Agriculture, Ecosystems and Environment 129 (2009) 43–51

Contents lists available at ScienceDirect

Agriculture, Ecosystems and Environment

journal homepage: www.elsevier.com/locate/agee

Irrigation of olive groves in Southern Italy with treated municipal wastewater: Effects on microbiological quality of soil and fruits

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ARTICLE INFO

Article history:
Received 10 January 2008
Received in revised form 10 July 2008
Accepted 14 July 2008
Available online 15 August 2008

Keywords:
Olea europaea L.
Faecal indicators
Health hazards
Agricultural recycling
Wastewater reuse

ABSTRACT

The use of municipal wastewater in agriculture requires a careful monitoring of a range of hygiene parameters. Yearly hygienic impact assessments on soil and fruit were made between 2000 and 2006 in an olive (*Olea europaea* L.) grove established near a municipal wastewater treatment plant in Southern Italy (Ferrandina–Basilicata region, 40°29′ N, 16°28′ E). The experimental grove was managed in two plots. The first plot, non-tilled, was drip irrigated daily with reclaimed wastewater. The second plot was unirrigated (i.e. rainfed) and subject to conventional management for the region. Samples of wetted soil from different depths and of treated wastewater were analysed for *Escherichia coli*, enterococci, sulphite-reducing *Clostridium* spores and *Salmonella* spp. Fruits were collected both from the canopy and from nets spread on the ground and analysed for faecal contamination. The average annual quantity of wastewater distributed was 293 mm. *E. coli* concentration in the wastewater varied considerably, being frequently above the stringent Italian mandatory limit of 10 CFU 100 mL^{-1} and also the WHO limit of 1000 MPN 100 mL^{-1}. *Salmonella* was never detected in the wastewater, the soil or on the fruit samples. Slight increases in the other bacteria were observed in the wastewater-irrigated soil during the irrigation season and especially in the top 10 cm. Soil resilience and bacterial mortality/inactivation probably explains the seasonal decrease of soil bacteria content over the 7 years of the study. Because of their high resistance to disinfection treatments and to environmental conditions, the spores of the sulphite-reducing bacterium *Clostridium* could be useful as an indicator of contamination in future guidelines that might be enacted for the use of wastewater in agriculture. No significant microbial contamination was recorded on fruit harvested directly from the canopy of the wastewater-irrigated trees. Contaminations on fruits sampled from the ground in the wastewater-irrigated plot were always low and usually similar to, or lower than those observed on drupes collected from the rainfed plot. In the rainfed plot, the recorded occasional contaminations were probably due to a number of factors, such as grazing of farm stock, presence of wild animals and surface water runoff from adjacent agricultural areas. This work confirms that, under suitable conditions, low-quality wastewater can be useful as an additional water resource for olive irrigation in water-scarce Mediterranean environments.

1. Introduction

Water shortage is a problem of high concern in the Mediterranean Basin (Marecos do Monte et al., 1996; Angelakis et al., 1999; Massoud et al., 2003; Hochstrat et al., 2006). Taking into account the scarcity of conventional water sources, due to water demand increases linked to population growth and to agricultural water usage (50–80% of total water consumption), there is an urgent need to make available alternative water sources for agriculture replacing the high-quality water required for human consumption (Marecos do Monte et al., 1996; Angelakis et al., 1999; Oron et al., 2001; Toze, 2006).

The reuse of municipal wastewater for irrigation could be a realistic way of reducing water shortage, as it has been demonstrated in many countries in the Mediterranean region such as Israel, Cyprus, Jordan and Tunisia (Angelakis et al., 1999). In Israel, for example, treated sewage effluent is expected be the main (~70%) source of water for irrigation by 2040 (Haruvy et al., 1999).

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doi:10.1016/j.agee.2008.07.003

On the other hand the use of wastewater in agriculture is often associated with significant health risks because of the presence of high concentrations of human pathogens, enteric in origin, such as bacteria, viruses, protozoa and helminths (Toze, 2006). In Italy, recent guidelines allow unrestricted crop irrigation with a bacteriological effluent quality of 10 Colony Forming Units (CFU) per 100 mL of *E. coli* (Decree No. 185, 12/06/2003, Ministry for Environment). No restrictions exist for surface waters, which are often badly contaminated with total coliform load usually ranging from 10^4 to 10^5 CFU 100 mL^{-1} (Bonomo et al., 1999). These are used for unrestricted irrigation of vegetables normally eaten uncooked. However, many farmers regularly use untreated wastewater unlawfully to satisfy crop water needs under extreme water shortage conditions (Ait Melloul et al., 2001; Campos et al., 2002; Capra and Scicolone, 2004).

A different approach was adopted by the World Health Organization (WHO) which recommends the more liberal threshold of 1000 CFU 100 mL^{-1} of faecal coliforms for unrestricted irrigation of crops to be eaten uncooked, sports fields and public parks (WHO, 1989). Although there are no hygienic standards concerning restricted irrigation of cereals crops, industrial and fodder crops, pasture and trees (WHO, 1989), Blumenthal et al. (2000) suggested a threshold from $\leq 10^3$ to $\leq 10^5$ faecal coliform bacteria 100 mL^{-1} on treated wastewater depending on the exposed groups and irrigation techniques.

The classification of treated wastewater in microbiological categories, and its use for irrigation, should take into account crops types (edible or not) and their human consumption (without or after processing), health hazards for risk groups (young, old, pregnant or immunocompromised consumers and operators such as farmers), water application technologies and the duration of the irrigation season. All this could allow a wider use of the treated wastewater for irrigation associated with minimal health and environmental risk (Blumenthal et al., 2000; Oron et al., 2001; Salgot et al., 2001, 2006; Campos et al., 2002).

Stringent microbiological quality standards require intensive and expensive sanification methods (complex treatment systems; high chemical usage). The simplified treatments, used in this experiment (Lopez et al., 2006), can result in a significant reduction of faecal agents in the effluent, but with considerable reductions of treatment cost. The schemes used were designed to produce water for irrigation reuse with higher levels of organic matter, nitrogen and phosphorus, all being important for soil fertility and plant metabolic activities and usually eliminated during the sewage treatment.

Even though some researches were done in order to better clarify the fate of various pathogenic microorganisms in the soil–plant system (Campos et al., 2000a,b; Oron et al., 2001; Pourcher et al., 2007), their survival in different climates, soil characteristics, irrigation systems, and agronomic practices need to be investigated further.

The main purpose of this study is to monitor the medium-term impact on the soil–plant system of drip irrigation with low-quality wastewater, reclaimed by simplified municipal treatment schemes. *E. coli*, enterococci and sulphite-reducing *Clostridium* spores were selected as indicators of faecal contamination, whereas *Salmonella* was selected as pathogenic bacteria. In addition, the research focuses on the use of reclaimed municipal wastewater for olive tree (*Olea europaea* L.) irrigation under safe conditions.

2. Materials and methods

2.1. Experimental field, pedological and climatic parameters

Trials were carried out for 7 years (2000–2006) in an olive grove located in Southern Italy (Ferrandina–Basilicata region, 40°29′ N, 16°28′ E). Mature trees (*O. europaea* L. -cv Maiatica, a double aptitude variety) were vase trained and planted at a distance of about 8 m × 8 m.

The climate in the area is classified as semi-arid. The annual rainfall of 561 mm (mean 1976–2006) falls mostly in the winter. The mean annual temperature ranges from 15 to 17 °C.

The soil of the experimental grove is a sandy loam (WRB: Haplic Calcisol). The top 60 cm soil layer had an average organic matter content (±standard deviation) of 12 ± 6.2 g kg^{-1}, total nitrogen 0.8 ± 0.2 g kg^{-1}, available phosphorus 11.7 ± 5.9 mg kg^{-1} and potassium 104 ± 70 mg kg^{-1}.

The key meteorological parameters (air temperature, rainfall, humidity, etc.) were measured daily by a standard weather station placed close to the trial area. The reference evapotranspiration (ETo), provided by SAL service of the Extension Regional Service (www.alsia.it), was the mean value coming from the application of Blaney–Criddle, radiation and Hargreaves methods for ETo estimation. Crop evapotranspiration (ETc) was calculated using the equation recommended by FAO: ETc = Kr × Kc × ETo (Kr = reduction coefficient; Kc = crop coefficient) (Doorenbos and Pruitt, 1977; Allen et al., 1998).

2.2. Irrigation treatment and horticultural practices

Wastewater was treated by a pilot unit according to simplified schemes (Lopez et al., 2006). The simplified treatment excluded biological processes for organic matter and nitrogen removal from the wastewater in order to recover them and use them as fertilising substances. The treatment schemes used avoided the cost incurred by the biological oxidation and the removal, dehydration and disposal of biological sludge. The pilot plant functioned according to three configurations, which drew wastewater from the municipal plant in three different points (sewer water, water from the pre-denitrification unit and the final sedimentation) (Lopez et al., 2006). Two disinfecting agents, peracetic acid and products based on chlorine, were tested at different doses and contact times thus producing water of diverse microbiological quality. Among the treatments tested that based on the flow squeeze from the pre-denitrification unit allowed to use water subjected to a reduction of only the rapidly biodegradable organic matter fraction, which is able to cause soil anoxic conditions. With respect to disinfection, the products based on chlorine showed less disinfection efficiency in water with more than 100 mg L^{-1} of BOD. Better results were obtained using peracetic acid with contact times exceeding 60 min and doses of 2.5 mg L^{-1}.

The treated wastewater was distributed daily by drip irrigation to a 0.66 ha part of the grove—the irrigated plot. In 2000, each tree was provided with six self-compensating drippers delivering 8 L h^{-1} of water at an operating pressure of 1.5 atm. Because wastewater quality strongly influences dripper efficiency (Taylor et al., 1995, Capra and Scicolone, 2004) a self-cleaning filter (120 mesh per inch, MOD. ODIS ser. 3040), equipped with an automatic flushing system, was used. During the irrigation season the filter was ulteriorly cleaned out by hand at 15-day intervals because the treatment schemes used provided wastewater with high organic particle content increasing the risk of emitter clogging. At the beginning of each irrigation season, the entire reticulation system was cleaned out and all emitters were checked and either cleaned or replaced.

Irrigation volumes were calculated by subtracting the effective precipitation (70% of total) from ETc. The efficiency of the irrigation system (0.9 for drip irrigation) was also taken into account. Irrigation was generally carried out from May to October and was discontinued 30–40 days before harvest.

The soil surface of the irrigated plot was covered by spontaneous weeds and grasses and mowed at least twice a year. Irrigated trees were lightly pruned each year. Crop residues and prunings were left on the ground as mulch. Fertilisers were applied by fertirrigation taking into account chemical analyses of the wastewater and of the soil and the mineral element balance in the orchard system (cover crops and pruning material contributions, amount of fruit removed from the grove) (Palese et al., 2006). Pest and disease control was performed according to the regional service recommendations for commercial olive groves.

A nearby rainfed plot, characterised by trees with similar features was taken as control. It was managed as conventional in the area namely by tillage, 2–3 times per year; mineral fertilisation carried out once per year by using granular product applied to the soil. Heavy pruning was carried out every 2 years and prunings were burned *in situ*.

2.3. Treated municipal wastewater, soil and olive fruit sampling

Wastewater was sampled three times during each experimental year's irrigation season. Water samples were collected randomly in the field from 5 to 8 emitters using 1000 mL sterile glass bottles and stored at 4 °C before microbiological analysis.

The odour test was performed on the water samples according to the APHA, AWWA, WEF methodology (1995).

To determine background soil contamination with faecal bacteria, soil was sampled in May 2000 before the experiment started. Soil samples were collected from the wetted area at different depths (0–5, 5–10, 10–30, and 30–60 cm). Soil sampling was in triplicate and performed three times during the irrigation period—before the irrigation season (May, BIS), in the middle (July, MIS) and at the end (October, EIS). Sample collection was done using a sterile manual auger. Soil samples were brought to the laboratory in sterile plastic bags and stored at 4 °C. Microbiological analysis was done within 24 h of sample collection. Soil moisture was determined on the same soil samples by drying at 105 °C for 48 h.

Each year, at the end of the irrigation season, 1.5-kg samples of olive fruits were collected by hand with sterile gloves directly from that part of the crown nearest to the drippers (the worst case condition). These were transported to the laboratory in sterile plastic bags and stored at 4 °C before microbiological analysis.

Fruit sampling was also carried out at harvest, 30–40 days after the last irrigation. Olives were harvested by a trunk shaker and samples were collected from nets placed directly on the ground under the emitters.

For comparison, analyses were carried out on soil and fruit samples collected, at the same dates and with the same methodologies, from the nearby rainfed plot.

2.4. Experimental design and data analysis

A repeated measures experimental design was adopted (Milliken and Johnson, 1984). The four dependent variables describing faecal soil contamination (*E. coli*, enterococci, sulphite-reducing *Clostridium* spores and *Salmonella* spp.) were measured each year over the irrigation season at irregular intervals: before irrigation started (BIS), in the middle (MIS) and at the end of the irrigation season (EIS). The dependent variables (bacterial density) were recorded in the soil samples randomly within two different blocks (the plot irrigated with municipal wastewater and the rainfed plot) at four different soil depths (0–5, 5–10, 10–30, and 30–60 cm). The experiment was repeated over 5 year trials (2000, 2001, 2002, 2005, and 2006).

The dataset was submitted to a univariate and multivariate repeated measures analysis of variance (proc glm) for a repeated measures experiment design using the Statistical Analysis Systems software (SAS-STAT V8 Version, 1999–2001). The analysis model adopted as a source of variation the irrigation treatments (two levels), soil depths (four levels) and years (five levels). Given the non-independence of repeated measures, the SAS "repeated" statement was adopted in order to carry out the test for sphericity of the covariance matrix structure (Khattree and Naik, 2003).

2.5. Microbiological analyses

2.5.1. Treated wastewater

E. coli and enterococci were enumerated in treated wastewater by a membrane filter procedure (APHA, AWWA, WEF, 1995; APAT, IRSA-CNR, 2003). Water samples were subjected to suitable dilutions and filtered through cellulose nitrate membrane filters with 0.45 μm pore size. The estimation of *E. coli* was performed using TBX medium (Oxoid, Basingstoke, UK). Plates were incubated for 24 h at 44 °C and blue colonies were counted as *E. coli*. Enterococci were enumerated on a Slanetz & Bartley medium (Oxoid). After plate incubation (for 48 h at 37 °C), membranes were placed on Bile Aesculin Azide Agar (Merck, Darmstadt, Germany) and incubated for 2 h at 44 °C. When any blacking of the medium occurred colonies were counted as enterococci. The density of both *E. coli* and enterococci were reported as CFU 100 mL^{-1}.

Sulphite-reducing *Clostridium* spores were determined according to APAT, IRSA-CNR (2003) and APHA (1998) methods. Water samples were pre-treated for 10 min at 80 °C. Spores were enumerated by means of a membrane filter technique using SPS agar (Merck). Plates were incubated for 24–48 h at 37 °C in an anaerobic jar with the anaerobic atmosphere generating system Anaerogen (Oxoid). The appearance of a black colony, surrounded by a black zone, following incubation was considered as sulphite-reducing *Clostridium* spores. Results were reported as CFU 100 mL^{-1}.

The determination for *Salmonella* spp. was performed as reported in APAT, IRSA-CNR (2003). One liter of treated wastewater was filtered through cellulose nitrate membrane filters (0.45 μm pore size). The membrane was then dipped in Buffered Peptone Water (BPW) (Merck) and incubated overnight at 37 °C. Afterwards a 100-μL aliquot of culture was transferred to 10 mL Rappaport Vassiliadis Soya Peptone Broth (RVSPB) (Oxoid) and incubated for 24 h at 42 °C. The broth was then streaked onto XLD agar (Merck) plates and Hektoen Enteric Agar (Merck). After 24 h at 37 °C, 3 colonies with typical morphology were inoculated in tubes with Triple Sugar Iron Agar (Oxoid). Presumptive *Salmonella* colonies were submitted to a slide agglutination test using Omnivalent Salmonella Antiserum (Mast Group, Merseyside, UK).

2.5.2. Soils and olive fruits

Soil samples were sieved at 2 mm under sterile conditions and then subjected to extraction–dilution step in 0.1% sodium pyrophosphate (pH 7.0) with glycerol added (1% final volume).

One-hundred grams of olives were homogenised with 900 mL of tryptone water (0.1%) by stomacher. Then 10-fold dilutions were carried out within the same medium.

E. coli and enterococci were counted using the Most Probable Number (MPN) method and following the 3 replications × 5 dilutions scheme (Woomer, 1994; APHA, AWWA, WEF, 1995). Enumeration of sulphite-reducing *Clostridium* spores was carried out according to the pouring plate method under anoxic conditions.

For *E. coli* counting 1 g fresh weight of soil or a 1-mL aliquot of the 10-fold dilutions was inoculated into tubes filled with 10 mL of Lauryl Tryptose Broth (Oxoid) and containing inverted vials.

After incubation for 24–48 h at 35 °C, a 0.1-mL aliquot of any broths showing gas bubbles was transferred to tubes containing EC Broth (Oxoid) and incubated for 24–48 h at 45 °C. A 0.1-mL aliquot was taken from tubes showing gas production (considered a positive reaction) and placed in a tube of Tryptone Water (Oxoid) to perform the indol test. After 48 h at 45 °C, several drops of Kovac's reagent were added to the broths agitating slightly: a cherry red colour, visible at the surface of the broth, was considered positive for indol confirming the presence of *E. coli*.

In order to measure enterococci soil and fruit samples and their dilutions were inoculated in tubes filled with Azide Dextrose Broth (Oxoid); tubes were incubated for 24–48 h at 37 °C. Subcultures were then inoculated in Ethyl Violet Azide Broth (Oxoid) and incubated for 48 h at 37 °C. Grey–violet colour detected in the bottom of the tubes was indicative of enterococci presence.

Soil and fruit results were expressed as MPN g^{-1}, respectively on dry and fresh weight basis using the MPN tables (de Man, 1983).

To assay the sulphite-reducing *Clostridium* spores an aliquot of 10 mL from soil and fruit sample dilution was incubated for 15 min at 75 ± 5 °C. Suspensions were rapidly cooled and further diluted in a sterile extraction solution. An aliquot of 1 mL of each dilution was inoculated in SPS agar plates (Merck) by the pour plate method; then plates were incubated for 24–48 h at 37 °C in an anaerobic jar with the anaerobic atmosphere generating system Anaerogen (Oxoid). Black colonies, surrounded by a dark halo and resulting catalase-negative, were identified as sulphite-reducing *Clostridium*. Soil and fruit results were expressed as CFU g^{-1}, respectively on dry and fresh weight basis.

Salmonella spp. determinations were carried on 10 g soil samples inoculated in 90 mL of BPW. Olive samples instead were combined with 900 mL of BPW medium. After overnight incubation at 37 °C, 100 μL aliquots were transferred to RVSPB. *Salmonella* spp. identification was performed according to the methods described before for wastewater.

3. Results and discussion

3.1. Climatic parameters, irrigation volumes and irrigation system efficiency

Climatic parameters measured over the experimental period are reported in Table 1. Over the years of the trials the ETo values were very similar (1113.4 mm ± 15.3 mean over the trial period ± standard error), while the amount and distribution of rain determined different levels of water deficit, e.g. in 2001 (1059.8 mm) and 2002 (670.3 mm). The weather in 2001 was characterised by a very dry summer with only 7.6 mm of precipitation between June and September whereas, in 2002, precipitation during the same period was 133.4 mm. Irrigation volume applied over the annual growth season averaged 293 mm between 2000 and 2006.

Over the years of the experiments no disagreeable odours were detected on wastewater collected during its distribution by drippers. Such concern is one of the most important in assessing the implementation of wastewater treatment facilities (Muttamara, 1996).

Table 1
Reference evapotranspiration (ETo), precipitation, water deficit (April to October period) and seasonal irrigation volume over the years of trials

	2000	2001	2002	2003	2004	2005	2006
ETo (mm)	1142.7	1155.8	1081.7	1183.3	1105.9	1070.6	1054.0
Precipitation (mm)	274.6	96.0	411.4	314.4	274.8	256.6	296.4
Water deficit (mm)	868.1	1059.8	670.3	868.9	831.1	814.0	757.6
Irrigation volume (mm)	223.1	314.4	270.9	292.1	278.1	327.8	347.8

Over the 7 years of the trial the number of emitters that had to be replaced (before irrigation started and during the irrigation season) represented only about 5% of the total. The use of a self-cleaning filter equipped with an automatic flushing system reduced the clogging of the emitters allowing both efficient and reliable irrigation and a reduction of system maintenance. Total suspended solids and Biochemical Oxygen Demand (BOD_5), measured in the reclaimed wastewater, were, respectively 5 and 100 mg L^{-1} (mean over the experimental years). These parameters are considered as the main factors in controlling irrigation efficiency (Capra and Scicolone, 2004).

3.2. Reclaimed wastewater quality

Water contamination by *E. coli* varied considerably from 0 to 78,000 CFU 100 mL^{-1} during the trials (Table 2). In most cases (80%) the *E. coli* count was over the current Italian threshold of 10 CFU 100 mL^{-1} (Decree No. 185, 12/06/2003). In the 20% of samples *E. coli* contamination was equal to or above the value of 1000 MPN 100 mL^{-1} fixed by the World Health Organisation (WHO, 1989) for "irrigation of crops likely to be eaten uncooked". According to the WHO guidelines (1989), wastewater reclaimed by the pilot plant could be used for fruit tree irrigation. Irrigation should be stopped 2 weeks before harvest and no fruit should be picked up off the ground.

No indication is given, either in Italian or in international legislation, about the maximum allowable concentration of enterococci and sulphite-reducing *Clostridium* spores. Salgot et al. (2006) estimated for *Clostridium perfringens* levels ranging from zero to 100 CFU mL^{-1} relating to the final use of reclaimed wastewater. In particular, for fruit trees irrigation (microbial category IV), Salgot et al. (2006) indicated a threshold of <10 CFU mL^{-1}. Enterococci and sulphite-reducing *Clostridium* are widely recognised, together with *E. coli*, as useful indicator for contamination, because of their resistance to disinfection and environmental factors and their ability to survive for long periods in the environment (Tallon et al., 2005; Salgot et al., 2006; Pourcher et al., 2007).

Considerable variability in the levels of enterococci and sulphite-reducing *Clostridium* spores were observed in the treated

Table 2
Microbiological hygienic quality of treated wastewater

Year	*Escherichia coli*		Enterococci		Sulphite-reducing *Clostridium* spores		*Salmonella* spp.
	Minimum (CFU 100 mL^{-1})	Maximum (CFU 100 mL^{-1})	Minimum (CFU 100 mL^{-1})	Maximum (CFU 100 mL^{-1})	Minimum (CFU 100 mL^{-1})	Maximum (CFU 100 mL^{-1})	
2000	0	78,000	0	3,900	100	4,000	Absent
2001	10	51,000	670	13,500	10	6,400	Absent
2002	25	24,000	10	7,500	80	5,200	Absent
2003	1000	7,800	250	8,300	56	6,800	Absent
2004	50	800	20	200	20	80	Absent
2005	100	700	330	800	630	3,400	Absent
2006	72	3,000	18,000	28,000	88	31,000	Absent

wastewater during the 7 years of the experiment—enterococci ranged from 0 to 28,000 CFU 100 mL^{-1} and spores of sulphite-reducing *Clostridium* from 10 to 31,000 CFU 100 mL^{-1}. Such wide variability depended mainly on changes in our simplified wastewater treatment schemes. As reported by Lopez et al. (2006) the pilot plant worked according to three different schemes producing water characterised by a variable content of total coliforms and enterococci ranging from 0 to 100,000 and 0 to 100 MPN 100 mL^{-1}, respectively. These values are indicative and strongly influenced by the types of disinfection agents used, their contact times and the resistance to disinfection of the faecal bacteria.

Salmonella spp. was never detected in wastewater samples (Table 2) or in the soil and fruit samples collected during the 7 years of the trials.

3.3. Hygienic quality of soil

The concentrations of *E. coli* (<1 MPN g^{-1} d.s.), enterococci (<1 MPN g^{-1} d.s.) and sulphite-reducing *Clostridium* spores (2 CFU g^{-1} d.s.) measured in the soil sampled before the trials started (May 2000) were very low (mean of the four sampled depths). No *Salmonella* contamination was recorded.

3.3.1. Escherichia coli

The sphericity test, performed on bacterial densities, was significant ($P < 0.0001$) and thus the null hypothesis of compound symmetry of the within-block measurement was rejected. It follows that the experimental design could not have been analysed using the univariate techniques applicable for a split plot design (Milliken and Johnson, 1984).

The MANOVA test for the repeated measures of *E. coli* was significant ($P < 0.0001$) for all the sources of variation (time intervals, irrigation treatment, depth of soil and year) as well as for all levels of interaction–combination of the sources of variation.

A slight increase in the concentration of *E. coli* was recorded in the irrigated soil during the irrigation season reaching a maximum value in the middle of the season (from 2 to 16 MPN g^{-1} d.s.) (Fig. 1a). Taking into account the quality of the treated wastewater (*E. coli* ranging from 0 to 78,000 CFU 100 mL^{-1}) (Table 2) and that water was distributed to the olive grove daily during the irrigation period (an average of 2.3 mm day^{-1}), the measured contamination should be considered as very slight. As reported by Campos et al. (2000b) 1 day after irrigation faecal coliform contamination in the soil was significantly reduced (up to four logarithmic units) depending on the quality of the wastewater and the type of irrigation system. Soil seems to be able to reduce human bacteria contamination and their associated health risk. From this point of view, land application could be considered as an efficient mean of wastewater disposal (Campos et al., 2000a,b). On the other hand in rainfed soil a significant increase in *E. coli* value (53 MPN g^{-1} d.s.) was recorded at the last sampling date. This was in agreement to Gessel et al. (2004) who found background levels of faecal coliforms, ranging from 30 to 90 MPN 100 mL^{-1}, in soil sampled before manure application. This contamination could be ascribed to grazing, common in the experimental site, and considered as a non-point (diffuse) source of contamination together with roaming wild animals and birds and runoff from agricultural areas (Mawdsley et al., 1995; Venglovsky et al., 2006).

At the beginning of each experimental year, we observed a decrease of *E. coli* concentration compared with the values measured at the end of the wastewater distribution in the previous year (Fig. 1b). Many researchers, even if starting from different sources of faecal bacteria (livestock manure, treated sewage sludge, pig slurry), reported survival times for *E. coli* ranging from 3 to 80 days depending on the wide variability of

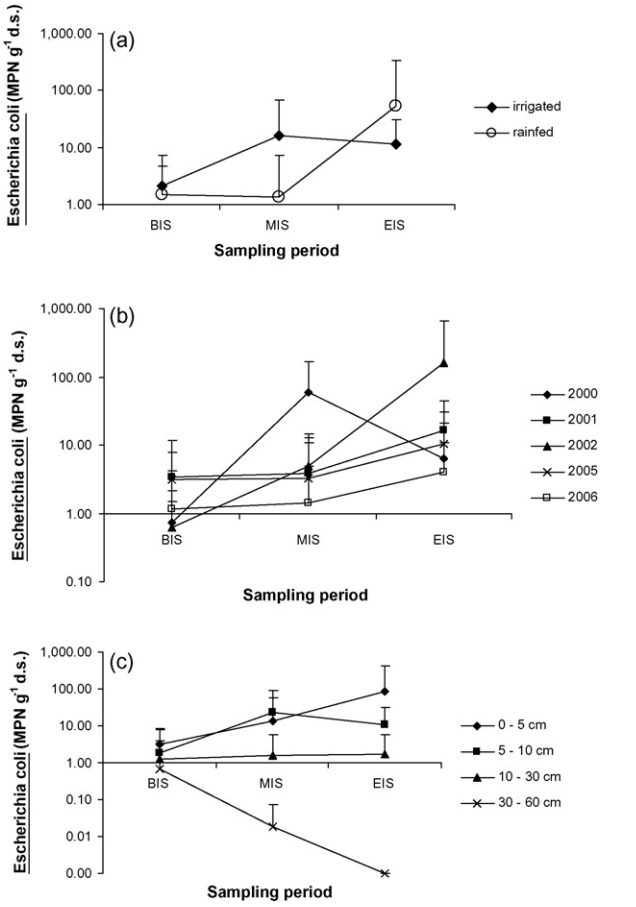

Fig. 1. *Escherichia coli* concentrations (as logarithmic values) measured before the irrigation season (BIS), in the middle (MIS) and at the end (EIS) according to the different treatments (a), the experimental years (b) and the soil depths (c). Vertical bars represent standard deviation ($n = 60$ replicates for treatments; $n = 24$ replicates for experimental years; $n = 30$ replicates for soil depth).

factors influencing it. Low temperatures and good soil moisture represent the best conditions for bacteria survival (Mawdsley et al., 1995; Ogden et al., 2001; Estrada et al., 2004; Nicholson et al., 2005; Pourcher et al., 2007). In general sandy soils have a negative effect on the survival of pathogens compared with clay soils, which makes ideal microhabitats for bacterial survival (adequate levels of water and nutrients, protection against predation) (Mawdsley et al., 1995; Cools et al., 2001; Pourcher et al., 2007). Organic matter also plays an important role in the survival of exogenous microflora. A sandy soil with 2.4% of organic matter makes better conditions for exogenous bacteria survival than loamy sand and loamy soils with low organic matter content (1.5 and 1.1%, respectively) (Cools et al., 2001).

E. coli prevalence was detected especially in the two upper soil layers (0–5 and 5–10 cm) (Fig. 1c). Decreasing concentrations were observed with depth with values measured at the deepest levels being negligible (ranging from 1.7 to <1 MPN g^{-1} d.s. which is equivalent to no contamination). We infer that soil acts as a filter, reducing bacterial concentration in the deeper soil layers. These findings are in agreement with the results of El Hamouri et al. (1996) and Oron et al. (2001). In particular El Hamouri et al. (1996) observed a gradual reduction of faecal coliform concentration through the soil profile, a silty clay type, and a complete disappearance of contamination beyond the limit of 25 cm, when raw wastewater bearing 1000 CFU of coliforms per 100 mL was used for irrigation. Straining, depending on the soil pores and

bacterial size, and adsorption onto soil particles are the most important factors influencing bacteria transport through the soil (Mawdsley et al., 1995; Campos et al., 2000b; Oron et al., 2001). Furthermore the presence of channels due to plant root systems and earthworm burrows can strongly influence vertical migration of pathogens through the soil profile (Mawdsley et al., 1995; Joergensen et al., 1998). On the other hand, the correct irrigation management (low water volumes distributed daily by drip irrigation system according to soil hydrological and physical parameters and climatic pattern) and the intense water absorption by the roots of both olive trees and cover crops, active in the wetted soil volume, excluded water logging by runoff and percolation to deeper soil layers avoiding aquifer pollution by faecal bacteria.

3.3.2. Enterococci

The sphericity assumption was met ($P < 0.0001$) but, unlike *E. coli*, the MANOVA test for repeated measures of enterococci was not significant except for the time intervals and year interaction ($P < 0.0001$).

Enterococci followed the same trend in both irrigated and rainfed soils even if in the former they showed higher contents (Fig. 2a). Such enrichment was clearly due to the distribution of wastewater which, during the experimental period, ranged from 0 to 28,000 CFU 100 mL^{-1}. A very slight enterococcic contamination was recorded in soils sampled from the rainfed plot.

Enterococcal concentrations during the irrigation season of the different experimental years did not indicate any clear trend, but wide variability in contamination was noticed (from 0.188 to 87.1 MPN g^{-1} d.s.) (Fig. 2b).

As reported for *E. coli*, enterococci were present particularly in the upper soil layers (0–5 and 5–10 cm) peaking in coincidence of EIS sampling (Fig. 2c); in the others layers enterococcal concentration tended to decrease with depth down to negligible values (always <1 MPN g^{-1} d.s. in the deepest levels).

3.3.3. Sulphite-reducing Clostridium spores

The sphericity test was significant ($P < 0.0001$) on all data. On the other hand the MANOVA test for repeated measures of the sulphite-reducing *Clostridium* spores was not significant.

A significant increase of spores was observed at MIS and EIS sampling dates in the irrigated soil with respect to the rainfed soil due to the distribution of the contaminated wastewater (Fig. 3a). A small degree of contamination was recorded also in rainfed soil. Pourcher et al. (2007) found a relatively high concentration of *C. perfringens* (around 10^2 spores g^{-1} d.m.) in soil sampled before sludge application evidencing the omnipresence nature of this bacteria.

No clear trend was shown by *Clostridium* spore concentrations during the seven trial years but a high variability between the years (Fig. 3b). *Clostridium* spores are able to survive for many years under unfavourable environmental conditions indicating remote faecal pollution (Cimenti et al., 2005).

With respect to the other microorganisms studied, sulphite-reducing *Clostridium* spores showed a slightly different behaviour in their distribution through the soil profile (Fig. 3c). Like *E. coli* and enterococci, the highest concentrations were recorded in the upper 0–5 and 5–10 cm layers, but the reduction of contamination in the deepest layers was less marked especially at the end of wastewater distribution. Schijven et al. (2003), studying the breakthrough curve of *C. perfringens* spores in effluent coming from laboratory columns (effluent samples taken at 0.4 m), verified that at first they attached very rapidly to the porous medium because of their relatively high collision efficiency (a parameter which indicates the intrinsic adsorption capacity of the soil); subsequently the previously attached spores broke through. The high detachment

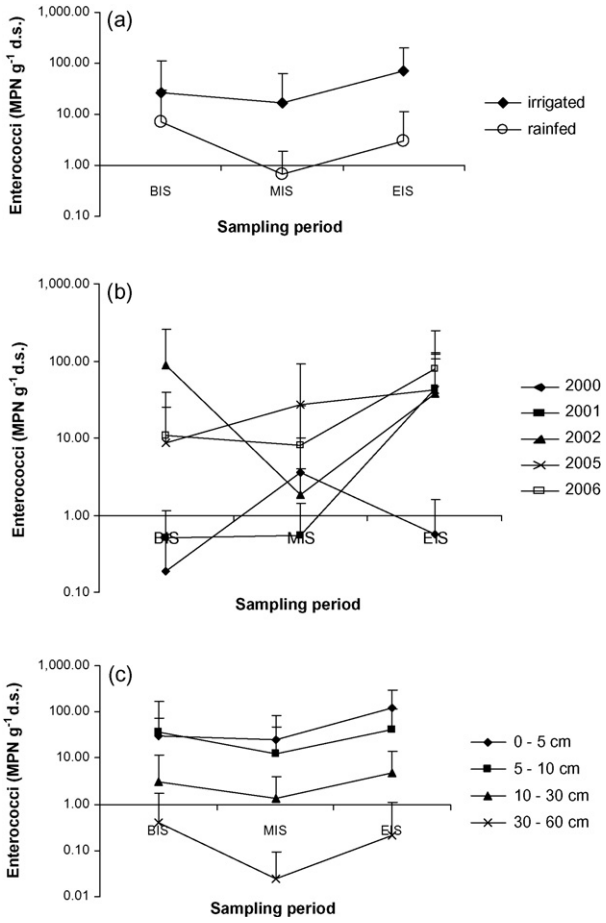

Fig. 2. Enterococci concentrations (as logarithmic values) measured before the irrigation season (BIS), in the middle (MIS) and at the end (EIS) according to the different treatments (a), the experimental years (b) and the soil depths (c). Vertical bars represent standard deviation ($n = 60$ replicates for treatments; $n = 24$ replicates for experimental years; $n = 30$ replicates for soil depth).

rate, evidenced by the high tail of the breakthrough curve, was indicative of reversible adsorption of spores. This condition, together with negligible spore inactivation, lead the authors to suggest that spores removal depends on the duration of contamination. Furthermore the high persistence of spores can allow significant subsurface transport. Such a condition suggests the need to consider sulphite-reducing *Clostridium* spores as further faecal indicators within the guidelines for wastewater reuse. On the other hand *Clostridum perfringens*, member of the sulphite-reducing *Clostridium* group, has been proposed as a faecal anaerobe indicator, due to its strong correlation with occurrence of pathogens in different matrices (Davies et al., 1995). Epidemiological studies of water, associated with illnesses, demonstrated a direct correlation between occurrence of gastrointestinal symptoms and the concentration of *C. perfringens*, *Aeromonas* spp. and *Vibrio cholerae* (non-O1) in beach water (Kueh et al., 1995) and Savichtcheva and Okabe (2006) included *C. perfringens* among the alternative indicators of water faecal pollution. These organisms have been also proposed as microbiological standards for drinking water (European Union, 1998). Payment and Franco (1993) indicated *C. perfringens* as suitable indicator for viruses and parasitic protozoa. In addition, the presence of sulfite-reducing *Clostridium* is commonly used to evaluate the hygienic status of sediments (Robles et al., 2000). Sulphite-reducing *Clostridium* were also found by Guzmán et al. (2007) to be the most resistant microorganisms in biosolids produced after mesophilic and

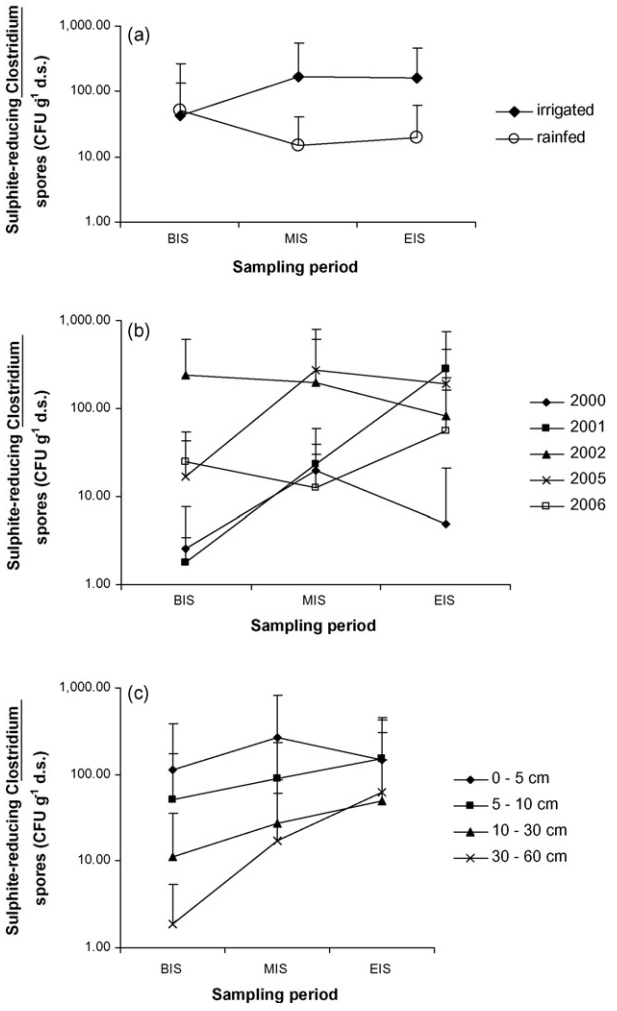

Fig. 3. Sulphite-reducing *Clostridium* spores concentrations (as logarithmic values) measured before the irrigation season (BIS), in the middle (MIS) and at the end (EIS) according to the different treatments (a), the experimental years (b) and the soil depths (c). Vertical bars represent standard deviation ($n = 60$ replicates for treatments; $n = 24$ replicates for experimental years; $n = 30$ replicates for soil depth).

thermophilic treatments. The same authors suggested that sulphite-reducing *Clostridium* may be an indicator for the presence of protozoan oocysts.

3.4. Hygienic quality of olive fruits

No surface contamination was recorded on the fruits picked at the end of the irrigation season from the irrigated treatment, except in 2001, when a very low concentration of Enterococci and sulphite-reducing *Clostridium* spores was recorded (Table 3). It is not easy to determine if this contamination was due to the fruits' contact with the wastewater, to an environmental pollution or to an accidental contamination occurring during sampling. The former possibility could be realistic because we operated in the worst case condition (drupe sampled from the part of the crown placed near the drippers) but the absence of pollution in the other experimental years does not provide a certainty to this hypothesis. On the other hand drip irrigation, especially when utilized on fruit trees, avoids aerosol spraying and contact among wastewater, fruits and leaves thus reducing or eliminating contamination.

The fruits collected from the nets at harvest time showed only in one case a very weak *E. coli* contamination (10 CFU 100 g^{-1} fresh weight) on irrigated drupes (Table 3) which fell within the quality recommendations ($\leq$100 CFU g^{-1} *E. coli* for pre-cut fruits and vegetables ready-to-eat) established by the European Commission (Commission Regulation 2073/2005). According to WHO guidelines (1989), in case of fruit tree irrigation with reclaimed wastewater, fruits should be not harvested from the ground. El Hamouri et al. (1996) found that cucumber (ground contact) was much more contaminated than tomatoes (grown using stakes), although both crops received the same volumes of irrigation with treated wastewater. Bastos and Mara (1995) found contamination levels on radishes and lettuces of the order of 10^3 to 10^4 *E. coli* per 100 g fresh weight using for irrigation an effluent within limits set by WHO (1989). Minhas et al. (2006) reported contamination for different types of crops irrigated with sewage water characterised by average faecal coliform counts of 1.5×10^8 100 mL^{-1}. Faecal coliform counts for vegetables, fodder and grain crops ranged between <2 and 9×10^5, 9×10^2 and 2×10^5 and <2 MPN 100 g^{-1} fresh weight, respectively. In addition, El Hamouri et al. (1996), Ait Melloul et al. (2001) and Minhas et al. (2006) stressed the importance of the exposure of the edible parts of the plants to solar radiation and dessication to reduce possible contamination

Table 3
Hygienic quality of olive fruits over the years of trials

Year	Treatment	Sampling procedure	*Escherichia coli* (CFU 100 g^{-1} fresh weight)	Enterococci (CFU 100 g^{-1} fresh weight)	Sulphite-reducing *Clostridium* spores (CFU 100 g^{-1} fresh weight)	*Salmonella* spp.
2000	Irrigated	Canopy	0	0	0	Absent
		Net	10	5	0	Absent
	Rainfed	Net	0	0	0	Absent
2001	Irrigated	Canopy	0	4	20	Absent
		Net	0	1	15	Absent
	Rainfed	Net	0	8	16	Absent
2002	Irrigated	Canopy	0	0	0	Absent
		Net	0	80	0	Absent
	Rainfed	Net	0	31	2	Absent
2005	Irrigated	Canopy	0	0	0	Absent
		Net	0	0	0	Absent
	Rainfed	Net	0	0	0	Absent
2006	Irrigated	Canopy	0	0	0	Absent
		Net	0	11	0	Absent
	Rainfed	Net	0	20	0	Absent

especially for pathogen bacteria less resistant to environmental conditions.

Enterococci and sulphite-reducing *Clostridium* spores measured on fruits sampled from the nets in the irrigated grove are reported in Table 3. The contamination by these faecal indicators was always weak and often comparable or lower than that showed by fruits collected from the rainfed plot (Table 3). Such a condition suggests that the found contamination could depend on environmental factors or post-harvest handling.

4. Conclusions

Municipal wastewater, reclaimed according to low cost simplified schemes, has been used successfully, under our specific experimental conditions, for the safe irrigation of the olives. The hygienic quality of the soil and of the products was preserved although *E. coli* content of wastewater was often over the limits set by the Italian Government and WHO. While *Salmonella* spp. was never detected in water, contamination by the other examined enteric bacteria (enterococci, sulphite-reducing *Clostridium* spores), not considered by the guidelines for wastewater reuse in agriculture, varied considerably. The wastewater distribution along the irrigation season slightly affected soil hygienic features especially in the soil top 10 cm. However a soil quality recovery was observed during the winter period. The guided management of the experimental grove (particularly irrigation and soil management practices) excluded water percolation and avoided exogeneous bacteria transport to the deeper soil layers; in addition, soil acted as a filter reducing bacterial concentration in the same layers. Among the studied bacteria, sulphite-reducing *Clostridium* showed a slightly different behaviour especially in distribution through the soil profile because of its resistance to the environment and the reversible adsorption mechanism of its spores in the soil. Such concern suggests that long-term safe reuse of low-quality wastewater for irrigation of olive trees (but also of other fruit crops) should be supported by guidelines which take into account more suitable indicators for the assessment and monitoring of microbiological quality of wastewater, soil and products.

No significant contamination was recorded on fruits harvested by irrigated olive trees even if we worked under the worst-case conditions. On the other hand this study has stressed the important role of other uncontrollable bacterial sources (grazing activity, roaming wild animals and birds, runoff from agricultural areas) probable responsible of the light contamination of soil and drupes in the rainfed plot.

Acknowledgments

This research was financed by the European Community and the Italian Government (AQUATEC Project "Innovative and sustainable technologies for facing water emergency in Southern Italy"). The authors thank Mr. Angelo Mossuto for his valuable support in sampling and experimental field management.

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