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Occurrence of Food-born Pathogen in Vegetables Irrigated with Sewage Water and Their Biological Treatment

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THE USE of untreated sewage water in irrigation of vegetables represents critical problem for the environment and human health. The present study was conducted to assess the extent of bacterial contamination of two vegetables (lettuce and tomato fruits) due to irrigation with sewage water in Belbais, Al-Sharqia Governorate, Egypt. Biological treatment process for the sewage water by two algal spp. (*Spirulina platensis* and *Chlorella vulgaris)* at two different concentrations of the algae (2.5ml/ and 10ml/500ml of sewage water) and at two different incubation periods (15 and 21 days) were done. Analysis of sewage and underground water for total coliform, fecal coliform, algae and heavy metals were done. Results showed that sewage water samples were highly contaminated than underground water. Twenty four vegetables samples (lettuce and tomato fruits) were collected from 2 fields, the first was irrigated with sewage water (6 lettuce and 6 tomato fruits samples) and the second with underground water (6 lettuce and 6 tomato samples). All samples were examined for *E. coli* and *Salmonella* spp*.* For lettuce irrigated with sewage 50% of samples were contaminated with *E .coli* while for tomato irrigated with sewage 66.6 of samples were contaminated with *E. coli* and *Salmonella spp.,* but the tomato samples irrigated with underground were 33.3 and 50% of samples were contaminated *with E. coli* and *Salmonella spp*., respectively. The highest effect of the *Spirulina platensis* and *Chlorella vulgaris* to reduce the bacterial count (total coliform and fecal coliform) and to remove Lead (Pb) concentrations almost was of the higher concentrations with long time, while to remove the Cupper (Cu) was at the lower concentrations with the long time for the same algae.

Keywords: *Salmonella* spp*., E. coli*, Lettuce, Tomato fruits, Sewage water, Biological, Treatment, *Spirulina platensis*, *Chlorella vulgaris.*

There is increasing evidence that consumption of fresh-served vegetables is a major factor contributing to human gastrointestinal illness, due to the potential for contamination with pathogenic microorganisms. Incidence of food-borne pathogens on vegetables and fruits varies by region and can be extremely high in some developing countries. However, substantial outbreaks continually occur in developed countries. The produce related illnesses cost USA up to $39 billion annually (Scharff, 2009). United States Environmental Protection Agency (USEPA, 2012) stated that members of two bacteria groups, coliforms and fecal coliforms, are used as indicators of possible sewage contamination because they are commonly found in human and animal feces. Wide spectrum of pathogenic organisms in low quality water poses the most immediate and direct risk to public health due to the transmission of fecal bacteria and diseases such as: bacterial diarrhea, dysentery, typhoid and cholera to consumers of wastewater-irrigated produce (Plauborg *et al.*, 2010). Consumption of fruit and vegetable products is commonly viewed as a potential risk factor for infection with enteropathogens such as *Salmonella spp.* and *Escherichia coli* O157, with recent outbreaks linked to lettuce, spinach and tomatoes (Heaton & Jones, 2008). Girmaye *et al*. (2014) detected total coliform in lettuce vegetable farm and fecal coliform in cabbage due to irrigation with polluted Awash River water. World Health Organization (WHO, 2013) stated that salmonellosis is one of the most common and widely distributed food-borne diseases. Millions of human cases are reported worldwide every year and the disease results in thousands of deaths. Goldfrank *et al.* (2006) concluded that most common causes of food-borne disease are bacteria *Salmonella spp*., *Shigella sp*., *Clostridium perfringens*, *Staphylococcus aureus*, *Campylobacter sp.*, *Bacillus cereus*, *Escherichia coli*, group A *Streptococcus*, *Clostridium botulinum*, *Vibrio-cholera*, viruses, *hepatitis, A,E,F* and *G*, Norwalkvirus, parasites: *Entamoeba histolytica*, *Giardia lamblia*, *Trichinella spiralis*, chemicals and heavy metals.

Rattan *et al.* (2005) reported that sewage effluents contain much higher amount of P, K, S, Zn, Cu, Fe, Mn and Ni compared to groundwater. Rajappa *et al.* (2010) found that concentration of lead (Pb), copper (Cu) and cadmium (Cd) were below the detectable level in most of the ground water. Pathogens can enter vegetable plants and become internalized, that is, colonize some plant tissues. Early studies suggested that *E. coli* could be transported into the edible part of lettuce from soil through root system (Solomon *et al*., 2002). *Salmonella* Newport could be transported from contaminated roots to the aerial parts of lettuce seedlings depending on the developmental stage of the plant (Bernstein *et al*., 2007). Pathogens may enter aerial portion of plants through stoma, scar tissue, or wounds as a consequence of irrigation water contacting leaf surfaces or from raindrop splashes from the soil surface (Kroupitski *et al*., 2009; Materon *et al*., 2007a Mitra *et al*., 2009).

Hossein *et al.* (2014) stated that phycoremediation is the process of employing macro or microalgae to reduce the toxic pollutants from the municipal wastewater, which has many advantageous over other conventional methods, as it is low cost and environmental friendly. Olguin (2012) stated that using microalgae in sewage treatment has several advantages such as microalgae are excellent at capturing CO_2 fixation, biodiesel resulted from microalgae is one of the very few bio-fuels with negative CO_2 emissions. Also, microalgae treat sewage water to make it used in irrigation purposes. Abdel-Raouf *et al.* (2012) found that the environmental factors which were favorable for algal growth were unfavorable for the survival of coliforms. **Doke *et al.* (2004)** reported that microalgae such as *Spirulina* could reduce the total bacterial count from sewage water. Chen & Pan (2005) stated that living cells of *Spirulina* were found to have high tolerance to lead and can be regarded as an attractive option for biosorption of heavy metal contaminant. And they added that the use of *Chlorella vulgaris* as a treatment provides high treatment efficiency. Bashan and Bashan (2010) evaluated *C.vulgaris* capacity to remove copper, nickel and lead from the wastewater.

The main objectives of this research included: a- analysis of sewage and underground water for total coliform, fecal coliform, algae and some heavy metals. b- To assess the extent of bacterial contamination of vegetables (lettuce and tomato fruits) due to irrigation with untreated sewage water. c- Biological treatment of sewage by the use of (*Spirulina platensis)* and (*Chlorella vulgaris)* to remove or decrease bacteria and heavy metals in the sewage water before using it in the irrigation of crops.

Materials and Methods

Water analysis

Samples collection

Eight water samples that used in irrigation of vegetables were collected from two water sources (sewage and underground water) in Belbais area in Al Sharqia Governorate in Egypt during July 2010 and analysis according to American Public Health Association (APHA, 2005).

Samples procedures

Enumeration of total coliform and feacal coliform were carried out by using MPN technique according to APHA (2005). Total coliform was detected on Lauryl tryptose broth (LTb). The positive tubes were confirmed on brilliant green lactose bile broth (BGLB). Feacal coliform was detected by using EC broth media and confirmed using EMB agar media.

Algal detection

All water samples (sewage and underground) were examined microscopically using a magnification of 10X and 40X for determining the algal flora in these samples according to Patrick & Reimer (1975) and Perscott (1978).

Heavy metals analysis

The analysis had been done in the Central Laboratories for the Ministry of Electricity and Power. All water samples (sewage and underground) were examined for four heavy metals (Cd, Al, Cu and Pb) using atomic absorption spectrophotometer according to APHA (2005).

Detection of *E.coli* and *Salmonella* spp. from vegetables (tomato fruit and lettuce)

Sample collection

Twelve samples of lettuce were collected from two fields (6 samples were irrigated with sewage water and 6 were irrigated with underground water). Another 12 samples of tomato fruit were collected from the same fields (6 were irrigated with sewage and 6 with under-ground) during July 2010. All samples were collected and immediately brought to the laboratory of microbiology for bacterial analysis. The investigatins were conducted within three hours of sample collection.

Detection of E. coli

Analysis of *E. coli* were done according to the bacteriological analytical manual (BAM) (Feng *et al*., 2002). Twenty five grams of tomato fruits or Lettuce were aseptically removed from each sample using a sterile scalpel and blended in 225ml of sterile peptone water for 2 min to make a1:10 dilution as a cell suspension and diluted. The samples (1ml) from each dilution series were transferred in triplicates into Lauryl tryptose broth (LTb) medium. All tubes with positive results (turbidity and gas) were sub-cultured in EC broth media. The positive tubes were confirmed on Eosin methylene blue Agar (EMBA) .Colonies that were characteristic of *E. coli* were Gram stained, streaked onto tryptic soy agar (TSA). Pure colonies were sub-cultured in peptone water, and tested for (IMVIC reactions) and Triple Sugar Iron .

Detection of Salmonella spp.

Salmonella spp. was detected according to bacteriological analytical manual (BAM) method (Feng *et al*., 2002). Samples (25g) were aseptically removed from each sample using a sterile scalpel and blended in 225ml of sterile buffered peptone water for 2 min as a cell suspension and incubated at 35^{o}C for 24 hr for the metabolic recovery of cells. Then inoculated into selenite cystine broth (SCB) to allow selective enrichment for *Salmonella spp*. Then streaked on bismuth sulphite agar (BSA), hektoen enteric agar (HEA) and Xylose Lysine Deoxycholate (XLD, Oxoid) agar. The plates were examined for typical Salmonella colonies. Two typical colonies from the selective media were inoculated into triple sugar iron (TSI) and lysine iron (LI) agar slants. Presumptive *Salmonella* from TSI and LI agar slants were inoculated into 10ml urea broth (UB), and then subjected to (IMVIC tests).

Biological treatment of sewage water by algae against bacteria and heavy metals

Two isolates of algal species (*Spirulina platensis)* and (*Chlorella vulgaris)* were used for the treatment process. Those isolates were prepared in the agricultural research center in Giza, Egypt. Two concentrations of Algae were 2.5 and 10 ml per 500ml of sewage and were incubated at 30^oC for two incubation periods 15 and 21 days). Total coliform and fecal coliform of sewage water were detected before and after treatment. The same procedure was repeated for detection of Pb and Cu after 15 and 21 days using atomic absorption spectrophotometer method after the treatment process with the algae.

Statistical analysis

Standard deviation has been calculated for the studied parameters. In addition, the obtained data were treated statistically using analysis of variance as described by Snedecor & Cockran (1969). Means were compared by LSD at 5% using SPSS program Ver. 16.

Results and Discussion

Detection of bacteria, Algae and heavy metals in sewage and underground water

Anukool & Shivani (2011), US Environmental Protection Agency (USEPA, 2012) and United States Geological Survey (USGS, 2012) stated that the members of two bacteria groups: total coliforms (TC) and fecal coliforms (FC) are used as indicators for sewage contamination because they are commonly found in feces of human and animal. This was agreed with the present study as shown in Table 1 where it found that sewage water was highly contaminated with (TC) and (FC) bacteria and results showed that TC and FC bacteria were (>50x 10^3cfu/100ml) in the sewage water samples. The results indicated that TC and FC count of the underground water were (32.5+8.19 and 43.25+3.86 /100ml), respectively, that exceeds the permissible limits of the Egyptian standards for drinking underground water, 2007, which must be free from coliform bacteria.

TABLE 1. Detection of total coliform (TC), fecal coliform (FC), Algal sp. and some Heavy metals for sewage and underground water.

Contamination type \ Water type		Sewage water	Under ground
Bacterial group MPN/100ml	Total Coliform	>50x10^3	32.5±8.19
	Fecal coliform	>50x10^3	43.25±3.86
Egyptian standards		-	ND
Algal sp		6 genus of algae	ND
Heavy metals(mg/l)	Lead Pb	0.2044	ND
	Copper Cu	0.057	ND
	Cadmium Cd	ND	ND
	Aluminum Al	ND	ND

-ND: not detected

The permissible limits of treated wastewater (10^3MPN/100ml) stated in National Regulatory Standards for wastewater

For algae in water, Xu *et al*. (2008) and Burford *et al*. (2012) stated that the excessive nitrogen loads can stimulate excessive rates of primary production and a higher incidence of harmful algal blooms. They added that sewage nutrients increased primary productivity rates of phytoplankton due to the sedimentation of unused organic matter as stratification develops and a change in the phytoplankton species composition due to alterations in ambient nutrient ratios and quantities. This report was in the same line of the present study where different species (6 species) of algae such as: *Chroococcus minutes, Oscillatoria tenuis, Anabena sp., Synedra ulna, Melosira granulate and Spirulena major* were detected in the sewage water. On the other hand, the underground water samples of the present study showed no growth for any algal species due to the low concentrations of nitrates and phosphates. For heavy metals in water the present study showed that the sewage water contained higher amounts of Pb and Cu (0.2044 and 0.057mg/l, respectively) while in ground water were not detected (table 1).This was in agreement with that of Rattan *et al.* (2005) who reported that sewage effluents contained much higher amount of P, K, S, Zn, Cu, Fe, Mn and Ni compared to ground water. For Cd and Al in the present study were not detected in each sewage or underground. This agree with that of Leung & Jiao (2006) and Rajappa *et al.* (2010) who detected heavy metals in very low concentrations in underground water samples .

Detection of Salmonella spp. and E. coli in vegetable

Statistical analysis indicated that there is significant difference between the types of irrigation water for the presence of pathogens, as well as there is significant difference between the percentage of the recorded pathogen (*E. coli* and *Salmonella spp.*) in the two vegetable crops**.** Results showed that 3 out of 6 samples of lettuce (50 %) that irrigated with sewage water were contaminated with *E. coli* but free from *Salmonella* spp. (Table 2). This was in the same line with that of Ali *et al.* (2013) who found that 3 out of 10 samples of retail lettuce stores (30%) were contaminated with *E. coli* and Girmaye *et al*. (2014) who found that the highest total coliform count 6.6×10^6 was recorded from lettuce vegetable farm and faecal coliform count 5.7×10^5 was recorded in cabbage due to irrigation with polluted Awash River water in India On the other hand Mieke *et al*. (2014) found that *Salmonella* spp. prevalence in fresh-served vegetables that irrigated with water from reservoir was very high, namely 42% (20/48) in lettuce farms and 29% (14/48) in strawberries. The present investigation showed that lettuce samples which were irrigated with underground water were free from each of *E .coli* and *Salmonella spp*. According to Steele & Odumeru (2004) and Halablab *et al*. (2011) who reported that ground water had a good microbial quality compared to other water sources contaminated with sewage and the level of aerobic bacteria on lettuce irrigated by river water showed statistically a higher bacterial load than samples irrigated using ground water.

TABLE 2. Percentage of contaminated Lettuce and tomato fruit samples with *E coli* and *Salmonella* spp. which irrigated with Sewage or Underground water.

Vegetable type	Number of samples	Irrigation water type	% *of E. coli*	% of *Salmonella* spp.
Lettuce	6	Sewage	50	-
	6	Underground	-	-
Tomato	6	Sewage	66.6	66.6
	6	Underground	33.3	50
LSD at 5%	Water type: 2.55	pathogen: 4.23	water type x pathogen: 8.46	

In the present study 66.6% of tomato fruits that irrigated with sewage water were contaminated with *E. coli* and *Salmonella spp*. (Table 2). These results were agreed with that obtained by Forslund *et al.* (2012) who found that *Salmonella* has been shown to survive and grow on the surface of tomatoes and also taken up internally through stem scar where it was isolated from the tomatoes irrigated with sewage water in Italy. Also Ali *et al.* (2013) found that 1 out of 10 samples of tomato (10%) was contaminated with *E. coli*. The present results of tomato fruits irrigated with underground water showed that 2 out of 6 (33.3%) and 3 out of 6 (50%) of samples were contaminated with *E. coli* or *Salmonella spp*. (Table 2). Manure applications have been often anecdotally implicated in creating differences in microbiological water quality of surface waters. Analysis of point source data in the survey of surface waters in southern Alberta Canada showed that predicted manure output from cattle, pig, and poultry feeding operations was directly associated with prevalence of these pathogens (Johnson *et al.,* 2003). Microbial quality of well water can be affected by the design of wells, nature of the substrata and depth to groundwater and rainfall (Gerba, 2009). Rainfall events inevitably increase concentrations of pathogens and indicator organisms in streams, reservoirs, and ponds due to surface run off into waterways and release of bacteria from bottom sediments (Pachepsky & Shelton, 2011). Lettuce and tomato fruits samples that irrigated with swage water appear significantly highly contaminated with *E. coli* and *Salmonella* spp. than that irrigated with underground water. In the same line, data showed that the number of Lettuce and tomato fruit samples which contaminated with *E. coli* were significantly high than that contaminated with *Salmonella* spp. (Table 2).

Biological treatment of sewage by algae is important to decrease the sewage water pollutant from pathogen and heavy metals before irrigation. Oilgae (2009), Olguin (2012) and Hossein *et al*. (2014) stated that algae are an important bioremediation agent that is already being used by many wastewater facilities. They added that purpose microalgae–bacteria based systems for treating wastewater. Abdel-Raouf *et al.* (2012) stated that the environmental factors which were favorable for algal growth were unfavorable for the survival of coliforms. So, the growth of some algal species led to decrease bacterial count. In the present study two algal species of *Spirulina platensis* and *Chlorella vulgaris* with two concentrations (2.5 and 10 ml/500ml sewage) at two incubation periods (15 and 21 days), for biological treatment of sewage water against bacteria (T C and FC) and heavy metals (Pb and Cu).The highest significant effect of the *Spirulina platensis* and *Chlorella vulgaris* to reduce total coliform and fecal coliform was at high concentration 10ml/500ml sewage with long time (21days). Where, *Spirulina platensis* and *Chlorella vulgaris* reduced 99.82 and 99.67%, respectively, from the total coliform and 100% from the fecal coliform. In the same line Ahmad *et al.* (2013) found that the maximum reduction in total coliform was 100% by *C. vulgaris* from wastewater. On the other hand, Doke *et al.* (2004) reported that *Spirulina sp.* could reduce the total bacterial count up to 75%. The present data (Table 3) indicated that *Chlorella vulgaris* is more efficient in removal heavy metals (Pb and Cu) from contaminated water compared to *Spirulina platensis*. *Spirulina platensis* and *Chlorella vulgaris* at retention time 21day with concentration 10ml/500ml significantly reduced sewage Pb by 96.6 and 99.02% respectively. In addition, the highest efficiency of *Spirulina platensis* and *Chlorella vulgaris* to remove Cu concentration were at low algal concentration (2.5ml/500ml sewage), where the removal percent is 74.04 and 84.21%, respectively.

In the same line, Mansor*et al.* (2011) found that *Spirulina* is capable of removing about 64% Pb and 63% Zinc. In addition, Bashan & Bashan (2010) evaluated the capacity of *C. vulgaris* to remove over 97% of copper, 91% of nickel and over 90 % of Pb from the wastewater. Chen & Pan (2005) stated that living cells of *Spirulina* were found to have high tolerance to lead and can be regarded as an attractive option for biosorption of heavy metals contaminant.

TABLE 3. Biological treatment of sewage water against total coliform (Tc) and fecal coliform (Fc) and heavy metals (Pb and Cu) by algal sp. *Spirulena platensis* and *Chlorella vulgaris* at (2.5 and 10 ml/500ml) concentrations for 15 and 21 days.

	Before treatment	**After treatment**							
Type of algae		**Blue-green alga(*Spirulenaplatensis)***				**Green alga(*Chlorella vulgaris*)**			
Incubation period		**15 days**		**21 days**		**15 days**		**21 days**	
Concentration of alga (ml/500ml)		2.5	10	2.5	10	2.5	10	2.5	10
Total coliform (MPN/100ml)	$2.4x10^5$	$24x10^3$	$24x10^3$	73	440	$24x10^3$	310	490	790
	% R zero	90.00b	90.00b	99.97a	99.82a	90.00b	99.87a	99.80a	99.67a
Fecal coliform (MPN/100ml)	$2.4x10^5$	$9x10^3$	520	zero	Zero	$19x10^3$	zero	zero	20
	% R zero	96.25b	99.78a	100a	100a	92.08c	99.99a	100a	100a
Pb(mg/l)	Concentration 0.2044	0.014	0.0136	0.011	0.008	0.0111	0.0097	0.0006	0.002
	%removal	93.2b	93.3b	94.6b	96.1ab	94.6b	95.6b	97.06ab	99.02a
Cu (mg/l)	Concentration 0.057	0.0234	0.0378	0.0148	0.0195	0.0149	0.0169	0.009	0.011
	%removal	58.95d	33.68e	74.04b	65.79c	73.86b	70.35b	84.21a	80.70 a

Values in the same row with the same latter not significant different at $P<0.05$
Percent (%) of Reduction(R) in bacterial count and percent (%) of removal in heavy metals

Conclusion

Sewage water highly contaminated by chemical and biological pollutants so the use of untreated sewage water in irrigation of vegetables represents critical problem for the environment and human health. The results of the study showed that the vegetables (lettuce and tomato fruits) that irrigated with sewage water were highly contaminated than that irrigated with underground water. Also the results indicated that use of *Spirulina platensis* and *Chlorella vulgaris* to remove of decrease bacteria and heavy metals in the sewage water lead to decrease in the remaining of each bacteria and heavy metals. So sewage water must be treated before using in irrigation.

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تواجد مسببات الأمراض المنقولة بالغذاء في الخضروات المروية بمياه الصرف الصحي و معالجتها بيولوجيا

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إستخدام مياه الصرف الغير معالجة فى رى الخضروات يمثل مشكلة خطيرة على البيئة وصحة الانسان. وقد أجريت هذه الدراسة لتقييم مدى التلوث البكتيرى لاثنين من الخضار (الخس و ثمارالطماطم) وذلك بسبب الري بمياه الصرف الصحي الغير معالجة في بلبيس ، محافظة الشرقية ، مصر. .وقد أجريت تحاليل على ثمانى عينات مياة منها أربعة عينات من مياه الصرف الصحي وأربعة عينات من المياه الجوفية ويشمل ذللك التحليل التالى: بكتريا القولون الكلية ، بكتريا القولون البرازية، الطحالب والمعادن الثقيلة. وأظهرت النتائج أن عينات مياه الصرف الصحي كانت ملوثة للغاية عن المياه الجوفية. كما تم جمع أربع وعشرون من عينات الخضار (الخس و ثمار الطماطم) من حقلين تم رى الأول بمياه الصرف الصحي (٦ عينات من الخس و٦ من ثمار الطماطم)، وتم رى الحقل الثاني بالمياه الجوفية (٦ عينات من الخس و ٦ من ثمار الطماطم) . وتم فحص كل العينات لبكتريا *ايشيريشيا كولاي وجنس السالمونيلا*. واظهرت النتائج ان عينات الخس المروية بمياه الصرف الصحي اعطت ٥٠% من العينات ملوثة ببكتريا ايشيريشيا كولاي بينما بالنسبة لثمار الطماطم المروية بمياه الصرف الصحي فقد اعطت٦٦,٦% من العينات ملوثه بكلا من بكتريا *ايشيريشيا كولاي والسالمونيلا* . ولكن عينات الطماطم المروية بالمياة الجوفية فقد اعطت ٣٣,٣% و ٥٠% من العينات ملوثا *بايشيريشيا كولاي و السالمونيلا* على التوالي. لذلك فقد تم اجراء عملية المعالجة البيولوجية لمياه الصرف الصحي مع اثنين من الطحالب وهما طحلب اخضر مزرق (*سبيرولينا بلاتينسيس*) وأخر طحلب أخضر (*كلوريللا فولجاريس)* بتركيزين مختلفين وهما (٢,٥ مل و ١٠ مل من الطحالب لكل ٥٠٠ مل من مياه الصرف الصحي) وعند فترتين تحضين مختلفتين هما (١٥ و ٢١ يوما). وقد كان أعلى تأثير للطحلبين *(سبيرولينا بلاتينسيس و كلوريللا فولجاريس)* للحد من العد البكتيري لكلا من بكتريا (القولون الكلية ، بكتريا القولون البرازية) وإزالة الرصاص كان عند التركيزات الكبيرة من الطحالب والزمن الأطول، في حين لإزالة الحجم الاكبر من النحاس كان عند التركيزات الأقل مع فترة التحضين الأطول لنفس الطحالب.