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Identification of Wastewater Bacteria Involved in the Degradation of Triclocarban and its Non-Chlorinated Congener

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Abstract

Triclocarban (TCC) is an antimicrobial additive of personal care products that is only partially degraded during wastewater treatment. Bacteria responsible for its transformation are unknown. We obtained wastewater bacteria capable of using as the sole carbon source TCC or its non-chlorinated analog, carbanilide (NCC). Enrichments established using activated sludge amended with TCC and NCC, respectively, were maintained for one year through successive transfers. Enrichments displayed exponential growth after two weeks, reaching stationary phase after one month. The NCC enrichment was shown to accumulate aniline. Denaturing gradient gel electrophoresis of amplified 16S rRNA genes indicated markedly reduced community richness compared to the inoculum and a single, prominent taxonomic unit emerged in both chlorinated and non-chlorinated carbanilide enrichment cultures. Cloned 16S rRNA genes showed both enrichments were dominated by a single genotype related to uncharacterized organisms within the *Alcaligenaceae*. Of ~30 sequences from each enrichment, no other organisms were detected in the TCC enrichment while a small, flanking community of Alpha Proteobacteria was detected in the NCC enrichment. Study results demonstrate that growth of wastewater bacteria on TCC and its lower chlorinated analog can be linked to bacteria within the family *Alcaligenaceae*. These organisms are promising agents for the bioremediation of hazardous phenylurea pollutants.

Keywords

Carbanilide; Alcaligenes; wastewater bacteria; phenylurea herbicide; antimicrobial; Triclocarban

1. Introduction

Triclocarban (TCC) is a diphenyl urea compound that is widely distributed in terrestrial and aquatic ecosystems [1,2]. It is added to soaps, detergents and other personal care products for its antimicrobial properties. In the U.S., TCC is a high production volume (HPV) chemical consumed at a rate of 2.60 ± 0.68 mg per capita per day [3,4]. Since TCC-containing personal care products are applied topically on the skin, the bulk of the mass enters sewage treatment facilities, where only about 21% is actually degraded or lost; over

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70% of the mass loading of TCC to wastewater treatment plants sorbs to sludge and persists during sludge digestion, thereby opening a path for deposition of TCC on land with biosolids applied in agriculture and forestry as a fertilizer and soil conditioner [1]. A much lower percentage (<3%), but environmentally relevant mass passes through the wastewater treatment plant to enter into effluent-receiving surface waters, where it accumulates in aquatic organisms and sediment at mg/kg concentrations [4,5]. Gaining a better understanding of the interactions between wastewater microorganisms and TCC-related carbanilides is critical for devising more effective treatment approaches for this common contaminant and related phenyl urea pesticides.

Information about bacterial transformation and mineralization of TCC and its higher and lower chlorinated congeners is limited. In the 1970s, Gledhill observed TCC to persist in wastewater but also demonstrated its biodegradability after a two-week lag phase in TCC enrichment cultures inoculated with secondary activated sludge [6]. The microorganisms in the culture produced monochloro- and dichloroanilines as primary degradation products. None of the responsible bacteria were identified, however. In aerobic soil, Ying et al. observed that TCC concentrations decreased from 1.07 to 0.63 mg/kg over 70 days, indicating a half-life of approximately 180 days [7]. No degradation was observed by Ying et al. under anaerobic conditions, however, other work provided evidence for slow dechlorination of TCC over a fifty-year time span in estuarine sediment [4].

More information on organisms potentially capable of TCC metabolism may be garnered from studies of bacterial degradation of similar phenyl urea compounds. Sorenson et al. [8] isolated a moderately halophilic *Marinobacter* sp. from contaminated desert streambed sediment able to grow on the non-chlorinated analog of TCC (NCC) as the sole carbon and nitrogen source. The organism completely degraded NCC with transient accumulation of aniline as the primary degradation product. A variety of studies have also reported degradation of phenyl urea herbicides (e.g., linuron and diuron) by mixed microbial consortia. These compounds are similar to TCC but lack one of the two phenyl rings. Dejonge et al. [9] enriched for a mixed assemblage of soil bacteria growing on linuron. An isolated *Variovorax* species was shown to grow on linuron as sole carbon and nitrogen source. The organism degraded linuron with transient accumulation of dichloroaniline. A variety of bacteria have been isolated that can degrade chloroanilines including *Pseudomonas*, *Commamonas*, *Nitrosomonas*, *Acinetobacter*, *Klebsiella*, and *Alcaligenes* species among others [9–13].

Since its discovery as a widespread environmental contaminant in the U.S. [2], TCC has emerged as one of the most frequently detected and most persistent pharmaceuticals and personal care products (PPCPs) in the natural and built water environment [14]. Whereas the compound is known to be transformed to a limited extent, it is presently unknown which organisms carry out this function and whether TCC metabolism can be linked to microbial growth. In this study, we enriched from activated sludge bacteria capable of using TCC or NCC as the sole carbon and energy source, and showed them to belong to a heretofore unknown taxonomic genotype, by using a combination of denaturing gradient gel electrophoresis and analysis of cloned 16S rRNA gene sequences.

2. Materials and Methods

2.1 Sampling and enrichment cultures

A two- liter activated sludge sample was taken from the secondary treatment basin of the Baltimore Wastewater treatment plant in June of 2006 for use as inoculum in TCC and NCC enrichment cultures. Substrates were added to empty flasks in methanol stocks and the methanol was allowed to evaporate in 2 hours at 50°C before adding 70 ml of M9, mineral

salt medium that contains only nitrogen sources but no carbon sources [15]. A 50-ml aliquot of each sludge sample was allowed to settle for 30 minutes in a 50 ml-conical tube to remove most protozoa, and only then 1 ml of the supernatant was added to each enrichment flask. Since TCC and NCC are sparingly soluble in water, they were added at concentrations in excess of their solubility at 1 mg/ml. Control flasks contained M9 medium and either substrates without inoculum or contained inoculum without substrate. Cultures were shaken in the dark at 30 °C and inspected for growth periodically by monitoring for increases in total protein in 50-μl samples using the bicinchoninic acid protein assay (Thermo; Rockford, IL). Upon reaching the stationary phase, 5-ml aliquots were transferred to fresh medium and on several occasions, 1-ml aliquots were taken for DNA extraction. Enrichments were continuously cultured for at least one year and 1-ml samples were periodically obtained and frozen at −80°C in 50% glycerol.

2.2 Microscopy

Enrichments were monitored under phase contrast microscopy in 10-μl wet mounts using an Olympus BX41 microscope fitted with epifluorescent optics and a color digital camera. The BacLight live-dead staining (Molecular Probes, Carlsbad, CA) assay was used to qualitatively assess survival of bacteria. A 50-μl aliquot of culture was stained according to the manufacturer's suggested protocol and 10 μl observed by epifluoresence microscopy at 400X magnification.

2.3 High Performance Liquid Chromatography (HPLC)

Aniline and carbanilide were qualitatively determined in cell-free supernatants on a Shimadzu HPLC equipped with a DGU-14A eluent degaser, two LC-10ADvp gradient pumps, an SCL-10Avp system controller (Shimadzu Corporation), and a UV detector at a wavelength of 254 nm. Compound separation was achieved on an Eclipse XBD C18 column (5 μm particle size, 2.1 × 150 mm; Restek Corporation; Bellefonte, PA). Chromatography was carried out using an isocratic method and 15:85 methanol:phosphate buffer (pH 2.5) running at 1 ml/min. Identification of peaks was based on co-elution of unknown peaks with those of authentic standards of aniline and NCC (Sigma-Aldrich) at a retention time of 3.0 and 10.1 min., respectively.

2.4 DNA Extraction, PCR and Denaturing Gradient Gel Electrophoresis (DGGE)

Cells were pelleted by centrifugation at 14,000 x *g* for 10 min., resuspended in TE buffer and lysed with 1 mg/ml lysozyme for 10 min at room temperature. Proteins were digested with 100 μg/ml proteinase K in 0.5% sodium dodecyl sulfate for 1 hour at 50 °C. Total community DNA was then extracted using phenol-chloroform followed by CTAB (cetyltrimethyl ammonium bromide) according to standard protocols [16]. The resulting extract was precipitated with 1 volume of cold isopropanol, washed with 70% ethanol, dried and resuspended in 50 μl of sterile double distilled H_2O.

Bacterial community composition was determined by DGGE analysis of ~400 bp portions of PCR-amplified 16S rRNA genes from most bacteria using a forward primer (1070f, ATGGCTGTCGTCAGCT), reverse primer with GC- clamp (1392r, ACGGGCGGTGTGTAC) and procedures previously described [17]. Sybr green stained polyacrylamide gels were scanned on a Typhoon 9410 scanner (GE Piscataway, NJ) with excitation at 488 nm, emission at 520BP, and an experimentally optimized PMT gain to prevent band saturation. Subsequently, the images were analyzed in Photoshop CS4. The vertical pixel position of bands was determined based on a reference point at the top of the gel and the average pixel intensity (0–254) of each band was determined using the marquee tool and histogram function. A table of band intensities or operational taxonomic units

(OTUs) by samples was then analyzed by hierarchical cluster analysis using the heatmap function in the R 2.7 statistical package.

2.5 Cloning and Sequencing

Samples of extracted DNA from each enrichment culture were used as template in a PCR to amplify the partial 16S rRNA gene using primers 8F (AGAGTTTGATCCTGGCTCAG) and 907R (CCCCGTCAATTCCTTTGAGTTT). All reactions were in triplicate using 1, 10 or 50 ng of pooled template DNA from each culture. Each 50-μl reaction mixture contained 5 μl of 10X buffer (Promega, Madison, WI), 6 μl of 25 mM $MgCl_2$, 2.5 μl of dNTP's (5 mM), 2 μl of forward and reverse primers (10 μM), 1 μl of pooled template DNA and 0.25 μl of Taq polymerase (5U/μl). The thermocycler protocol consisted of 95 °C for 5 min., followed by 30 cycles of denaturation (95°C for 30s), annealing (55 °C for 45s) and extension (72 °C for 60s); and ending with a final extension at 72 °C for 10 min. The amplified 16S rRNA gene regions were cloned into Escherichia coli cells using the Topo TA cloning kit per the manufacturer's instructions and bidirectionally sequenced by SeqWright (Houston, TX) using M13R and M13F primers on an ABI Prism 3730xl DNA sequencer.

Base calling, quality trimming, and masking of cloning vector sequences was accomplished using Phred and Crossmatch [18] with default cutoff values. Putative chimeric sequences were detected using Bellerophon [19] or the Chimera Check program [20] including sequences from this study in the analysis. The top five Blast hits were retrieved using the blastn program provided with the NCBI blastcl3 package [21]. All sequences were imported into ARB [22] and aligned with a recent version of the Silva ARB Ref database (updated 4-10-08) containing quality-checked, pre-aligned small subunit rRNA gene sequences [23]. The ends of the alignment were masked along with column gaps. Distance matrices were calculated by DNADIST in Phylip [24] using the Jukes Cantor model [25].

2.6 Tree construction

Neighbor-joining and maximum likelihood trees were initially generated in ARB using ~700 bp portions of aligned sequences. Enrichment sequences and phylogenetically- related reference sequences were then exported from ARB in a single alignment file in Phylip format and used to generate a final maximum likelihood tree with CIPRES (http://www.phylo.org/sub_sections/portal/) implementing Randomized Accelerated Maximum Likelihood for bootstrapping [26] and a general time reversible gamma distributed model. The number of bootstrapping replications was determined automatically by the program and the maximum likelihood search function was selected to identify the best-scoring tree. Other trees were constructed by parsimony with PAUP [27] or by Bayesian inference using MrBayes 3.1.2 [28].

2.7 Accession Numbers

Partial 16S rRNA genes obtained during this study were submitted as a phylogenetic study with an alignment to Genbank under the accession numbers HM045847 - HM045909.

3. Results

3.1 Growth of wastewater bacteria on TCC and NCC

Wastewater bacteria inoculated into media containing either TCC or NCC as the sole carbon source grew exponentially after a lag phase of two weeks as indicated by an increase in total protein (Fig. 1A). No growth was observed in control flasks containing M9 medium and substrates without inoculum or containing M9 medium and inoculum without substrates (data not shown). At the onset of exponential growth, aniline was detected by HPLC in the NCC enrichment (Fig. 1B), and a brownish-yellow color developed in this culture (Fig. 1C).

As total protein increased, reductions in the height and area of peaks corresponding to NCC were also evident (Fig. 1B). Both enrichments achieved approximately equal levels of total protein (140 – 170 μg/ml) at the stationary phase of growth and exhibited doubling times estimated at 1.49 and 2.13 days for the NCC and TCC enrichment cultures, respectively. This growth cycle did not change when cells from late exponential phase were used to inoculate fresh media, or during one year of continuous culturing.

Microscopic observations showed that the bacteria formed biofilms around TCC or NCC crystals (Fig. 1D and 1E). In general, BacLight live-dead staining showed that cells closest to these crystals fluoresced in red, orange or yellow color, indicating they had been stained by propidium iodide and suggesting a lack of membrane integrity. Living cells colored by Syto9 stain and appearing in green were evident in patches within the biofilms and in the pelagic population.

3.2 Comparison of enrichment bacterial community composition

Bacterial community profiles generated by denaturing gradient gel electrophoresis (DGGE) showed a ~60% reduction in community richness from the inoculum (Fig. 2). A total of 23 OTUs were detected in the secondary activated sludge sample used as the inoculum. In contrast, the TCC and NCC enrichments produced 14 and 15 OTUs, respectively, and when the TCC enrichment was sub-cultured on NCC as the sole carbon source, the number of OTUs decreased to 11.

Hierarchical cluster analysis grouped the 50 OTUs detected in Fig. 2A into eight groupings based on occurrence (Fig. 2B). Several OTUs occurred exclusively in only one culture. Nineteen OTUs occurred only in the inoculum and were not detected in any of the enrichments. Of the 15 OTUs detected in the NCC enrichment, 12 were exclusive to this culture and three were detected in the inoculum. Similarly, seven of 14 OTUs were exclusive to the TCC enrichment culture and only one was detected in the inoculum. More than half of the OTUs in the TCC enrichment and TCC enrichment sub-cultured on NCC were shared, as would be expected. Three OTUs were detected in one or more of the enrichment cultures that were not detected in the inoculum, suggesting they were not abundant in the inoculum. Only one of these occurred in all three enrichment cultures and was not abundant at a relative band intensity of <0.1.

Overall, one OTU was dominant in all samples. The OTU 242 occurred in all three enrichment cultures, was visible in the inoculum, and had the greatest band intensity in all cultures at > 0.1 relative intensity (Fig. 2B). This OTU appeared to remain abundant in at least the NCC enrichment over two consecutive months of monitoring (Fig. 2C). While slight shifts in the profile were evident, OTU 242 remained dominant. Taken together, the data suggest that bacteria represented by OTU 242 are the most abundant in these enrichment cultures. To identify the abundant bacteria, we cloned and sequenced 16S rRNA genes from the TCC and NCC enrichment cultures.

3.3 Phylogenetic analysis of TCC and NCC utilizing bacteria

A single genotype (TCCNCC1) dominated clone libraries from both the TCC and NCC enrichments with an average, within group identity of 99.5% across 58 sequences. Of 32 sequences of a length of ~700 bp recovered from the TCC enrichment, all were of the TCCNCC1 genotype while 26 of 30 sequences recovered from the NCC enrichment were of this genotype. In addition to the TCCNCC1 genotype, four other sequences were detected in the NCC enrichment. Due to the dominance of the TCCNCC1 genotype further sequencing was not accomplished.

Phylogenetic analysis of the TCCNCC1 genotype indicated that it falls within an uncharacterized lineage of the Beta subclass of Proteobacteria (Fig. 3). The most closely related characterized organisms were in the family Alcaligenaceae including *Pusillimonas*, *Pelistega*, and *Alcaligenes* species, but percent sequence identity over the ~700 bp was low (<95% on average). In addition, the TCCNCC1 grouping is clearly separated from these organisms by high bootstrap values. The most similar strains (i.e., Bacterium RM1 and Bacterium RM17) were previously detected in wastewater [29]. None of the other >1 million reference sequences in the Silva database were more similar to those already shown and overall tree topology was conserved using Neighboring Joining, Parsimony and Bayesian analysis (data not shown).

Other organisms detected in the NCC enrichment were similar to Alpha Proteobacteria. NCCE5 and NCCH5 were nearly identical (99.7%) and similar to Phylobacteriaceae previously shown to be involved in denitrification or nitrate reduction in an aquaculture treatment system [30]. NCCG3 was most similar to the chemolithoautotroph, *Oligotropha carboxidovorans* and to unknown Alpha Proteobacteria within a waste gas-degrading community [31]. NCCB6 was most similar to an unknown organism (AJ534616) from a uranium mining waste treatment facility. Overall, these analyses show that bacteria detected in enrichments resemble other bacteria previously detected in wastewater and that the bacterial communities in both carbanilide enrichment cultures are dominated by a single genotype.

4. Discussion

Enrichment culturing methods have proven useful in the isolation of bacterial strains possessing phenotypes not easily detected in the natural environment. The production and use of TCC over the last half century has led to the accumulation of this compound in environmental compartments, including soil, sediment and living organisms, primarily resulting from a lack of complete mineralization of TCC during wastewater treatment [4,5,7]. In this study, we have used the enrichment culturing technique in an attempt to isolate and identify at least some of the bacterial species capable of growing at the expense of TCC in secondary activated sludge. Our study provides the first identification of bacteria that could be involved in TCC or NCC degradation and points toward a group of bacteria that may be targeted in future studies.

Both enrichments were dominated by a single genotype, suggesting that organisms represented by the TCCNCC1 genotype are most abundant in these cultures. However, it is also possible that these bacteria are dominant because they are consuming breakdown products produced from other bacteria in the culture. This seems less likely given the extreme dominance of the TCCNCC1 genotype in clone libraries and the presence of a sole enrichment substrate, as well as the long period over which the cultures could be stably maintained. Further sequencing of clones may have revealed greater diversity and while cloning and PCR bias can affect bacterial diversity, it is unlikely that proportions of taxa would change substantially with further sequencing. This conclusion is supported by DGGE analysis that showed a single, dominant OTU was shared across all enrichment cultures and inoculum (Fig. 3); no other OTUs were shared across all cultures and inoculum.

The TCCNCC1 genotype is most closely related to other clones or strains previously detected in water and wastewater. Specifically, Bacterium rM1 and rM17 were the most similar organisms by Blast analysis and in maximum likelihood trees. Bacterium rM1 and rM17 were previously detected in activated sludge after it had been treated with phenol at 1 g per liter per day [29]. While any physiological similarities between rM1 or rM17 and the TCCNCC1 genotype are unknown, it is interesting that these phylogenetically related

organisms appear in wastewater in response to high levels of recalcitrant carbon sources generally thought to be toxic or inhibitory to other organisms. Based on live/dead staining results, it is possible that overcoming cytotoxicity of carbanilides may be a significant factor for growth of wastewater bacteria on these antimicrobials.

Other bacteria related to the TCCNCC1 grouping include *Pusillimonas* and *Pelistega* species. *Pusillimonas noertemannii* (previously strain BN9) degrades 5-aminosalicylate and *Pelistega europeaea* is an avian pathogen [32,33]. Based on a phylogenetic analysis of 16S rRNA genes, these genera along with *Brackiella*, *Kerstersia*, *Oligella*, *Sutterella* and *Taylorella* form a coherent cluster [33–35]. In our analysis, the TCCNCC1 grouping is clearly separated from this cluster and other *Alcaligenes* spp., but is still most closely associated with Alcaligenaceae (Fig. 3). Based on 16S rRNA gene phylogeny, the TCCNCC1 grouping along with Bacterium rM1, rM17, and other clones (e.g. EU528218) may represent a novel genus within the Alcaligenaceae. However, confirmation of this will require further phenotypic analyses.

Bacterial degradation and growth on TCC and related phenyl urea compounds appears to be rare. Essandoh et al. reported 56% biodegradation of TCC applied to packed soil columns, but this activity stopped after 9 days and chemical oxygen demand removal was dramatically inhibited. Similarly, Snyder et al. report that <4% of TCC in biosolids amended soils was mineralized over a period of 7.5 months [36]. The authors attribute this low activity to a loss of bioavailability of the compound. In this study, we show that growth of bacterial populations on TCC is sustainable, but only in mixed culture and only after prolonged enrichment. Whereas attempts to isolate a pure culture capable of mineralizing TCC failed here, there stable cultivation of carbanilide-metabolizing microbial consortia, as demonstrated here, represents an important milestone in the quest for TCC degraders.

Results of his study should help in efforts to exploit bacterial metabolic capabilities for the bioremediation of chlorinated carbanilides as well as related phenyl urea herbicides, as these compounds may be metabolized by a similar pathway. Such a pathway is proposed in Figure 4. It is likely that triclocarban and phenyl urea herbicides are initially degraded by bacteria through hydrolysis of the urea bridge, thereby producing 4-chloroaniline and 3,4-dichloroaniline (Fig. 4, Reaction 1). In most bacteria, 4-chloroaniline is deaminated and hydroxylated by an aniline dioxygenase producing 4-chlorocatechol [37–40] (Fig. 4, Reaction 2). The degradation of 3,4-dichloroaniline is less clear, but may proceed via dehalogenation (Fig. 4, Reaction 4). The resultant monochloroaniline may undergo deamination and hydroxylation to 4-chlorocatechol by a dioxygenase enzyme [10–12,40,41] (Fig. 1, Reaction 2). Intermediate 4-chlorocatechol is then degraded in *ortho*- or *meta* cleavage pathways via 1,2- or 2,3- catechol dioxygenase enzymes producing 3-chloro-*cis,cis*-muconate or 5-chloro-2-hydroxymuconic acid semialdehyde, respectively (Fig. 4, Reactions 3 and 5) [42,43]. These products are then degraded by intermediary metabolic pathways resulting in carbon and nitrogen assimilation. In wastewater, TCC occurs at concentrations in the parts-per-billion range and are thus too low to select for and sustain microbial growth on this compound. In contrast, digested sewage sludge applied on land as fertilizer contains TCC at 3–5 orders of magnitude higher concentrations, levels that are sufficiently high to support microbial growth but also could be inhibitory.

In conclusion, this study demonstrates that the hazardous compound TCC and its congener NCC can serve as substrates for the growth of newly identified environmental bacteria. Bacterial growth was linked to the presence of a single dominant genotype related to the Alcaligenaceae. These organisms deserve further study, due to their remarkable ability to utilize as carbon and energy sources anthropogenic carbanilide congeners carrying up to three chlorine substituents. This metabolic capability suggests that these organisms could

serve for the remediation of environments containing toxic carbanilide and phenyl urea pollutants.

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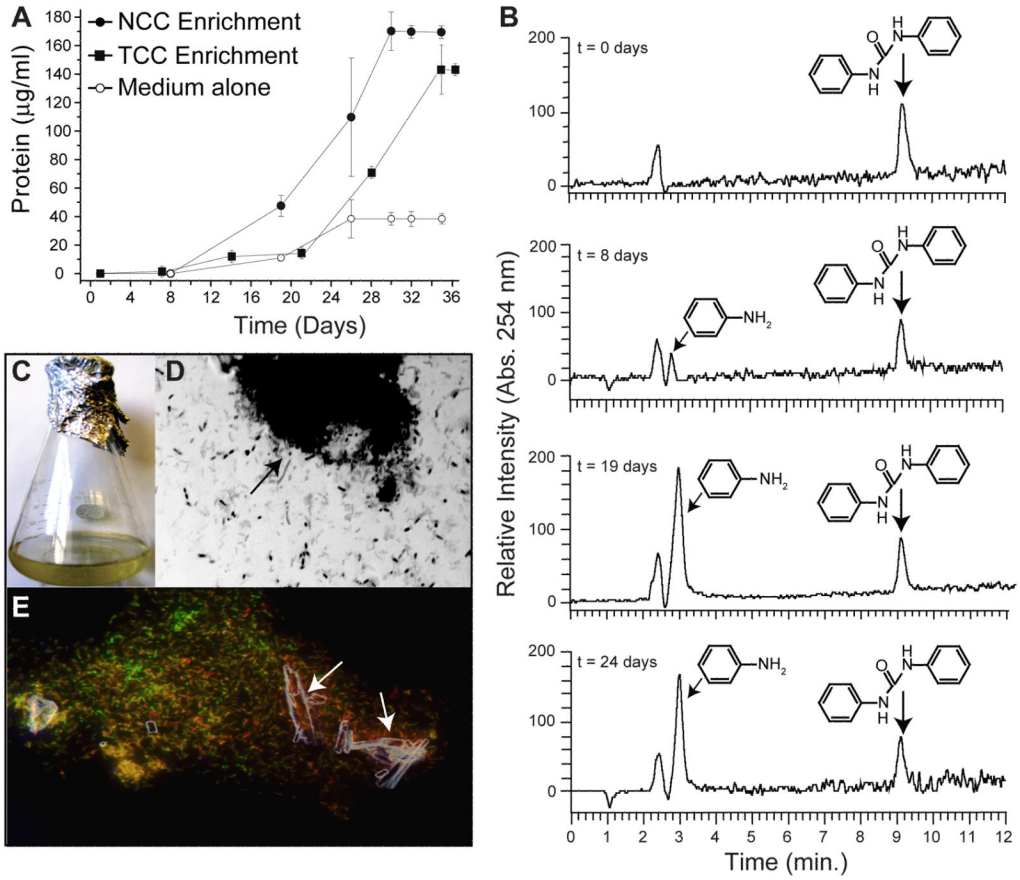

Figure 1.

A) Growth of wastewater bacteria on NCC and TCC as the sole carbon source. **B)** HPLC chromatograms showing aniline and carbanilide in the NCC enrichment. The peak area and height of aniline increases at the expense of carbanilide during exponential growth. **C)** The NCC enrichment during exponential growth. A yellowish/brown color is indicative of aniline production. **D)** Phase contrast image of bacteria in the TCC enrichment. Arrow points to a biofilm formed around TCC crystals. **E)** A composite micrograph (400X magnification) showing a bacterial biofilm around TCC crystals (arrow) with live/dead fluorescent staining. Brightfield illumination shows TCC crystals. Green cells (“live”) are stained with the membrane permeable Syto9 and red, orange to yellow cells (“dead”) are stained with both Syto9 and membrane- impermeable propidium iodide.

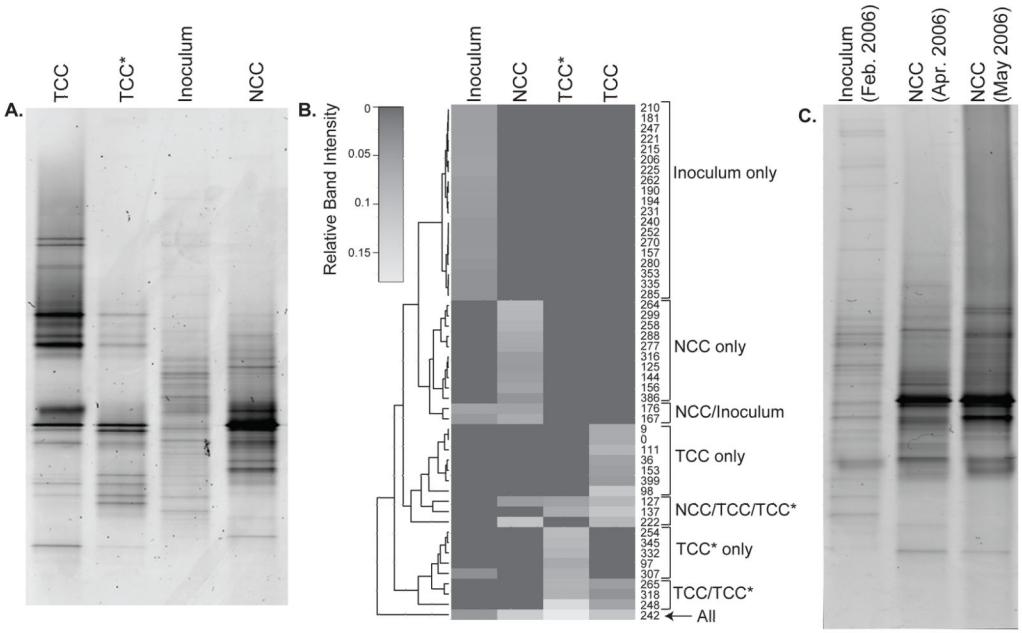

Figure 2.

A) Comparison of bacterial community profiles from the inoculum, NCC, TCC and TCC enrichment cultured on NCC (TCC*). Partial 16S rRNA genes amplified by PCR were separated on polyacrylamide gels containing 40 – 60% denaturant. **B)** hierarchical cluster analysis of OTU occurrence patterns based on relative band intensity. Heatmap shading denotes relative band intensity. The dendrogam (left) clusters OTUs into 7 groups listed on the right. Only one OTU occurs in all enrichments (OTU 242, arrow) and had the greatest band intensity. B) Comparison of bacterial community profiles from the inoculum and NCC enrichment over two consecutive months of culturing. Slight shifts in the NCC profile are evident, but the major band at OTU 242 remains dominant.

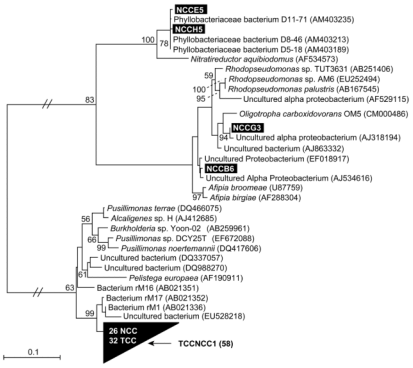

Figure 3.

Maximum likelihood tree analysis of ~700 bp partial 16S rRNA gene sequences recovered from the TCC and NCC enrichment cultures and reference sequences. Numbers next to nodes represent confidence in bootstrap replications. A single genotype related to an uncharacterized branch of the Alcaligenaceae dominates both enrichment cultures while a flanking community of Alpha proteobacteria is present in the NCC enrichment culture.

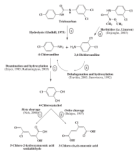

Figure 4.
Proposed pathway for TCC degradation based on previous studies. See text for details.