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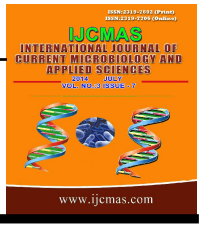


**Original Research Article**

# Coliforms carriage by nematodes in agricultural crop fields irrigated with fresh and recycled water

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**A B S T R A C T**

It has been proposed that nematodes are involved in the transmission of food-borne pathogens from contaminated soil to plants. To examine this hypothesis, a survey was conducted in two experimental research stations in Israel, which utilize domestic recycled- and fresh-water for irrigation of fields in the north (NR) and south (SR) regions of the country. The number of nematodes isolated during the survey varied considerably throughout the survey and between sites. However, in the SR but not in the NR site, more nematodes were detected in fresh-water irrigated soil. Microbiological analysis of nematodes revealed a higher prevalence of coliforms' carriage in nematodes derived from recycled water irrigated fields (34% vs. 26%) in SR fields, whereas NR fields showed comparable prevalence. Enumeration of the nematodes-associated coliforms in SR samples revealed a significant higher concentration (0.46 ± 0.54 CFU/nematode) in recycled- versus fresh-water (0.16 ± 0.24 CFU/nematode) irrigated fields. The findings from SR (but not NR) fields are in agreement with the notion that nematodes could serve as potential vector for food borne pathogens in fields irrigated with recycled water.

## Introduction

Shortage of fresh water in many countries, particularly in semi- and arid regions, has led farmers to increasingly utilize recycled water for irrigation of agricultural crop fields. For example, in Israel about 67% of wastewater is recycled and used for irrigation, in India 25% and in South Africa 24% of waste water is recycled (Gijzen, 2000). Studies on the nutritional value of reclaimed water have demonstrated their capacity to promote crop yields (Gori et al., 2000; Ouazzani et al.1996). Nevertheless, treated effluents have been shown to contain pathogenic microorganisms (Bitton et al., 1984), which resist disinfection in the treatment plants apparently due to the presence of high organic load (Chang, 1961; Ding et al., 1995).

Consequently, long term irrigation of crop fields with waste water might increase the microbial biomass into the soil (Filip et al., 2000). There have been several reports showing that fecal coliforms are capable of surviving and even growing in the environment (Davies et al., 1995; Desmarais et al., 2002). Escherichia coli, fecal coliforms, and Enterococci have been found in freshwater, soil, and plant samples derived from fields irrigated with treated water (Jones, 1999; Buncic et al., 2004).

Nematodes are ubiquitous in soils and represent one of the most abundant life forms on our planet (Heip et al., 1985). A top layer of soil can contain as much as $10^6$ nematodes per square meter (AWWA, 1995). Under optimal growth conditions, certain species have reproductive cycles of less than 5 days (Poinar, 1991). Free living nematodes nourish themselves on bacteria, organic detritus, decomposing organisms, diatoms, and other living organisms (Poinar, 1991). Accordingly, nematodes are also most abundant in reclaimed water (1 Chang et al., 1960; Mott et al., 1981; Lupi et al., 1994).

Outbreaks related to consumption of fresh produce have been increasingly reported in recent years (Brandl, 2006; Heaton and Jones, 2008). Although, contamination of fresh produce might occur during harvest and post-harvest processing, in several cases contamination was reported to occur in the field. It has been proposed that nematodes might serve as a vector for transmitting human pathogens to plants (Gibbs et al., 2005). In support of this notion, several studies have demonstrated the capacity of nematodes to carry human pathogens under in vitro conditions. The free-living, bacterivorous nematodes, *Caenorhabditis elegans* was shown to transport Salmonella spp., Listeria spp. and Escherichia coli from contaminated soil to the surface of fresh produce (Kenney et al., 2006). A related free-living nematode found more commonly in the rhizosphere of agricultural soils, Diploscapter spp. strain LKC25 was also reported to attract, ingest and shed Salmonella, *E. coli* and Listeria (Gibbs et al., 2005). Although parasitic nematodes cannot ingest bacteria, they can carry soil bacteria on their cuticle and potentially transmit them to susceptible host plants. Indeed, several species of plant-parasitic nematodes are well-known vectors of soil-borne plant diseases (McClure and Spiegel, 1991). In our study, we have found that under in vitro conditions, the plant-parasitic nematode, Meloidogyne javanica could carry *E. coli* on its cuticle and thus may serve as a temporary reservoir (Maghodia et al., 2008). To explore a potential role of soil nematodes in transmission of food-borne pathogens in fields irrigated with recycled water, we have examined potential differences in 1 the abundance of nematodes and carriage of coliforms and *E. coli* in fields irrigated with recycled and fresh water.

## Materials and Methods

### Details of the survey

Soil samples were collected from fields in two agricultural research stations representing different types of soils, climates and agricultural crops. One station is located in the northern part (NR) of the central coast of Israel (average annual rainfall is 550 mm with Mediterranean climate), and the other in the south region (SR) of the country (average annual rainfall is 231 mm). The soil in the NR site contain 57.7% clay, 28.6% silt, 13.8% sand, with 3.1% CaCO3, and 38.6 available NO3-N mg kg-1, while the soil in the SR fields was sandy soil containing 53% sand, 20% clay, 27% silt,

0.62% organic matter, 16.4% CaCO3. Soil samples were taken from an orchard planted with persimmon (grafted on Vergiana persimmon rootstock), in the NR station, and from a field planted with Sunflower (SR). Both sites are used for ongoing studies on the effect of secondary effluent irrigation on crops' yield and contain nearby fields irrigated with fresh water (control). The NR site was irrigated with secondary effluents derived from a rural domestic wastewater treatment plant for five successive years. The SR site received water from a large municipal wastewater treatment plants that also received industrial discharges.

Irrigation with the recycled water was carried on for 10 successive years. The survey in the SR site was conducted from June, 2006 to December, 1 2007, while the survey in the NR site was from March, 2007 to July, 2008.

## Soil Sampling

Soil samples of 500-1000 g were collected from a depth of 10-15 cm near the rizhosphere put into sterile polypropylene plastic bags and transferred to the laboratory on the same day. Samples were immediately processed for nematodes extraction and the rest of the soil was kept at 4 oC. Microbiological analysis was performed within 24 h.

## Enumeration of nematodes

Five soil samples from each sampling date were evaluated for the presence of nematodes. Nematodes enumeration was performed by the method of Rose et al (1996). Briefly, 50 g of soil were taken into 80 □m sieve and kept in a funnel filled with double-distilled water (DDW) for 48 h at 25 oC. The funnel is stop cocked at the bottom and due to high specific gravity, nematodes settled down towards the end of the funnel and after two days they were collected and counted under Olympus Optical SZX12 microscope (Tokyo, Japan) at 1X magnification. Total nematodes concentration is presented as the average number of nematodes per 1 g of soil. In some experiments, the 5 samples were mixed together and the number of nematodes was determined in a 50 g soil portion derived from the composite sample.

## Detection of coliforms and *E. coli* in nematodes

To enumerate nematode-associated coliforms, nematodes were first washed with sterilized DDW in 1.5 ml conical polypropylene tube by centrifugation at 500 X g for two min to remove unattached bacteria. The nematode pellet was crushed with a disposable polypropylene pestle (Kimble-Kontes Product, Vineland, NJ) and resuspended in 100 µl of sterilized DDW following inoculation on violet red-bile agar (VRBA; Conda Laboratories, Madrid, Spain) plates. The plates were incubated at 37oC for 24 to 48 h, and large pink to purple colonies (diameter, > 0.5 mm) were considered to be coliforms (Sela et al., 2005). To recover potentially injured coliforms from negative samples, 100 µl of nematodes' extract was enriched in 10 ml Lennox broth (LB; Acumedia, Inc. Lansing, MI) for 18-24 h at 37 oC. Ten-fold dilutions of the enriched culture were spread-plated in duplicate on VRBA and positive or negative coliform-containing samples were scored, as described above. To determine the presence of *E. coli*, coliforms were further streaked on Chromocult® TBX agar (Merck & Co., Inc., Whitehouse Station, NJ) and incubated for 18-24 h at 37 oC. Blue-green colonies were presumptively identified as *E. coli*. Indole test was used to confirm the identification of *E. coli* (MacFaddin, 1980).

### Detection of coliforms and *E. coli* in soil

Soil samples (50 g, each) were suspended in 450 ml buffered-peptone water in sterile 3.5 L stomacher bag (SM3-01, Plastiques Gosselin, Borre, France). Samples were pummelled with hands for 5 min and 100 µl of the suspension was spread-plated in duplicate on VRBA agar. Enumeration of coliforms, enrichment 1 and detection of E.coli were performed as described above.

## Result and Discussion

Occurrence of nematodes in fresh- and recycled-water irrigated fields The abundance of nematodes in NR soil was low (2.5±1.4 and 1.32±1.1/g soil) in fresh- and recycled-water irrigated fields, respectively at the beginning of March 2007 and increased steadily to reach a maximum (20.4±7.4 and 32.3±35.7/g soil) in June 2007 (beginning of summer). The population then declined rapidly during the month of August, 2007 to 11.4 and 11.1 nematodes per gram soil, and again, increased in the middle of October, 2007 and reached a maximum (144.8 and 71.8 nematodes per gram soil) in fresh- and recycled-water irrigated fields, respectively in January, 2008. However, at the beginning of March, 2008 the nematodes population declined rapidly (83.8 and 42/g soil) with further reduction until June, 2008 (38.8 and 19.6/g soil) in fresh- and recycled-water irrigated fields, respectively. In July, 2008 the nematodes population again increased up to 85.2 and 50.6 /g soil in the two types of fields, respectively (Fig. 1A). In contrast to NR fields, SR soil contained generally higher nematodes population throughout the survey. The average nematode numbers were low at the beginning of June, 2006 (31.4±18.9 and 5.2± 2.9/g soil in fresh- and recycled-water irrigated fields, respectively), and then increased in January, 2007 (85.8 ± 20.4 and 41.4 ± 23.6/g soil) and reached a maximum (426 and 108 /g soil in fresh- and recycled-water irrigated fields, respectively) at the beginning of October, 2007. The nematodes population then declined gradually 1 in the middle of October, 2007 (279 and 171 /g soil in fresh- and recycled-water irrigated fields, respectively) with further reduction until November, 2007 (197 and 75 /g soil, respectively in fresh- and recycled-water irrigated fields, respectively). In December, 2007 the nematodes population again increased up to 210 and 103 /g soil in fresh- and recycled-water irrigated field sites, respectively (Fig. 1B).

All together, NR soil samples harboured similar number of nematodes (26.4 ±35.1 and 20.8 ± 27.8/g soil) with no significant difference in fresh- and recycled water irrigated fields, respectively, while SR fields contained significantly higher nematodes population (87.9 ± 69.8 vs. 41.4 ± 29.3/g soil) in fresh- and recycled-water irrigated fields, respectively (Table 1).

### Occurrence of coliforms in soil

To assess possible impact of irrigation water quality on the coliforms population in the various fields, the number of coliforms was determined (Table 1). In NR soil samples the total number of coliforms was 1.23 x 103 (± 1.73 x 103) CFU/g in recycled-water and 1.27 x 103 (± 1.82 x 103) CFU /g in fresh-water irrigated fields respectively. The total coliforms in SR soils was 1.45 x 103 (±3.29 x 103) and 1.07 x 103 (±9.73 x 102) CFU/g in recycled- and fresh-water irrigated fields, respectively (Table 1). No significant difference in coliforms population was observed in fresh and recycled-water irrigated fields in both sites (paired t test; P=0.5). None of the soil samples was found to contain viable *E. coli*.

Carriage of coliforms 1 and *E. coli* by nematodes To examine whether the type of irrigation water have affected carriage of Coliforms by nematodes, a microbiological analysis of the nematodes collected from NR and SR sites was performed. Recycled water irrigated fields exhibited higher prevalence of coliforms-carrying nematodes in SR samples (34% in RW-irrigated fields vs. 26% in FW-irrigated fields). In contrast, similar carriage (22% and 21%) was detected in NR samples.

All together, carriage of coliforms was slightly higher in RW- compared to FW-irrigated fields (Table 1). Enumeration of nematodes' associated coliforms in SR fields revealed that RW-irrigated soil samples contained significantly higher numbers of coliforms compared to FW-irrigated soil samples (0.46±0.54 vs. 0.16±0.24 coliforms per nematode). No significant difference in coliforms carriage was observed in nematodes derived from NR fields. However, the number of samples containing viable counts by direct plating was rather low (Table 1). All together (NR+SR samples), no significant difference in the carriage of coliforms by nematodes was scored. None of the nematode samples was found to carry *E. coli* either by direct plating or following enrichment.

Israel is part of a transition zone between the Mediterranean region and the great deserts of Arabia and the Sahara. It is divided into three physiographic lithologic regions: the coastal region, the mountain and hill region, and the valleys, plains, and plateaux. Climate ranges from Mediterranean in the north to extremely arid in the south. Depending upon climatic conditions in 1 each of the physiographic lithologic regions, climatogenic soils range from those characteristic of the Mediterranean region (non calcic brown soils, terra rossa, brown rendzina, grumusols) in the north to desert soils (serozem, hammada) in the south (Dan and Koyumdjisky, 1963). Israel, like other countries in the semi-arid Mediterranean region, is suffering from scarcity of water resources and the demand for water for agriculture usage is increasing rapidly. In Jordan, recycled water has been extensively used for irrigation purpose of agricultural crops and it has been observed that soils irrigated with treated wastewater harbored higher total coliforms and fecal Coliforms as compared to soils irrigated with potable water (Malkawi and Mohammad, 2003).

Free-living, bacterivorous nematode *Caenorhabditis elegans* has been shown to ingest food-borne pathogens (Anderson et al., 2003; Caldwell et al., 2003) and observed to transmit *Salmonella enterica* serotype Newport from contaminated soil to the surface of fresh produce (Kenney et al., 2006). The survival and persistence of Escherichia coli O157:H7 and the *S.enterica* serotypes Newport and Poona were observed in the gut of C. elegans, which led to the conclusion that this nematode can serve as a temporary reservoir of food-borne pathogens (Kenney et al., 2005). In order to investigate the potential role of nematodes in vectoring food-borne pathogens, we have performed a survey in RW- and FW-irrigated fields in two geographical regions in Israel and examined the occurrence of nematodes, presence of coliforms in soil and carriage of coliforms by nematodes.

A considerable variation in the population size 1 of nematodes was found during the survey, possibly due to differences attributed to climate change, soil characteristics, geographical regions and effluent quality. It has been shown that free living nematodes nourish themselves on bacteria, organic detritus, and decomposing organisms (Poinar, 1991).

**Figure.1** Monthly nematode abundance in fresh- and recycled-water irrigated field
3 sites in the Northern (A) and Southern (B) regions of Israel. The data represent the
4 number of nematodes per gram soil at individual sampling date. Error bars represents
5 standard deviation

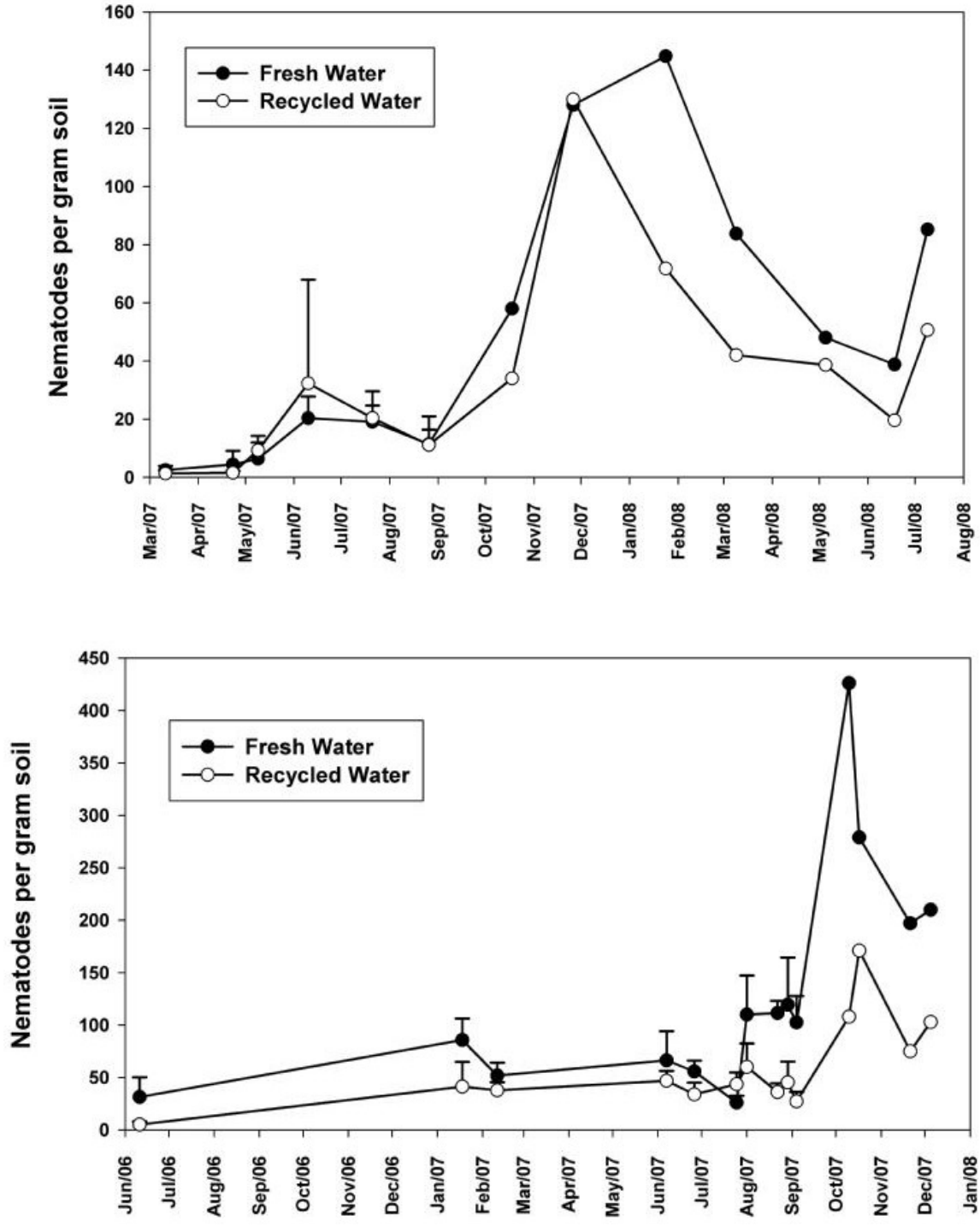

**Table 1**. Abundance of coliforms in soil and in nematodes from fresh- and recycled-water irrigated fields in different regions of Israel

| Site | No. of Samples | | Nematodes /g soil Mean ( ± SD) | | Total Coliforms (CFU/g soil); Mean ( ± SD) | | Prevalence of Coliforms carriage by nematodes (%) | | No. of Coliforms (CFU/nematode) Mean ( ± SD) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FW | RW | FW | RW | FW | RW | FW | RW | FW | RW |
| NR | 62 | 65 | 26.4 (35.1)$^{ns}$ | 20.8 (27.8)$^{ns}$ | $1.27 \times 10^3$ ($1.82 \times 10^3$)$^{ns}$ | $1.23 \times 10^3$ ($1.73 \times 10^3$)$^{ns}$ | 21 | 22 | 0.11 ± 0.10$^{ns}$ (N=6)[a] | 0.03 ± 0.01$^{ns}$ (N=2)[a] |
| SR | 73 | 73 | 87.9 (69.8)* | 41.4 (29.3)* | $1.07 \times 10^3$ ($9.73 \times 10^2$)* | $1.45 \times 10^3$ ($3.29 \times 10^3$)* | 26 | 34 | 0.16 ± 0.24* (N=12)[a] | 0.46 ± 0.54* (N=14)[a] |
| All | 135 | 138 | 64.9 (66.1)* | 33.3 (30.3)* | $1.16 \times 10^3$ ($1.45 \times 10^3$)$^{ns}$ | $1.34 \times 10^3$ ( $2.61 \times 10^3$)$^{ns}$ | 24 | 28 | 0.14 ± 0.20$^{ns}$ (N=18)[a] | 0.40 ± 0.52$^{ns}$ (N=16)[a] |

*Significant at $P < 0.05$, according to two-tailed, paired Student's *t* test, ns: non-significant

[a]Number of sample with detectable levels of coliforms by direct plating on VRBA agar.

Since, long-term wastewater irrigation often results in an increase of microbial counts, total biomass and soil enzyme activities (Filip et al., 2000), it was expected that RW-irrigated fields will contain higher number of bacteria and nematodes. Nevertheless, we did not find significant difference in either nematode count or total coliforms count in RW- and FW-irrigated NR fields. In contrast, FW-irrigated SR fields contained significantly higher nematode count compared to RW-irrigated fields. Since fresh water sources in both regions are likely to be comparable (Mekorot Water Company, Israel), the different results might be explained by differences in the source of recycled water and the history of effluent irrigation (see above). Indeed, SR site received recycled water from a large municipal treatment plant containing industrial effluents, which might contain low levels of toxic compounds (Chiou, 2008). Furthermore, the irrigation history was much longer in SR versus NR sites, favoring accumulation of toxic compounds in the soil, potentially harmful to nematodes.

Microbiological analysis of soils derived from the various fields displayed comparable numbers of coliforms in both RW- and FW-irrigated fields. While, coliforms represent only a small fraction of the total microflora of the soil, the comparable numbers of coliforms in RW- and FW-irrigated fields might reflect other ecological factors. It has been shown that the nature of 1 the soil, such as moisture holding capacity, pH and organic matter, plays an important role in determining survival and retention of microorganisms (Gerba and Goyal, 1984). Thus, the comparable coliforms counts obtained in our study might reflect that complex

environments exist in the examined fields, rather than the irrigation water type. Still, a higher prevalence of coliforms carriage was detected in nematodes derived from RW-irrigated SR-fields, which is in agreement with the notion that nematodes might provide protection for microbial soil inhabitants and hence may serve as a temporary reservoir for bacteria in the field.

In summary, the different results obtained in NR and SR fields, point to the presence of variations in the ecology of each ecosystem in the different fields and call for expansion of the study, before a conclusion regarding the role of nematode as potential vector for food-borne pathogens could be drawn.

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