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RESEARCH ARTICLE

Hydrothermal carbonization of livestock mortality for the reduction of pathogens and microbially-derived DNA

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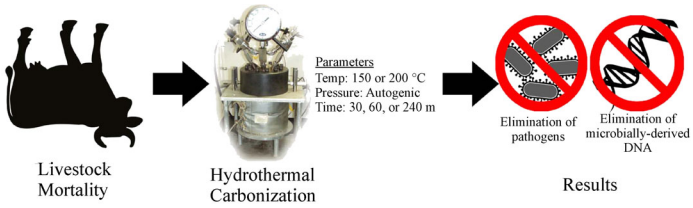
HIGHLIGHTS

- Hydrothermal carbonization treatment eliminates pathogens and microbial DNA.
- Hydrothermal carbonization treatment worked at both 150°C and 200°C.
- Hydrothermal carbonization treatment worked in both bovine bone and tissue.
- 30 minute treatment was sufficient for pathogen kill and complete DNA degradation.

GRAPHIC ABSTRACT



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ABSTRACT

Hydrothermal carbonization (HTC), utilizing high temperature and pressure, has the potential to treat agricultural waste via inactivating pathogens, antibiotic resistance genes (ARG), and contaminants of emerging concern (CEC) in a environmental and economical manner. Livestock mortality is one facet of agricultural waste that can pose a threat to the surrounding environment. While several methods are utilized to treat livestock mortality, there remains a paucity of data on the elimination of microbially-derived DNA in these treatment practices. This DNA, most notably ARGs, if it survives treatment can be reintroduced in agricultural environments where it could potentially be passed to pathogens, posing a risk to animal and human populations. HTC treatments have been successfully utilized for the treatment of CECs, however very little is understood on how ARGs survive HTC treatment. This study aims to fill this knowledge gap by examining the survivability of microbially-derived DNA in the HTC treatment of livestock mortality. We examined three treatment temperatures (100°C, 150°C, and 200°C) at autogenic pressures at three treatment times (30, 60, and 240 min). We examined the amplification of a plasmid-borne reporter gene carried by *Escherichia coli* DH10B introduced to both beef bone and tissue. Results indicate that while all three temperatures, at all treatment times, were suitable for complete pathogen kill, only temperatures of 150°C and 200°C were sufficient for eliminating microbial DNA. These results serve as the basis for future potential HTC treatment recommendations for livestock mortality when considering the elimination of pathogens and ARGs.

1 Introduction

Livestock mortality in animal production systems may pose a potential threat to surrounding environments with emissions of nutrients, organic matter, malodorous volatile organic compounds, and pathogens – most often affecting air, surface and groundwater, and soil. A treatment method that can adequately process livestock mortality, that contains or eliminates infection potential while reducing environmental pollutants, is critical for the sustainability of animal production systems. Current methods of disposal of livestock mortality involve pit burial, composting,

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incineration, and rendering [1]. Both pit burial and composting run the risk of pathogen survival and contamination of groundwater and soil [2,3]. Incineration is expensive, and has the potential to aerosolize infectious particles, further exacerbating the problem.

Hydrothermal carbonization (HTC) is a thermochemical process that converts solid biomass at elevated temperature and pressure – in the presence of water and absence of oxygen. Under high temperature and pressure environments, organic matter in the biomass is decomposed and a carbonaceous solid called hydrochar is produced [4]. Therefore, HTC treatments may prove to be an efficient, economical, and biosecure treatment alternative.

Along with pathogen reduction, the fate of microbial DNA after agricultural waste treatment remains an area of active research [5]. While many treatments are sufficient to kill pathogens and antibiotic resistant bacteria (ARB), their ability to degrade DNA to the point where the genetic information encoded is unusable often remains undetermined. This raises the potential of significant amounts of cell-free, microbially-derived DNA carrying pathogenic factors such as antibiotic resistance genes (ARGs), being introduced to agricultural environments [6]. In turn, this DNA can then be transformed directly into pathogenic organisms, which can potentially pose health risks to animals and humans, or into non-pathogenic organisms, which may then serve as a reservoir for future transfer into pathogenic microbes [7].

The use of HTC treatment methods for municipal and agricultural systems is steadily gaining acceptance as a rapid and efficient process [8,9]. These methods also have the advantage of saving time and energy expense by avoiding dewatering and drying processes, as well as being able to sequester CO_2, and destroy contaminants of emerging concern (CEC) which include a number of organic molecules such as pesticides, antibiotics and other pharmaceutically active compounds, and endocrine disruptors. The solid HTC product (hydrochar) can also be used as a soil amendment to improve soil quality [10] and an environmental sorbent to remove pollutants from water [11,12].

Despite the heavy emphasis on the use of hydrochar produced from hydrothermally carbonizing agricultural wastes, very little work has been done to understand the effects of HTC on nucleic acids [13]. The work that has been performed in this area has primarily tested the survivability of DNA as a contaminant in sterilization procedures in clinical or research settings. For example, a study by Suyama and Kawaharasaki [14] demonstrated that purified DNA can survive steam autoclave conditions (121°C; 0.10 MPa) for over 60 min before reaching undetectable levels.

Therefore, given the paucity of data in this area of research, the aim of this study was twofold: 1) to confirm that HTC methods result in total pathogen kill; and 2) determine if HTC methods result in the elimination of DNA of microbial origin. We studied HTC treatments conditions of 150°C, and 200°C at autogenic pressures. These treatments were tested on the bone and tissue of stillborn beef calves (*Bos taurus*), spiked with *Escherichia coli* carrying a novel gene fragment in a high copy plasmid. For comparative purposes, we also looked at treatment of bone and tissue at 100°C. The survival of microbially-derived DNA was determined by amplification by polymerase chain reaction (PCR). This study is the first in documenting the effects of multiple HTC temperatures and pressures on the elimination of pathogens and reduction of microbially-derived DNA on livestock mortality.

2 Materials and methods

2.1 Material

A stillborn beef calf (*Bos taurus*) carcass (examined by a veterinarian as disease free) was frozen whole until processing. The intact frozen carcass was then sectioned into strips that ranged in width from 5 to 10 cm using a food-grade band saw. Carcass strips were then randomly selected for further sectioning into cubes of 2.54 cm× 2.54 cm (1 inch×1 inch) dimensions. Carcass cubes were stabilized for storage until they could be processed by freeze drying until a stable weight was achieved. Cubes were sorted by type (e.g., bone or tissue), and were kept dehydrated until they were ready to process at which point they were rehydrated.

2.2 Bacterial strain and plasmid

Escherichia coli (Ec) strain DH10B (F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *φ*80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L *end*A1 λ^-) was the representative "model" strain for this study. The plasmid used in this study was pCPDnosZ1 (GenBank Accession: HQ674784), a pCR4.1-TOPO construct carrying a 460 base pair (bp) *nosZ* gene fragment amplified from *Paracoccus denitrificans* PD1222 [15]. This pCR4.1-TOPO construct was selected for use in this study because it contains the pUC origin of replication, resulting in high (>400) copy numbers per cell [16], and *nosZ* is not a gene found natively in Ec DH10B, thereby allowing us to trace any amplifiable DNA of this product directly back to the plasmid. Since pCR4.1-TOPO carries two antibiotic resistance genes, selection for Ec carrying the pCPDnosZ1 plasmid was performed with either ampicillin (100 μg $\cdot mL^{-1}$) or kanamycin (50 $\mu g \cdot mL^{-1}$).

2.3 Experimental Design

Prior to each experiment, an inoculant culture of Ec carrying pCPDnosZ1 was grown for 16 h at 37°C in 500 mL of Luria-Bertani (LB) broth supplemented with

kanamycin (50 μg·mL^{-1}). A total of 1 mL was used for serial dilutions and plating to determine starting colony forming units (CFU) on LB agar (Sigma Aldrich, St. Louis, MO) supplemented with kanamycin (50 μg·mL^{-1}). The remaining culture was then pelleted by centrifugation (10 min for 2500 rpm) and supernatant was discarded.

For experiments examining beef bone, carcass cubes consisting of bone were manipulated with a 1.3 cm diameter forstner bit to create a core (1.3 cm diameter by 1.9 cm depth) inside the bone cube. This core was packed with pelleted Ec and capped with a "bone cap" created by using a 1.3 cm diameter plug cutter (see Fig. 1). Any pelleted Ec remaining that could not fit into the core of the bone cube was placed on the outside of the cube. The cube was then submerged in 50 mL of phosphate buffered saline (PBS) and allowed to incubate for 30 min at room temperature before being subjected to HTC treatment.

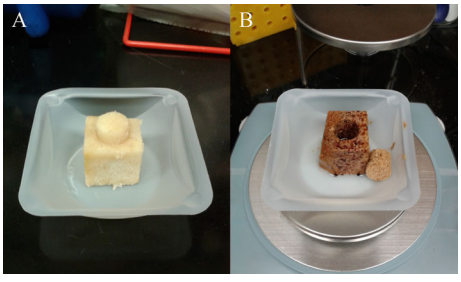

Fig. 1 Example of bone sample for testing pathogen and microbially-derived DNA reduction. Panel A represents pre-treatment, Panel B represents post-treatment.

For experiments examining beef tissue, carcass cubes consisting of tissue only were reconstituted in 45 mL of PBS for 30 min prior to HTC treatment. Pelleted Ec was then resuspended in 5 mL of LB broth, added to the resuspended beef tissue carcass cubes, mixed thoroughly by hand. The Ec and cube mixture was allowed to incubate for an additional 30 min at room temperature before being subjected to HTC treatment, providing time for the Ec to associate with the beef tissue [17].

Beef bone and tissue experiments, were performed at two HTC treatment temperatures (150°C, and 200°C) at autogenic pressures, with an additional treatment experiment performed at 100°C and 0.10 MPa. All experiments were conducted at three treatment times (30, 60, and 240 min). For each temperature, a control was performed, with setup as described above, but instead of being subjected to HTC, the experiment was incubated for 4 h at 37°C in a water jacketed incubator.

All bone and tissue experiments were placed in 250 mL Pyrex beakers and inserted inside a 1-L non-stirred T316 stainless steel reactor with an external heater (Parr Instruments, Moline, IL). The reactor was heated to either 150°C or 200°C with a heating rate of 7°C·min^{-1}. The reactor temperature was maintained for treatment times of 30, 60, or 240 min under autogenic pressure (Fig. 2). For experiments performed at 100°C, the 250 mL Pyrex beakers were placed in a Tuttnauer 2540EA autoclave (Tuttnauer, Hauppauge, NY) at treatment times of 30, 60, or 240 min at a pressure of 0.1 MPa. Since autoclave treatment times are limited to 99 min, the 240 min experiment was performed in three cycles of 80 min.

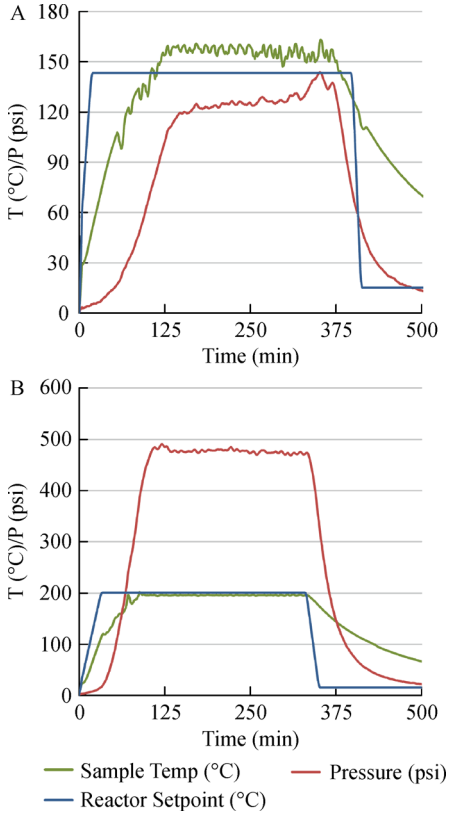

Fig. 2 Representative temperature and pressure profiles from Parr reactor for 150°C (Panel A) and 200°C (Panel B) with four hour treatment times.

2.4 Verification of Pathogen Elimination

While all analyses were performed above known sterilization pressure and temperature thresholds, pathogen counts were performed as a quality control measure to provide assurance of the HTC process in regards to pathogen reduction. After treatment, samples – and control reactions – were collected and analyzed for growth of Ec on LB agar with and without kanamycin (50 μg·mL^{-1}). Starting with undiluted sample liquid, serial dilutions down to 10^6 were performed. Ec was enumerated by plating a total volume of 50 μL from the sample, and each dilution, using the spread plate technique. Enumerations were performed in triplicate and incubated at 37°C, and plates were examined for visible colonies at 24, 48, and 72 h post-plating and CFUs were calculated.

2.5 Verification of DNA Reduction

To determine the amount of DNA reduction, DNA was isolated from samples using a PowerViral Environmental DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The *nosZ* gene fragment contained on pCPDnosZ1 was amplified via polymerase chain reaction (PCR) using both *nosZ*-specific primers (nosZF – 5′ CGYTGTTCMTCGAC AGCCAG 3′ and nosZ01622R – 5′ CGSACCTTSTTGC CSTYGCG 3′) and M13 (–20) Forward (5′ GTAAAACG ACGGCCAG 3′) and M13 Reverse (5′ CAGGAAAC AGCTATGAC 3′) primers. PCR amplification was carried out using GoTaq Green Master Mix (Promega, Madison, WI) in total volumes of 50 μL. Final reagent concentrations per reaction were as follows: 1X Master Mix; 200 nM of each forward and reverse primer; and 10 μL of DNA extraction. PCR reaction conditions were as follows: (1) an initial denaturation at 95°C for 5 min; (2) 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s; (3) a final extension at 72°C for 7 min. PCR reactions were visualized by gel electrophoresis on a 1% agarose gel strained with SYBR Safe (Invitrogen, Carlsbad, CA). Gel electrophoresis images were captured on a SafeImager 2.0 Blue Light Transilluminator (ThermoFisher Scientific, Carlsbad, CA) using VisionWorks LS Image Acquisition Analysis Software (UVP, Upland, CA) in a CCD camera-equipped EpiChemi II Darkroom (UVP, Upland, CA).

DNA extracted from HTC treated samples (150°C and 200°C) were further examined using a high sensitivity DNA kit (Agilent Technologies, Santa Clara, CA) analyzed on a 2100 Electrophoresis Bioanalyzer (Agilent Technologies, Santa Clara, CA). Analysis was performed according to manufacturer recommendations. Briefly, a total of 1 μL of DNA extraction was loaded into each sample well alongside 5 μL of high sensitivity DNA marker. Chromatograms were then visually examined.

3 Results and discussion

3.1 Pathogen elimination

As expected, all samples undergoing HTC treatment at 150°C and 200°C demonstrated total pathogen kill (Fig. 3). Additionally, samples treated in the autoclave at 100°C similarly demonstrated 100% pathogen reduction (Fig. 3). All three temperatures examined are known to be above sterilization temperatures required for inactivation of vegetative bacterial pathogens [18], however they serve as a quality assurance step to demonstrate pathogen kill via HTC treatment. Comparisons between the Ec inoculant cultures (mean: 1.87×10^{11} CFU) and the controls grown at 37°C for 4 h (mean: 3.72×10^{10} CFU), demonstrated roughly a log fold reduction in the control cultures; this reduction is potentially due to slight die-off during incubation with PBS [19].

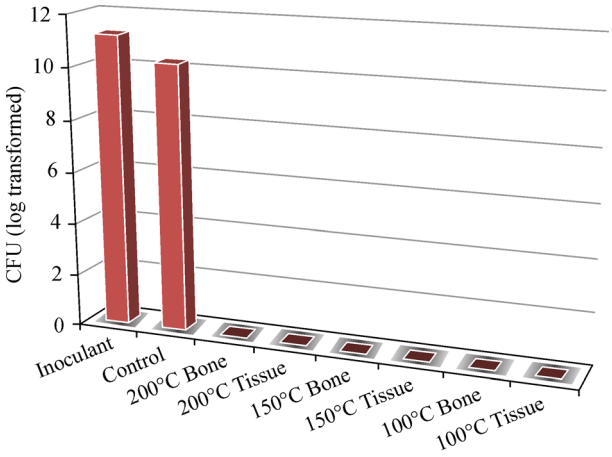

Fig. 3 *Escherichia coli* DH10B CFU after HTC treatment. Inoculant and control samples were grown at 37°C.

These results confirm claims by a number of HTC reviews that the treatment process leads to elimination of pathogens [4,9]. It should be noted the livestock material used during this study was of small dimensions (2.54 cm x 2.54 cm) due to restraints in reactor size, thereby making sample internal temperatures relatively easy to achieve and maintain at desired levels. For treatment of whole animal carcasses, the carcass will have to be comminuted so internal temperatures can reach the desired treatment temperatures for the required treatment times.

3.2 Reduction of microbially-derived DNA

Results of attempts to amplify extracted DNA from both bone and tissue samples, post-HTC treatment, can be seen in Figs. 4 and 5, respectively. For both 150°C and 200°C, DNA was undetectable by amplification via PCR at 30, 60

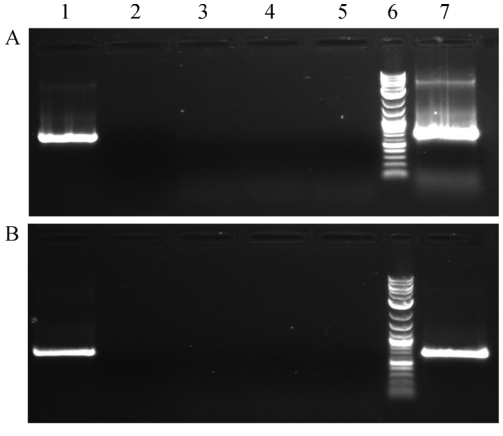

Fig. 4 Amplification results from DNA extractions after HTC treatment of bovine bone samples at 150°C (Panel A) and 200°C (Panel B). Lanes are as follows: 1) inoculant; 2) negative control; 3) 30 min; 4) 60 min; 5) 240 min; 6) New England Biolabs 2-log DNA ladder; 7) positive control.

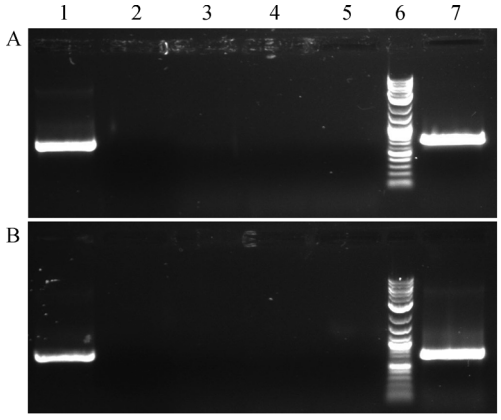

Fig. 5 Amplification results from DNA extractions after HTC treatment of bovine tissue samples at 150°C (Panel A) and 200°C (Panel B). Lanes are as follows: 1) inoculant; 2) negative control; 3) 30 min; 4) 60 min; 5) 240 min; 6) New England Biolabs 2-log DNA ladder; 7) positive control.

and 240 min treatment times. Control samples treated in a similar fashion during all stages of the experiment showed amplification products of the expected ~460 base pair length. These results indicate that temperatures of 150°C or greater, and pressures of at least 0.79 MPa (115 psi) for approximately 30 min (Table 1) were sufficient to eliminate microbially-derived DNA in both the tested bone and tissue livestock mortality samples. For samples treated for 240 min, achieving minimal temperatures of 137±3°C and pressures of 0.61±0.03 MPa (88±5 psi) were sufficient for eliminating microbially-derived DNA from both bone and tissue livestock mortality samples.

Further analysis of extracted DNA using a high-sensitivity DNA kit on an Agilent Bioanalyzer revealed that no DNA was present in the HTC treated samples (Fig. 6). These results indicate that HTC treatment temperatures of 150°C and 200°C at autogenic pressures are enough to eliminate microbially-derived DNA in animal mortality samples, most likely due to hydrolysis [20].

Samples treated at 100°C can be seen in Fig. 7, and both bone and tissue samples show DNA capable of being amplified by PCR at all treatment times – though amplifiable PCR products are markedly reduced at 240 min. These runs were originally intended to serve as a control, to show pathogen kill while retaining the ability to amplify DNA released from the killed bacteria. Reduction in amplifiable DNA in the 240 min samples correspond with results which show significant rates of depurination of DNA at temperatures of 100°C at extended treatment times [20]. Therefore, extended treatment times, beyond 240 min, at 100°C may result in total elimination of microbially-derived DNA, and may prove useful especially if desired hydrochar byproducts require reduced treatment temperatures. The benefits of lower temperature treatment, particularly at atmospheric pressure, however

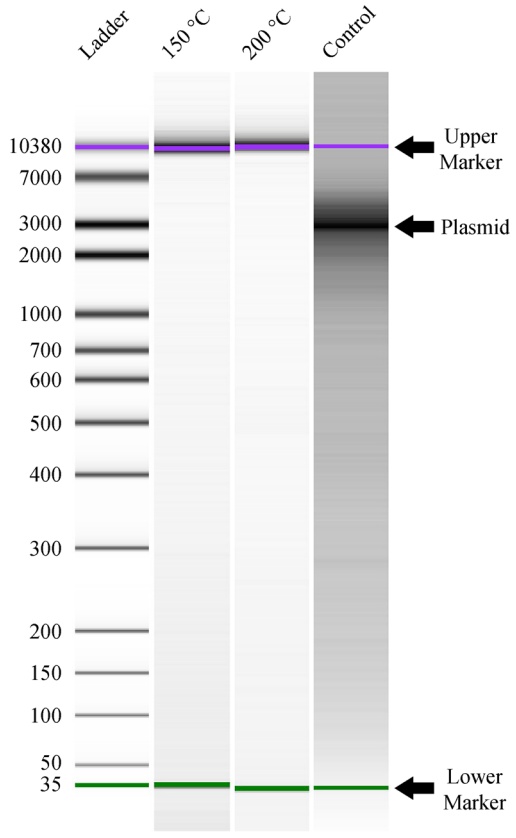

Fig. 6 Representative Bioanalyzer results from DNA extractions after HTC treatment for 30 min at 150°C and 200°C, along with untreated plasmid. The lower marker is 35 bp while the upper marker is 10,380 bp in length. Ladder lengths (in base pairs) are on left hand side.

must be weighed against the potential for bacterial spore survival [21]. These spore-forming bacteria, some of which may be pathogens in their own right, could result in propagation of antibiotic resistance genes, the very objective HTC treatment sought to eliminate [22].

With the emergence of colistin resistance in animal operation facilities [23,24], waste treatment technologies such as HTC have the potential to play a greater role in waste management. The ability of HTC treatment to rapidly inactivate both pharmaceutically active compounds (i.e., antibiotics) and the DNA that confers resistance to these compounds provides it an advantage over many other treatments. These advantages can potentially include an increased range of treatable contaminants, as well as reductions in operation costs and time. Operational costs are affected by both temperature and treatment time. Our studies demonstrate that for elimination of pathogens and their DNA, minimum temperatures of 150°C with treatment times of at least 30 min prove sufficient. Other contaminants however, such as pharmaceutically active compounds – and in particular for animal mortality, prions – may require both higher operational temperatures, pressures, and extended treatment times [25]. A study by

Table 1 Conditions (sample temperatures, reactor pressures, and treatment times) during HTC treatment of livestock mortality bone and tissue samples

	30 min			60 min			240 min		
	TT† /min	T/°C	P/MPa	TT/min	T/°C	P/MPa	TT /min	T /°C	P/MPa
bone 150°C	35	152±4	1.03 ±0.06	81	165±2	1.14 ±0.17	282	137±3	0.61±0.03
tissue 150°C	31	153±3	0.79 ±0.06	67	155±4	0.79 ±0.05	246	156±3	0.86±0.06
bone 200°C	36	222±12	1.55 ±0.17	71	208±5	4.40 ±0.63	244	196±1	3.26±0.09
tissue 200°C	35	198±5	3.30 ±0.52	67	213±4	4.87 ±0.40	240	202±1	3.93±0.07

Note: † TT, treatment time; T, sample temperature; P, reactor vessel pressure

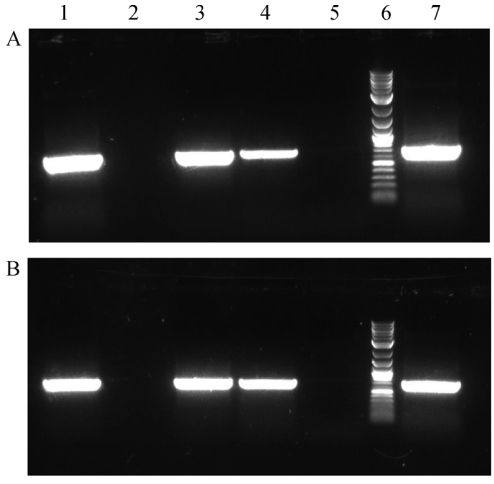

Fig. 7 Amplification results from DNA extractions after treatment of bovine bone (Panel A) and tissue (Panel B) samples at 100°C. Lanes are as follows: 1) inoculant; 2) negative control; 3) 30 min; 4) 60 min; 5) 240 min; 6) New England Biolabs 2-log DNA ladder; 7) positive control.

Taylor [26] reported instances of prions withstanding autoclave temperatures of 132°C–138°C; these surviving subpopulations were additionally capable of surviving consequent rounds of autoclave treatment. A similar study, which tested short treatment times with temperatures between 120°C–130°C and pressures of 600–700 MPa, likewise found reductions in prion infectivity, but not complete elimination [27]. Incorporation of an alkaline agent such as sodium hydroxide, in conjunction with autoclave conditions, was enough to inactivate prion activity [28], however these agents have the potential to corrode and weaken the structural integrity of HTC treatment equipment.

An additional consideration for HTC treatments is their ability to eliminate viruses. Several reports have documented the ability of viruses to survive in traditional agricultural waste treatment systems [29]. For the elimination of viruses in animal mortality, composting has been proposed as a suitable treatment practice [30,31]. However when compared to HTC treatment, composting requires: a significantly larger footprint – especially when considering large-sized carcasses, or large numbers of animal mortality; considerably longer treatment times – upwards of a month or more per pile; and can experience incomplete biological inactivation if treatment temperatures are not properly achieved and maintained – a scenario that can lead to pathogen survival and regrowth [32]. Additionally, some viruses, such as porcine parvovirus, are heat tolerant and able to survive temperatures of 55°C for a week or more [33].

The agricultural use of antibiotics presents a continuing challenge to the industry [34,35]. Barring sweeping regulatory changes, antibiotics will continue to be utilized in animal production systems, and the link between this use and potential negative impacts on human health will continue to be discussed [36]. As previously mentioned, the data on elimination of ARB and ARG in agricultural waste treatment streams is sparse. The literature that has been reported however indicates that while many biological, physical, and chemical disinfectant treatments can result in exponential decreases in numbers of ARB and ARG, they often do not result in complete removal [37]. When placed into a more hospitable environment (e.g., a living animal, or soil) the surviving ARB and ARG can then reestablish. Additionally, a number of wastewater treatments also generally fail to completely remove a large number of other hazardous compounds [38]. Given current research trends, HTC treatment may provide a suitable alternative to traditional waste treatment systems, allowing for elimination of ARBs, ARGs, and CECs.

4 Conclusions

This work serves a proof of concept that hydrocarbon thermalization of animal mortality may prove a suitable treatment option when considering total pathogen kill and elimination of microbially-derived DNA. We have arrived at these conclusions based on the inability to recover the model organism Ec DH10B, or amplify or detect plasmid-

borne DNA, post HTC treatment. After HTC treatment, the resulting byproducts (i.e., hydrochar, and the residual treated water) can be utilized without concern of reintroducing pathogens or ARGs to the environment. Further studies involving HTC treatment on animal mortality are necessary to optimize conditions that result in complete elimination of ARBs and ARGs across all animal species. This work can provide a basis for further work focusing on other waste streams, including manures and wastewaters. These studies are necessary to produce minimal operation conditions that achieve full elimination of ARBs, ARGs, and potential CECs, such that treatment can be performed efficiently and prove economically advantageous for producers worldwide.

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