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The Effects of E-beam Irradiation and Microwave Energy on Eastern Oysters (*Crassostrea virginica*) Experimentally Infected with *Cryptosporidium parvum*

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ABSTRACT. Shellfish have been identified as a potential source of *Cryptosporidium* infection for humans. The inactivation of *C. parvum* and other pathogens in raw molluscan shellfish would provide increased food safety for normal and at-risk consumers. The present study examined the efficacy of two alternative food-processing treatments, e-beam irradiation and microwave energy, on the viability of *C. parvum* oocysts in Eastern Oysters (*Crassostrea virginica*), which were artificially infected with the Beltsville strain of *C. parvum*. The effects of the treatments were evaluated by oral feeding of the processed oyster tissues to neonatal mice. Significant reductions ($P<0.05$) in infectivity were observed for in-shell and shucked oysters treated with e-beam irradiation at doses of 1.0, 1.5, or 2 kGy vs. untreated controls. A dose of 2 kGy completely eliminated *C. parvum* infectivity and did not adversely affect the visual appearance of the oysters. Oyster tissue treated with microwave exposures of 1 s (43.2 °C), 2 s (54.0 °C), and 3 s (62.5 °C) showed a reduction in *C. parvum* mouse infectivity, but the effects were not significantly different ($P>0.05$) from controls. Microwave energy treatments at 2 and 3 s showed extensive changes in oyster meat texture and color. Thus, because of lack of efficacy and unacceptable tissue changes, microwave treatment of oysters is not considered a viable food-processing method.

Key Words. E-beam irradiation, microwave, shellfish.

THE protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium hominis* cause diarrheal disease and gastrointestinal illness in humans in the United States and other countries worldwide (Fayer, Speer, and Dubey 1990; Morgan-Ryan et al. 2002; O'Donoghue 1995). *Cryptosporidium* is recognized as a significant contributor to morbidity and mortality in immunocompromised persons, such as AIDS patients (Fayer et al. 1997a). Other vulnerable populations are young children, pregnant women, and patients treated with immunosuppressive drugs (Guerrant 1997). Public health concerns about *Cryptosporidium* were heightened in the early 1990s because of waterborne-disease outbreaks of cryptosporidiosis that were linked to surface-water drinking sources (Kramer et al. 1996). For example, in 1993 in Milwaukee, WI, an outbreak of cryptosporidiosis sickened more than 400,000 people and killed over 100 people (MacKenzie et al. 1994). Surveys have found widespread distribution of *Cryptosporidium* in surface waters in the U.S. and Canada (LeChevallier, Norton, and Lee 1991; Rose et al. 1997). *Cryptosporidium* is considered an important biological water contaminant in the U.S. and has caused massive waterborne epidemics around the world (Fayer et al. 1997a; Fricker and Crab 1998; McLauchlin et al. 2000; Moore et al. 1993). Cryptosporidiosis outbreaks associated with the consumption of food have been reported from freshly pressed unpasteurized apple cider (Millard et al. 1994), unpasteurized apple juice, and chicken salad (Duffy and Moriarty 2003).

Major sources for *Cryptosporidium* contamination are wastewater treatment facilities and animal farms, especially ruminant farms (Fayer, Speer, and Dubey 1997b; Graczyk 1997; Thompson 2003). Rainfall, floods, and sewage overflows wash *Cryptosporidium* oocysts into rivers, bays, and oceans, and as a result contaminate fresh and marine waters with *Cryptosporidium* oocysts. This contamination can find its way into some aquatic organisms, such as shellfish, which may become part of the human food supply.

Oocysts of *C. parvum* have been detected in shellfish worldwide (Chalmers et al. 1997; Fayer, Trout, and Jenkins 1998; Freire-Santos et al. 2000b; Gomez-Bautista et al. 2000). All commercial oyster-harvesting sites tested in Maryland tributaries to the Chesapeake Bay (Fayer et al. 1998; Graczyk et al. 2000) were found contaminated. Because oysters are filter feeders and are often consumed raw, Fayer et al. (1998) concluded that *C. parvum* oocysts in oysters pose a potential public health problem and possible source for food-borne disease. A more recent and geographically wider survey found *Cryptosporidium* species in commercial shellfish (oysters and/or clams) from 64.9% of sites sampled in 13 Atlantic coast states from Maine to Florida and New Brunswick, Canada (Fayer et al. 2003). There have been no documented cases of cryptosporidiosis from consumption of raw oysters. Reports of food-related outbreaks of this emerging disease are difficult to document and are most likely under reported (Fayer, Morgan, and Upton 2000).

Customary processing operations may not completely eliminate *C. parvum* oocysts from the gills and other parts of the oysters. The parasite is able to survive in the tissue of fresh shellfish from harvest through consumption and is resistant to treatment with many toxic chemicals (liquids and gases) (Barbee et al. 1999; Fayer et al. 1996; Wilson and Margolin 1999) and to refrigeration temperatures (Fayer et al. 1998; Freire-Santos et al. 1999, 2000a).

The objectives of this study were to identify the effects of two alternative commercial food-processing treatments (e-beam irradiation and microwave energy) on the viability of *C. parvum* oocysts in Eastern oysters. The neonatal mouse model was used to test for oocyst infectivity (Lindsay, Blagburn, and Upton 1999).

MATERIALS AND METHODS

Oocysts. *Cryptosporidium parvum* oocysts (Beltsville isolate) were obtained from the feces of experimentally infected dairy calves (Fayer and Ellis 1993) at the Environmental Microbial Safety Laboratory, U.S. Department of Agriculture, Beltsville, MD. Purified oocysts were shipped on cold packs to the Center for Molecular Medicine and Infectious Diseases, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. Oocysts were stored at 4 °C and were less than 2-mo old when used for experiments.

Infection and sampling of oysters. Eastern oysters (*Crassostrea virginica*) were obtained from a commercial source

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(Cowart Seafood Corp., Lottsburg, VA), and used in this study. Oysters were 2–3 cm thick and 9–12 cm in length. Oysters (12–15 per 76-L aquarium) were acclimated for 2 wk in artificial sea water (Coral Life salt, Energy Savers Unlimited, Inc., Carson, CA) (salinity, 12–15 ppt) at the Department of Biomedical Sciences and Pathobiology (DBSP), Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. The oysters were alive before inoculation with *C. parvum* and then prepared after 24-h postexposure (PE) for e-beam irradiation treatment or microwave energy exposure. Batches of oysters were not checked for naturally occurring *Cryptosporidium* oocyst contamination. We used non-exposed control oysters in all experiments in the present study and had no indication that natural infections were present at detectable levels in our source oysters (see results below).

The pH and water temperature were checked daily and maintained between pH 8.0 and pH 8.2, and 16–19 °C. Oysters were fed two times per week with ''Instant Algae'' (3.1 billion cell/ml, Reed Mariculture/Instant Algae Products, San Jose, CA). The aquaria filters were removed for 24 h to prevent possible entrapment of oocysts in the filter material during the inoculation period, but the aeration system (air stones) functioned constantly during each experiment. A dose of 2×10^7 *C. parvum* oocysts was added to each aquarium to infect oysters prior to the treatments (Table 1).

E-beam irradiation. Oysters were removed from aquaria at 24-h PE and shipped overnight on cold packs to a commercial facility for e-beam irradiation (SureBeam Corporation, Glendale Heights, IL). Commercial e-beam irradiation treatment was applied to in-shell (n = 37) and shucked (n = 36) oysters at doses of 0 kGy (1 kilogray = 1×10^5 RADS) (control), 1.0, 1.5, and 2.0 kGy (Table 2). Each treatment was replicated three times. Irradiated and control oysters were shipped back to DBSP on cold packs. Oysters were stored at 4 °C and examined for infectious oocysts within 72 h.

Microwave irradiation (energy). Oysters were removed from aquaria 24-h PE and used for microwave treatment. Infected oysters (n = 37) were shucked and exposed to microwave energy (Amana Commercial Radarange Microwave Oven, Model RC20SE [designed to deliver 2100 W of microwave power to the oven cavity with a frequency of 915 MHz], Amana Refrigeration, Inc., Amana, IA). Three to four oysters were placed separately and not touching each other on a microwave safe plate for experimental treatments. Microwave treatment exposures were 0 s (control)/22.4 °C, 1 s /43.2 °C, 2 s /54.0 °C, and 3 s /62.9 °C (Table 3). Each treatment was replicated three times.

Table **1.** Total counts of oocysts of *Cryptosporidium parvum* identified by fluorescence antibody staining from individual Eastern oysters exposed for 24 h to 2×10^7 oocysts in seawater.

Treatment	Group	Replicate	Oyster A	Oyster B	Oyster C	Oyster D
Unshucked oysters	Control	1	9,346	12,350	8,750	NA[a]
		2	457	3,645	0	NA
		3	6,847	11,070	6,240	NA
E-beam	1.0 kGy	1	954	11,910	15,030	4,200
		2	3,379	3,240	675	NA
		3	4,200	2,548	1,765	NA
E-beam	1.5 kGy	1	728	790	12,425	NA
		2	1,253	0	540	NA
		3	13,709	3,286	960	NA
E-beam	2.0 kGy	1	2,475	2,620	3,495	NA
		2	1,843	10,257	6,175	NA
		3	15,318	4,708	954	NA
Shucked oysters	Control	1	325	10,327	13,325	NA
		2	240	1,640	2,175	NA
		3	805	0	460	NA
E-beam	1.0 kGy	1	12,750	965	11,425	NA
		2	943	4,640	875	NA
		3	3,097	1,385	5,220	NA
E-beam	1.5 kGy	1	240	13,426	9,340	NA
		2	7,400	4,327	2,790	NA
		3	3,510	460	875	NA
E-beam	2.0 kGy	1	7,360	4,325	12,286	NA
		2	950	10,565	3,748	NA
		3	9,830	640	1,475	NA
Microwave energy	Control	1	240	8,210	10,580	NA
		2	7,820	3,975	1,320	NA
		3	1,805	625	3,467	894
	1 s/43.2 °C	1	4,073	11,574	15,975	NA
		2	635	2,140	840	NA
		3	10,672	6,375	1,325	NA
	2 s/54.0 °C	1	11,573	975	10,290	NA
		2	2,974	4,350	7,365	NA
		3	1,823	1,547	640	NA
	3 s/62.9 °C	1	780	5,325	12,325	NA
		2	940	0	3,570	NA
		3	1,720	2,140	3,280	NA

[a] Not applicable.

Table **2.** Effects of various doses of e-beam irradiation on the infectivity of *Cryptosporidium parvum* from Eastern oysters using the neonatal mouse bioassay.

Dose e-beam irradiation	No. of oysters pooled[a]	No. of mice positive/total[b]	% of mice infected
Unshucked oysters			
Control	3	8/10	80
	3	10/10	100
	3	10/10	100
Total	9	28/30	93
E-beam 1.0 kGy[c]	4	3/10	30
	3	5/10	50
	3	5/10	50
Total	10	13/30	43
E-beam 1.5 kGy[c]	3	4/10	40
	3	2/10	20
	3	5/10	50
Total	9	11/30	37
E-beam 2.0 kGy[c]	3	0/10	0
	3	0/10	0
	3	0/10	0
Total	9	0/30	0
Shucked oysters			
Control	3	10/10	100
	3	10/10	100
	3	10/10	100
Total	9	30/30	100
E-beam 1.0 kGy[c]	3	4/10	40
	3	8/10	80
	3	4/10	40
Total	9	16/30	53
E-beam 1.5 kGy[c]	3	4/10	40
	3	2/10	20
	3	2/10	20
Total	9	8/30	27
E-beam 2.0 kGy[c]	3	0/10	0
	3	0/10	0
	3	0/10	0
Total	9	0/30	0

[a] Number of oysters pooled and used to infect neonatal mice.
[b] Number of mice positive for *C. parvum*/total number of mice inoculated.
[c] Treatments are significantly different ($P < 0.05$) from control.

Table 3. Effects of various doses of microwave energy on the infectivity of *Cryptosporidium parvum* from Eastern oysters using the neonatal mouse bioassay.

Dose microwave time (sec)	Temperature (°C) Initial (± SD)/ final (± SD)[a]	No. of oysters pooled[b]	No. of mice positive/ total[c]	% of mice infected[d]
Control	22.4(0.20)/22.4(0.20)			
		3	10/10	100
		3	10/10	100
		4	10/10	100
Total		10	30/30	100
Microwave 1 s	22.3(0.12)/43.2(0.18)			
		3	8/11	73
		3	5/10	50
		3	9/10	90
Total		9	22/31	71
2 s	22.4(0.85)/54.0(1.70)			
		3	6/10	60
		3	8/10	80
		3	6/10	60
Total		9	20/30	67
3 sec	22.3(0.10)/62.9(0.16)			
		3	3/10	30
		3	6/10	60
		3	7/10	70
Total		9	16/30	53

[a] Initial temperature (standard deviation)/final temperature (standard deviation).
[b] Number of oysters pooled and used to infect neonatal mice.
[c] Number of mice positive for *C. parvum*/total number of mice inoculated.
[d] Microwave treatments were not significant ($P > 0.05$) in reducing infectivity of *C. parvum* for neonatal mice.

Processing of infected oysters. Oysters were individually washed in tap water to remove surface-contaminating oocysts. Oysters were processed individually using the general methods described by Fayer et al. (1998). Hemolymph, gill washes, and oyster tissues were processed separately and then recombined before examination for *C. parvum* oocysts using a commercial immunofluorescent antibody (IFA) test kit (MERIFLUOR, Meridian Diagnostics, Cincinnati, OH) (Fayer et al. 1998). Briefly, hemolymph was collected by drilling a hole in the anterior part of the upper valve and inserting an 18-gauge needle (fitted to a 5-ml syringe) into the adductor muscle to aspirate the hemolymph. Hemolymph from each oyster was placed in a 15-ml centrifuge tube and particles were concentrated by centrifugation at 3,000 *g* for 10 min. The gills were removed and placed in a 15-ml centrifuge tube with 5 ml of phosphate-buffered saline (PBS) and were vortexed on a Thermolyne Maxi-Mix-1 mixer (Barnstead Thermolyne, Dubuque, IA) for 15 s. The gills were discarded and the gill washes were concentrated by centrifugation as for the hemolymph. The oyster tissue was placed in a Stomacher bag containing 10 ml PBS and homogenized in a Stomacher machine (Seward Medical London, Stomacher 80 Lab Blender, London, UK) for 2 min and filtered several times through cheesecloth to remove large particulate material. The pellets from the hemolymph and gill washes were mixed with the homogenized oyster tissue. The final total volume was recorded (typically 12–15 ml) and 200 µl of this were placed in each of three 12-mm diam. circular wells on a glass (Teflon™ coated) microscope slide. The slides were processed using the IFA test kit and examined for the presence or absence of *C. parvum* oocysts using an Olympus BX60 epifluorescence microscope. This was performed to determine if oysters were infected and to estimate the numbers of oocysts present in each oyster (Table 1). The numbers of oocysts present were estimated by counting the numbers of oocysts in each of three wells, calculating the mean per 200 µl, and multiplying this mean by 5 to estimate the number of oocysts per milliliter of sample. The total number of oocysts in each oyster was then estimated by multiplying the mean count by the total volume of homogenate for that oyster.

For each replicate, all homogenates of the oysters (three to four) in a treatment were combined and concentrated by centrifugation. The final concentrated material was used to inoculate neonatal mice.

Mouse infectivity assay. Infectivity of the processed oyster material (hemolymph, gill washings, and oyster tissue homogenate) was examined using the neonatal mouse bioassay (Lindsay et al. 1999). Briefly, 5-d-old suckling CD-1 mice were fed 100 µl/mouse of test material by oral lavage. Estimated doses ranged from 7×10^2 to 1.8×10^3 of *C. parvum* oocysts per tested mouse (see below). All mice in each litter were killed at 5-d PI by decapitation. The intestinal tissue (duodenum, jejunum, ileum, cecum, proximal, and distal colon) was removed from each mouse pup and placed separately in a 15-ml centrifuged tube with 9.0 ml of Hank's balanced salt solution containing 1000 U/ml penicillin and 1000 µg/ml streptomycin (pH 7.2) and kept at 4 °C. Intestines were homogenized using a Stomacher machine within 48 h of collection. The homogenate of this tissue from each mouse (200 µl on triplicate wells) was examined for *C. parvum* using the IFA test kit as outlined above.

Statistical analysis. Significant differences ($P < 0.05$) were determined with PROC GLM of SAS statistical software (SAS Institute, Inc., Cary, NC). When significant effects ($P < 0.05$) were found to be present, pairwise comparisons were performed using Bonferroni's *t*-test on adjusted mean inactivation (loss of infectivity) levels generated by the generalized linear model.

RESULTS

Experimental infection of oysters. Infection rates of oysters exposed to 2×10^7 *C. parvum* oocysts for 24 h ranged from not detectable to a maximum of 15,975 oocysts (Table 1).

Effects of e-beam irradiation on *C. parvum* infectivity. The e-beam irradiation of in-shell and shucked oysters at all dose levels was significant ($P < 0.05$) in reducing infectivity of *C. parvum* for neonatal mice (Table 2). Complete inactivation of *C. parvum* infectivity was achieved at 2 kGy. No grossly visible changes were observed in oyster tissues after e-beam treatments.

Effects of microwave treatment on *C. parvum* infectivity. Microwave irradiation of in-shell and shucked oysters was not effective ($P > 0.05$) in reducing the infectivity of *C. parvum* in oysters for neonatal mice (Table 3). Additionally, microwave treatment showed extensive changes in oyster meat texture and color changes starting at 2 s (54.0 °C) to 3 s (62.5 °C) indicating cooking had occurred. This cooking appearance was manifested as curling of the gills and the oyster meat becoming white at the edges.

DISCUSSION

Microwave and ionizing irradiation (γ, X-ray, or e-beam) are two treatments that have been investigated to control infectivity of food-borne bacteria, viruses, and parasites in various foods, including some seafood. Most of the irradiation research has used γ radiation. Research indicates that high-energy ionization, whether produced by γ, X-ray, or e-beam radiation, has generally similar chemical and biological effects (Wilkinson and Gould 1998). Kilgen (2001) summarized research on the effects of low, medium, and high doses of ionizing radiation on fresh, frozen, and processed seafood products. Kilgen (2001) noted that (1) in gen-

eral, Gram-negative bacteria are more sensitive to ionizing radiation than Gram-positive bacteria, and (2) a dose of 4 kGy was found sufficient to eliminate non-spore-forming pathogens in many kinds of foods, including frozen seafood. In regard to oysters, Kilgen (2001) summarized studies and reported that radiation doses of 1.0–1.5 kGy are necessary to reduce bacterial numbers to a non-detectable level. The following energy levels were found to be effective: 1.5 kGy to eliminate *Escherichia coli*; 1.2 kGy to eliminate *Vibrio* spp.; 1.5 kGy to eliminate *Vibrio vulnificus*; 1.0 kGy to eliminate *Vibrio cholerae*; and 1.0 kGy to eliminate *Vibrio parahaemolyticus* seeded in *Cr. Virginica* (Hu et al. 2005). Alternative food-processing treatments may be implemented in seafood processing if they provide a 5-log reduction of the pertinent pathogen (FDA 2004). Tauxe (2001) reported that elimination of 99.999% of bacteria takes five times the irradiation dose needed for a 1-log kill and would reduce a million bacteria to ten. For example, to reduce *Campylobacter* in meat it takes 0.2 kGy with one decimal log or 1 kGy to reduce it by 5 decimal logs.

Gamma irradiation has been recognized as a most effective preservation technique for several decades and has been used to extend food shelf-life and to assure the safety of food supplies (Farkas et al. 1997). The effects of γ radiation with ^{137}Cs at 0.4–0.8 kGy were tested on unsporulated and sporulated *Toxoplasma gondii* oocysts (Dubey et al. 1998). Results showed that irradiation at 0.5 kGy was effective in killing oocysts on fruits and vegetables. The results were used to develop a model system for sterilization of fruit contaminated with oocysts of other coccidians, such as *Cyclospora* or *Cryptosporidium*. Gamma irradiation of oocysts with doses of 450 or 500 Gy prevented *C. parvum* oocyst (Iowa strain) defecation in calves fed treated oocysts (Jenkins et al. 2004). In comparison, our 100% effective dose was 2 kGy in the present study. The differences in dose needed to achieve significant inactivation may be as a result of the animal models used, the matrix to contain oocysts, or the sources of irradiation.

The e-beam electrons have a limited penetration depth of about 5 cm or less as compared with X-rays that have significantly higher penetration depth (60–400 cm) depending upon the energy used. However, this limited penetration fits the size of oysters used in the present study and is suitable for treating similar-sized oysters. The present study indicates that oysters received a uniform distribution of e-beam penetration sufficient to kill *C. parvum* oocysts. The results of this study indicate that irradiation doses equal to, or more than, 2.0 kGy may serve in a commercial process to eliminate the enteric protozoan pathogen *C. parvum* in fresh oysters, shucked or in shell. The U.S. Food and Drug Administration (FDA) has received a request from the National Fisheries Institute Inc. to allow irradiation of shellfish at a maximum of 2.0 kGy (Martin, R. E., pers. commun.). Our results indicate that irradiation up to 2 kGy would maintain the appearance of fresh oysters and be in the range of acceptable quality for fresh oysters. Our results are similar to those of Jakabi et al. (2003) who demonstrated that irradiation up to 3 kGy did not affect the sensory characteristics of oysters (*Cr. brasiliana*).

In contrast to ionizing radiation, the microwave produces non-ionizing radiation and is too weak to break chemical bonds. The microwave radiation is absorbed at the molecular level, and results in heating. Microwave heating has been used to destroy microorganisms in food, but there is a long-standing debate about the thermal and non-thermal effects produced by microwave treatment of food (Anantheswaran and Ramaswamy 2001). In a recent review of this controversy, Banik, Bandyopadhyay, and Ganguly (2003) provided examples that claim to show non-thermal effects that induced various physiological effects. However, the research did not always specify the mechanisms to account for these non-thermal effects. A study of the effects of microwave radiation on developmental and infective stages of three parasites (*Eimeria nieschulzi, Strongyloides ratti*, and *Taenia taeniaeformis*) showed that microwave radiation was extremely effective in killing or preventing development of helminth and protozoan parasites without unduly distorting eggs or developmental stages (Conder and Williams 1983).

Bouchet and Boulard (1991) studied the effect of microwaves on the ultrastructure of oocysts of *Eimeria magna*. The oocysts were exposed to microwave energy (2,450 MHz; 600 W) for different durations (10, 15, and 20 s). The microwave treatment progressively destroyed the three layers of the oocyst wall and damaged internal structures. Adams et al. (1999) studied the relationship between temperature and survival of the nematode *Anisakis simplex* in fillets of arrow-tooth flounder (*Atheresthes stomias*) subject to microwave processing. Fillets, 14 cm long, 4.5 cm wide, and approximately 1.75 cm high, were inoculated with 10 live nematodes and then microwaved. No viable nematodes survived microwave processing at 71 °C or 77 °C. Smaller fillets required only 65 °C, probably because of fewer cold spots during microwave processing, while larger whole fillets required 77 °C to kill nematodes (Adams et al. 1999). Adams et al. (1999) indicated that damage to the nematode's cuticles was apparent after treatment. Inactivation of the parasites was attributed to the thermal effects of the microwave treatment (Adams et al. 1999). Many factors influence microwave thermal processes (mass of objects, shape of objects, specific heat, thermal conductivity, etc.) that are the same as conventional heating (Heddleson and Doores 1994).

In our study, microwave treatment reduced the infectivity of *C. parvum* in oysters but did not completely prevent infectivity at the levels tested. The oyster tissues appeared cooked at treatment time/temperature of 2 s (54.0 °C) or 3 s (62.5 °C). According to the FDA, oysters exposed to temperatures exceeding 49 °C (temperature at which the gills curl) cannot be sold as raw oysters. Therefore, microwave energy treatment at energies that render *C. parvum* non-infectious would render oysters unsuitable for marketing as a raw product.

The present study demonstrates that e-beam irradiation using current technology is capable of eliminating infectivity of *C. parvum* oocysts from oysters and that the oysters retain their uncooked characteristics after irradiation (Jakabi et al. 2003, present study). The doses of e-beam irradiation used will also remove other potentially harmful pathogens that might be present in fresh uncooked oysters. The amount of microwave energy needed to produce a lethal effect on *C. parvum* also damages the oyster tissue (cooks it) making it non-acceptable by consumers as a fresh (raw) product. E-beam irradiation is a tool that may help producers provide safer products to consumers.

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