%). The treated WP in the WTP presented lesser removal

percentages of BOD (7.15%) and COD (2.63%) when compared to the proposed treatment (Table 2).

Table 2. Mean values of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and removal efficiency, in relation to the initial values, of the coffee wastewater, after biological treatment with microorganisms, in the Wastewater Treatment Plant (WTP) on pilot scale and real scale.

[1]Time	BOD		COD	
	Means (mg L^{-1})	Efficiency of treatment (%)	Means (mg L^{-1})	Efficiency of treatment (%)
0 horas	3965 a		6793 a	
3 horas	3059 c	22.86	5485 b	19.25
24 horas	3125 c	21.18	4891 c	20.11
48 horas	2820 d	27.61	5540 b	18.44
72 horas	2644 d	33.32	4925 c	27.49
96 horas	2634 d	33.58	5059 c	25.53
8 dias	2591 d	34.66	5083 c	25.17
9 dias	2889 c	27.13	5847 b	13.92
10 dias	3629 b	7.86	6450 a	5.05
[2]WTP	3654 b	7.15	6554 a	2.63

Note: Means followed by the same letter do not differ from each other, within the columns, by the Scott-Knott test with significance of 5%.

[1]Times in hours and days correspond to WP samples treated at the WTP in pilot scale; [2]WTP corresponds to the WP sample treated at the real WTP.

Determination of the content of acids, carbohydrates, and alcohols. Different carbohydrates, alcohols, acids, and other compounds were quantified to evaluate the effect of biological treatment by bioaugmentation (Table 3). Except for sucrose, malic

acid, oxalic acid, and isobutyl acid, all analyzed compounds were detected for all evaluated times.

Table 3. Mean concentrations of different compounds in coffee wastewater quantified by HPLC.

Origin	Compound	Concentration in samples (mg L^{-1})				
		[1]0hours	3hours	8 days	10 days	[2]WTP
	Carbohydrates					
CP	Sucrose	139.49 ± 8.29	16.19 ± 1.25	nd	nd	nd
CP	Glucose	360.43 ± 45.05	114.26 ± 2.87	199.94 ± 6.87	231.71 ± 1.40	125.21 ± 0.50
CP	Fructose	858.19 ± 42.14	14.50 ± 1.37	271.51 ± 8.63	395.98 ± 5.38	314.76 ± 5.16
	Total carbohydrates	1218.68	268.25	471.45	643.88	439.97
	Alcohols					
MA	Ethanol	74.67 ± 7.45	62.38 ± 4.17	65.15 ± 2.63	72.46 ± 11.39	89.06 ± 11.39
MA	Glycerol	672.50 ± 22.61	18.71 ± 0.64	749.19 ± 20.09	711.27 ± 15.78	765.33 ± 0.96
	Total alcohols	747.17	81.09	814.34	783.73	854.39
	Acids					
CP	Chlorogenic acid	0.16 ± 0.02	0.29 ± 0.02	0.15 ± 0.005	0.18 ± 0.009	0.19 ± 0.0003
MA	Oxalic acid	nd	nd	2.47 ± 0.14	1.60 ± 0.14	1.51 ± 0.03
MA	Citric acid	40.19 ± 11.82	14.29 ± 2.23	11.60 ± 2.59	73.91 ±0.53	79.33 ± 0.11
MA	Tartaric acid	472.29 ± 40.03	157.39 ± 1.63	132.77 ± 37.16	150.31 ± 11.98	161.85 ± 1.99
MA	Malic acid	nd	6.01 ± 0.88	3.49 ± 0.67	5.15 ± 0.82	7.02 ± 0.21
MA	Lactic acid	1243.34 ± 94.75	256.91 ± 26.78	2095.31 ± 64.21	450.22 ± 40.97	2055.66 ± 5.24
MA	Acetic acid	764.39 ± 81.57	1464.68 ± 20.09	644.44 ± 79.16	1117.58 ± 15.89	1230.27 ± 0.71
MA	Propionic acid	57.95 ± 7.62	63.71 ± 0.28	172.29 ± 18.79	438.62 ± 46.11	246. 56 ± 7.65
MA	Isobutyl acid	50.33 ± 2.93	nd	nd	nd	nd
MA	Butyl acid	11.85 ± 0.60	122.69 ± 18.21	37.71 ± 1.13	199.99 ± 0.47	178.24 ± 23.65
MA	Isovaleric acid	120.29 ± 14.79	130.01 ± 23.85	186.25 ± 1.02	180.25 ± 12.13	216.57 ± 9.39
	Total acids	2760.65	2215.98	3286.48	2617.81	4177.20
	Others					
CP	Trigonellina	90.72 ± 2.81	95.89 ± 5.10	94.18 ± 12.16	91.94 ± 3.07	102.78 ± 0.66
CP	Caffeine	17.75 ± 1.05	15.11 ± 0.13	22.41 ± 3.09	22.69 ± 1.15	26.15 ± 0.13
MA	1,2-propanediol	nd	8.39 ± 0.22	9.34 ± 0.94	7.52 ± 0.94	8.76 ± 0.87
	Total others	105.83	122.03	125.93	122.15	137.69

CP= Coffee processing; MA= Microbial activity.

[1]Times in hours and days correspond to WP samples treated at the WTP in pilot scale; [2]WTPcorresponds to the WP sample treated at the WTP in real scale.

In general, the smallest values for each compound were observed after 3 h of bacterial inoculation and the largest values were observed after 10 days of treatment, which is similar to the initial values (without treatment). The lactic acid was the compound with a larger concentration in all analyzed samples, with a maximum of 2095.31 mg mL^{-1} after 8 days of treatment.

In the WP from the pilot-scale WTP, the fructose was the carbohydrate founded with a larger initial concentration (858.19 mg mL^{-1}), which remained high throughout the treatment; however, it reduced owing to the microbial activity. Among the alcohols, ethanol presented a low variation over time (62.38 to 74.67 mg mL^{-1}), and glycerol presented a maximum of 749.19 mg mL^{-1} with a significant drop (18.71 mg mL^{-1}) after 3 h. Caffeine and trigonelina presented small variations over the treatment time, from 90.72 to 95.89 mg mL^{-1} and from 15.11 to 22.69 mg mL^{-1}, respectively.

In the WP from the real-scale WTP, fructose was also the carbohydrate in larger concentration, and sucrose and isobutyl acids were not detected. The total concentration of each compound class of WP from the real-scale WTP was greater than the concentration of the samples treated by bioaugmentation.

Principal Component Analysis (PCA) was also conducted to evaluate the performance of the quantified compounds over the time period of the biological treatment. The 19 compounds detected in the five samples of analyzed WP were organized by PCA, in which the principal compounds represented 77.52% of the total data variation. Compound 1 corresponded to 47.54%, and Compound 2 corresponded to 29.98% of the total variability (Figure 4).

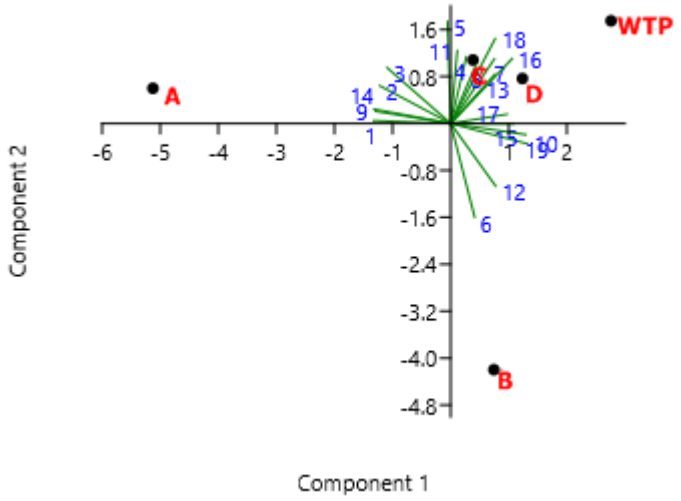

Figure 4. Principal components analysis (PCA) of the compounds detected by HPLC in five wastewater samples from processing of coffee fruits in five different sites of wastewater treatment: Points A to D= corresponded to time in hours of treatment after bioaugmentation in which the samples were collected, being: A= 0h, B= 3h, C= 8h, D= 10h; Point WTP= Wastewater after treatment in wastewater treatment plant in real scale. Numbers from 1 to 19 in vectors corresponded to compounds, being: 1= Sucrose, 2= Glucose; 3= Fructose, 4= Ethanol, 5= Glycerol, 6= Chlorogenic acid, 7= Oxalic acid, 8= Citric acid, 9= Tartaric acid, 10= Malic acid, 11= Lactic acid, 12= Acetic acid, 13= Propionic acid, 14= Isobutyl acid, 15= Butyl acid, 16= Isovaleric acid, 17= Trigonellina, 18= Caffeine, 19= 1,2-propanediol.

PCA analysis revealed that the compound profiles after 0 and 3 days are different from those of the other samples. The profiles after 8 and 10 days and the profiles of the samples of the treated WP in the real-scale WTP were grouped closer.

Acids and 1,2-propanediol were the components that most influenced the samples of 3 days, while acids and carbohydrates had more influence on samples of 0 days. The alcohols and some acids influenced on the grouping of the remaining samples.

Analysis of subchronic toxicity of ARC

The subchronic phytotoxicity on the *A. cepa* was utilized as a way to evaluate the efficiency of biological treatment of WP in the prototype and in real-scale WTPs.

The seeds of *A. cepa* demonstrated low sensitivity in contact to the WP. Induction of the germination (GI > 80%) occurred in the exposed seeds, the treated WP, and the non-treated WP when in low concentration (below 50%) (Figure 5).

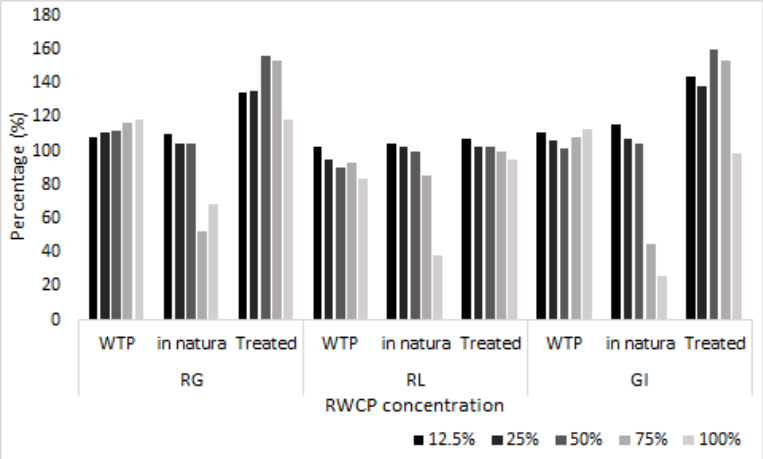

Figure 5. Percentage of relative germination (RG), root length (RL) and germination index (GI) *Allium cepa* (onion) over influence of untreated ARC and treated ARC in real and pilot scale WTP.

The best results were observed in the exposed seed and the treated WP in the pilot-scale WTP. The RG of the seeds in 50% and 75% of treated WP in the pilot-scale WTP were 160.45% and 153.69%, respectively, which reinforced the influence of the treatment.

The exposed seeds to WP without treatment presented small values of RG (52.26% to 68.77%) and GI (44.79% to 26.20%), which were observed in the presence of 75% and 100% of WP.

The seeds in the treated WP in the real-scale WTP exhibited intermediate values and small variation of the concentrations to RG and GI of 108.28% to 118.73% and 101.35% to 114.63%, respectively.

RL presented low variation between the evaluated WPs and between different concentrations. Statistical difference was observed in the seeds placed in 100% of non-treated WP, which exhibited the smallest tax (38.09%).

Discussion

Between 20 and 45 kg of wastewater per kilogram of coffee beans is generated during the wet and semi-dry coffee processing. Wastewater from coffee processing is rich in organic materials in suspension, organic, and inorganic constituents in the solution, such as sugars, proteins, pectin, cellulose, small quantities of natural coloring, and lipids (Dias et al. 2014). Owing to these characteristics, the effluent has high polluting power, and the reduction of these polluting compounds by aerobic biological treatment using bioaugmentation for the disposal or its reutilization can be an alternative to avoid the negative impacts on the environment and meet the regulations of the law (Li 2013; Pei et al. 2016). Several biological and biochemical parameters such as conductivity, suspended dissolved and total solids, total nitrogen, pH, total phosphorus, and bioassay for assessing whole effluent toxicity can be utilized as indicators for the efficiency of WP biological treatment of effluents (Howard et al. 2004) by bioaugmentation with native microorganisms in a pilot-scale WTP.

The efficiency of the microbial bioaugmentation process itself can be evaluated by analyzing the microbial population in the effluent (Herrero and Stuckey 2015; Zhao et al. 2016). The analysis of the bacterial population in the WP showed that *A. indonesiensis, C. flavescens, and S. marcescens* responded positively to the

bioaugmentation. The inoculated bacteria remained in the WP and the richness of the population did not present extensive variation over time, indicating that the microorganisms introduced in large population density did not interfere in a damaging way to the native bacterial community. These characteristics are fundamental to the success of the bioaugmentation (Tribedi and Sil 2013).

The variation observed in the composition of the bacterial community over time can be attributed to the fact that the feed system of the effluent in batches in the WTP makes the bioreactor of activated sludge an environment with opened community (Lee et al. 2015), where there is a bacterial local community (Leibold et al. 2004) and the immigration and emigration of bacteria occur continuously (Leibold et al. 2004; Lee et al. 2015). Thus, the activeness of the bacterial community is determined by the balance between the increase and decrease in the bacterial population in the bioreactor, the entry of population derived from wastewater, and the discharge of the unstable populations in the treated water (Hashimoto et al. 2014). The bioaugmentation of WP in the pilot-scale WTP was probably the main factor determining the difference in the microbiome with respect to the real-scale WTP.

The composition of the bacterial population influenced the reduction in the BOD and COD values. The period of greater reduction in BOD (~33%) and COD (~25%) (72 h to 8 d) coincided with the presence of seven endogenous species and the mixed inoculum. The maintenance of the highest reduction rate of BOD and COD in the sample of 8 days, even after the alteration in the bacterial community, can be attributed to a phenomenon named functional redundancy. This phenomenon, already observed in systems of biological treatment, in different species allowed the activated sludge to maintain the potential to degrade organic compounds in effluents, independent of the fluctuations in the microbial community (Hashimoto et al. 2014). The presence of

extracellular enzymes secreted by microorganisms, capable of hydrolyzing organic compounds, could promote the continuity in the reduction of BOD and COD values (Kurade et al. 2012; Theerachat et al. 2017; Frigo et al. 2017).

Despite the percentage of reduction in the BOD and COD, the real values obtained (2,591 mg L^{-1} and 4,925 mg L^{-1}, respectively) were still high and did not meet the law requirements (The government environmental agencies in Brazil are COPAM and CONAMA, and WHO regulates worldwide standards). The Resolution 430 of 2011 of the CONAMA determines the maximum concentrations of 60 mg L^{-1} and 180 mg L^{-1} for BOD and COD, respectively (CONAMA 2011). The process needs to be enhanced in order to achieve legally acceptable levels of BOD and COD. Increasing the operation time of the pilot-scale WTP in the feed batch could be an alternative for achieving considerable reduction in the values of BOD and COD in the WP, considering that the greater efficiencies in the removal of organic matter occur in the period that the WTP was operated in this system.

The greatest removal of BOD and COD taxes, reflective of the largest degradation of the organic matter, correlated with the increase in the DO tax in the final half of the treatment. This was attributable to the reduction in the organic matter with lower microbial activity and lower oxygen consumption (Zaveri et al. 2015). The level of DO in the water could be affected by the entry of WP in the treatment system, whereas the quantity of organic biodegradable pollutants could have been changed, and this factor influenced the dissolved oxygen.(Sant'anna 2013) The DO is fundamental to the good performance of the process of biological aerobic treatment, and the median value of 4.31 mg L^{-1} indicated that the pilot-scale WTP has a DO value that above the security limitation of 2 mg L^{-1} for O_2 (Sriwiriyarat et al. 2008).

The monitoring of temperature is important, since it directly reflects and affects the microbial activity (Andersson and Nilsson 2001; Dijkstra et al. 2011) and the DO during the biological treatment (Sant'anna 2013). The median temperature of 20°C verified in the WP during the operation of the prototype WTP is within the range of appropriate temperatures for the biological treatment (10°C–40°C), whose efficiency was optimized between 0 and 35°C (Sant'anna 2013). However, it can be suggested that the microbial activity and, consequently, the pollutant degradation can be maximized in slightly more elevated temperatures, closer to 35°C, which is the temperature of maximum activity of mesophilic microorganisms (Qiu et al. 2005).

The microbial activity was verified indirectly by the quantification of carbohydrate, alcohols, acids, and other compounds in the WP. *Acetobacter indonesiensis*, *S. marcescens*, and *C. flavescens* have the ability to oxidize different sugars and alcohols and therefore colonize various organic substrates (Brennan et al. 2002; Huang et al. 2014; Montero-Rodríguez et al. 2016). *C. flavescens* and *S. marcescens* are capable of metabolizing nitrogen compounds (Bacilio-jim et al. 2003; Gtari et al. 2012; Wang et al. 2016). Thus, the variations in the compound concentrations in the WP, detected by HPLC, can be attributed to the action of the microbial activity. Besides, the grouping of the samples by the proifile of the bacterial community, mainly in the final stages (8 and 10 days), can reinforce the influence of microorganisms on the WP composition during the treatment.

The reduction in the values of carbohydrates, alcohols, some acids, and caffeine after 3 h of treatment, suggested that these compounds could have been rapidly degraded by the microbial action of the inoculum that presented CFU mL^{-1}. The increase in the concentrations of the analyzed compounds from day 8 of treatment was probably due to the variations in the bacterial community during this period, which may

have led to the reduction of the capacity of degradation and/or in outworking of the effluent entry in the pilot-scale WTP containing greater quantities of these compounds.

Bacteria from gender *Acetobacter*, including *A. indonesiensis*, are responsible for the oxidation of ethanol into acetic acid (Yetiman and Kesmen 2015). Therefore, the reduction in the concentration of ethanol and increase in acetic acid, after 3 h, can indicate the action and metabolic activity of *A. indonesiensis* inoculated in the pilot-scale WTP.

The toxicology analysis must also be utilized as a parameter to evaluate the efficiency of biological treatment, which can reflect the biological effect of the contaminants (Brennan et al. 2002). The phytotoxicity analysis of the WP is important, since the WP is commonly just dumped into the ground.

In general, the WP presented low toxicity to onion seed. The toxic effect of the fresh WP only at higher concentrations indicates that the *A. cepa* seeds are probably slightly sensible to the toxic compounds present in the WP, or that these are not sufficient concentrations to cause damage to the seeds. The onion seeds did not show as good indicator of toxicity to the fresh WP. The absence of the toxic effect of the treated WP in turn indicated that the existence of toxic compounds were reduced by the biological treatment and that the produced metabolites after biodegradation are less toxic than in the fresh effluent (Vijayalakshmidevi and Muthukumar 2015).

Considering the results of phytotoxicity of *A. cepa*, it can be affirmed that the WP after biological treatment could be discharged in its concentrated form in the environment without causing toxic effects, which in the year 2016 corresponded to the estimated volume of 16.6 billion L of waste (ICO 2016). However, without biological treatment, the minimum dilution of the WP without toxic effect to *A. cepa* is 50% of the

effluent and, therefore, half of this generated volume could be discharged on the environment without damaging plants.

The variation in the microbiota was influenced by carbohydrates, acids, alcohols, trigonelina, caffeine, and 1,2-propanediol after day 10 of treatment in the pilot-scale WTP. Lower values of BOD, COD, and phytotoxicity were observed as compared to the fresh WP. However, these results were inferior to the ones obtained for treated WP in the pilot-scale WTP.

Conclusion

The bacteria *A. indonesiensis*, *C. flavescens,* and *S. marcescens* remained in the WP throughout the biological treatment, and they do not interfere in a damaging way to the native bacterial community. The biological treatment by bioaugmentation allows the reduction of organic compounds in the WP, apart from reducing their toxic effects on the plants, as compared to the spontaneous treatment and fresh WP. However, the process needs to be enhanced in order to achieve legally acceptable levels of BOD and COD. The biological treatment by bioaugmentation proposed was more efficient than the currently used spontaneous biological treatment. The bioaugmentation proved to be useful to accelerate the removal of pollutants and to improve the performance of the wastewater treatment.

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