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Effect of sewage on *Caenorhabditis elegans*

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Abstract
Dumping untreated sewage into the environment can be harmful as well as serious health risk. This includes other contamination potentials such as contamination of drinking water sources. Sewage can also directly and indirectly affect human health. The potential toxicity of dumped sewage to free-living organism *Caenorhabditis elegans* was studied at four different locations within dumping area. The findings showed that all worms were completely paralyzed especially at 100%, 50% and 25% of all the tested locations, after 24 hours of incubation. After 48 hours of incubation, sewage showed higher toxicity in *C.elegans*, as it produced ~90% mortality in *C.elegans* with observed paralysis on mobility. There was no significant difference among locations B, C and D on the mortality of *C.elegans*. Finally, this study gave initial and serious warning to avoid dumping untreated or poorly treated sewage into the environment.

Key words: Sewage, *C.elegans*, environment, ecosystem, pesticides, nematode.

Introduction
Daily, tens of million litres of sewage go down from homes, schools, factories and other business activities which are then flown through the sewer lines to sewage water plant. Sewage undergoes quickly five processes: preliminary treatment, primary treatment, secondary treatment, disinfection and finally sludge treatment. These processes can be done naturally (a couple of weeks) but cannot handle huge volume of sewage. Therefore, sewage must be fully treated before is being returned into the environment. The full explanation of this topic is beyond the scope of this study.

Unfortunately, >90% of urban sewage is directly discharged into surface water without being treated or after primary treatment only in most of developing countries [23]. However, discharging poorly treated or untreated sewage is still concern in many parts of the world.

Sewage is quite recently being dumped closer to local farms which may increase serious issues. Dumping sewage is often not sustainable and can lead to many problems such as contamination of drinking water sources, contamination of land and farm lands, loss of nutrient resources for farming, contamination of water sources using for drinking, farming and bathing and bad smells. Contamination of drinking water sources can be occurred from septic tank, raw sewage overflow, land application of sludge partially treated and leaking sewer lines [20].

Leakages of sewer lines result in the introduction of microorganisms, organics, chlorides and trace metals and chemicals which might cause disease and foul odor or taste in drinking water[20]. Therefore, a direct health risk can be occurred by sewage from different kind of pathogens such as viruses (e.g. hepatitis, enteroviruses, poliovirus, Norwalk), parasites (e.g. protozoans such as *Giardia* and *Cryptosporidium*, helminthes) and bacteria (e.g. *E.coli*, Cholera, Salmonella, Shigellas) [22, 23]. This includes small health risks, such as eye, skin or ear throat, when people contact to sewage. However, contamination of marine environment by sewage can also indirectly affect human health by consuming fish or shellfish rendered toxic by bacteria, organic compounds or metals [21], or exposure to contaminated water during recreational activities [9].

Contamination by sewage can cause eutrophication which leads to radical changes in productivity and biodiversity [7]. Mixture contents of sewage (nitrate, phosphate and organic matter of human waste) can also serve as a food for bacteria and algae leading to the overpopulations. It can then increase oxygen-demanding of most dissolved oxygen results in threating survival of other aquatic organisms [13, 15]. Adding sewage sludge to soil with lower organic content is widely used to increase fertility of soil that might increase the potential of heavy metal pollution of soil such as cadmium (Cd) especially when use partially treated sewage sludge [14]. Thus, high concentration of heavy metals can be harmful to organisms and can lead to physiological stress and diseases [12].

Caenorhabditis elegans has been successfully used as a test organism in several environmental and ecotoxicological studies to investigate soil toxicity [2, 8, 10]. *C.elegans* has water-permeable cuticle that allows uptake of water soluble materials such as dissolved metals [18].

Several endpoints have been examined in *C.elegans* such as changes in behaviour, gene expression, lethality, feeding and mortality to investigate toxicological effects of chemical compounds including heavy metals and pesticides [4, 5]. Paul and Manoj [17] used mortality of *C.elegans* to test toxicity of five pesticides carbaryl, malathion, endosulfan, methyparathion and chloropyriphos. In addition, *C.elegans* has successfully detected traces of toxicant in field soil which can be used as biomarker in initial monitoring [1].

A little is known about the toxicity of sewage to free-living organisms and other environmental impacts as sewage is being dumped without treatment. In Saudi Arabia for example, sewage is mostly dumped in nearby valley. In Al-Qassim region, most treated and untreated sewage is dumped in Al-Rummah valley. Therefore, this study would initially address the potential toxicity of sewage to free-living organism and *C.elegans* was selected to be a model organism for testing.

Materials and Methods

Twelve samples were collected from 4 different locations of fourth sewage treatment plant in Al-Qassim region. Three samples were randomly collected from each location. Clean and sterilized glass bottles were used to avoid contamination. The first three samples (called location A) were collected from the pool of primary treatment (before outflowing). Dumping sewage in Al-Rummah valley results in making an artificial river with approximately 3 km in tall and 50-100 m in width that is still increasing (Fig. 1).). see words was delected. The second three samples (called location B) were collected from estuary of sewage into the valley. The third three samples (called location C) were collected from the half way of the artificial river (1.5 km). The fourth three samples (called location D) were collected from the end of the artificial river. All samples collected from these locations were labeled and transferred to the laboratory at the college of agriculture and veterinary medicine at Qassim University for testing.

Caenorhabditis elegans was cultured according to Brenner [6]. It was grown in nematode growth media (NGM) (sodium chloride 3.0 g, agar 17.0 g, peptone 2.5 g, cholesterol 5 mg, distilled water 975 ml, calcium chloride 1 mM, magnesium sulphate 1 mM, potassium phosphate 2 mM). The plates were seeded with *Escherichia coli* (*E. coli*) strain OP50 and incubated at 37ºC for 24 hours [19]. Worms were either added to plates by “chunking” or single worms were transferred using a sterile platinum pick. Chunking was carried out when plates became crowded or the bacterial food supply was used up and involved cutting and transferring a small cube of agar from an old NGM plate of *C.elegans* to a new plate using a heat flamed stainless steel scalpel blade. The plates were incubated for three days at 20ºC allowing worms to grow. Worms were harvested by rinsing the plate with K-medium (potassium chloride 2.39 g and sodium chloride 3.099 g in 1 litre distilled water).

Figure 1. Dumping area of sewage at fourth sewage treatment plant in Al-Qassim region.

The sewage was initially tested as 100%, and then diluted to 50%, 25% and 10%. *C.elegans* was exposed to sewage in liquid media. Then 1.5 ml of sewage was transferred to labelled 6 well plates + 20 worms N2 (wild-type) *C.elegans*. Plates were incubated at 20°C for 24 and 48 hours. After 24 or 48 hours direct observations were taken on each well using a microscope for manually counting the dead worms in each treatment.

Dead worms were counted manually by direct observation under a microscope. For each treatment the mortality of 20 worms was determined and expressed as the mean mortality ± SEM.

The data obtained was calculated by using Microsoft Excel. Dilution-response curves for the mortality assay were plotted using Graph Pad Prism version 7. Data points were the mean ± SEM of each sewage dilution and the graphs were fitted using a nonlinear regression with a four parameter logistic equation where the upper plateau was set to 100% and the lower plateau set to 0.

Results

The effect of sewage on *C.elegans* was tested. Based on the mortality of *C.elegans* (Tables 1 and 2, Fig. 2) after incubating whether for 24 or 48 hours. After 24 h of incubation, directed observations under the microscope showed that all worms were completely paralyzed especially at 100%, 50% and 25% of all tested samples but worms are a bit mobile at 10% of all tested samples with 0% of mortality compared with control. After 48 hours of incubation, however, all samples tested here A, B, C, and D including their dilutions 100%, 50% 25% and 10% had significant effect on mortality of *C.elegans* ($P<0.05$, F test Graph Pad Prism 7). On the other hand, there is no significant difference among locations B, C and D on the mortality of *C.elegans* ($P>0.05$, F test Graph Pad Prism 7) except location A had significant difference compared with B, C and D ($P<0.05$, F test Graph Pad Prism 7). Furthermore, alive nematode seems to be unwell as they are completely paralyzed.

Table 1. Direct observations of *C.elegans* movement after incubating for 24 h with100%, 50% 25% and 10% of locations A, B, C and D compared with control.

	Locations / Observations			
Dilution %	A	B	C	D
10	M.S*	M.S	M.S	M.S
25	C.P**	C.P	C.P	C.P
50	C.P	C.P	C.P	C.P
100	C.P	C.P	C.P	C.P

*M.S means worms move slowly. **C.P means worms are completely paralyzed.

Table 2. The mortality percentage of *C.elegans* after incubating for 48 h with100%, 50% 25% and 10% of locations A, B, C and D compared with control (0% mortality).

	Locations / Percentage of mortality			
Dilution %	A	B	C	D
10	12.50	38.75	35.00	41.25
25	61.25	75.00	66.25	61.25
50	75.00	86.25	80.00	82.50
100	93.75	91.25	92.50	90.00

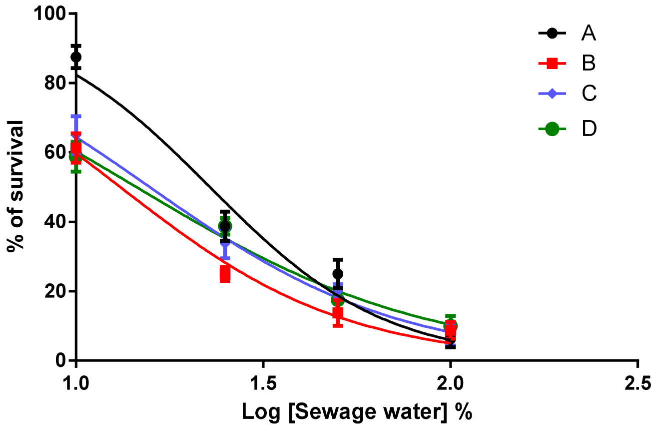

Figure 2. Comparison of the effects of sewage collected from four different locations A, B, C and D after 48 hour incubation on mortality of N2 expressed as % of the control mortality in distilled water. Each point is the mean ± SEM of 4 replicates, but in most cases the error bars are smaller than the symbols used. The lines were fitted using a non-linear regression in Graph Pad Prism 7 with the maximum plateau as 100% and the minimum as 0%.

Discussion

Early concerns were raised about leakages from sewer lines which generally untreated. Furthermore, the deep location of these lines can allow sewage to enter underground water directly. In contrast to the above statement, dumping untreated or poorly treated sewage in valleys is also direct contamination potentials not only for underground water but also microorganisms, birds, reptiles and animals. These include drinking water sources as well as crops in the nearby farms. In the experiments designed in this study *C.elegans* was used as a model organism to test the potential toxicity to free-living organisms. In *C.elegans*, Paul and Manoj [17] found out that > 90% mortality in carbaryl at 24 mM, > 90% mortality in malathion at 6 mM, 90% mortality in endosulfan at 5 mM, > 90% mortality in methyparathion at 2 mM and 90% mortality in chloropyriphos at 1 mM. Obtained results here show that sewage can produce similar percentage of mortality in *C.elegans* as 100% of locations A, B, C and D gave 93.75%, 91.25%, 92.5% and 90%, respectively, after 48 hours incubation although *C.elegans* can eat harmful bacteria such as *E.coli* [4]. It is not clear what mechanism of mode of action would be here, although at these dilutions worms are completely paralyzed after 24 h of incubation. At lower dilution 10% of location D 41.25% mortality was found to be the most toxic dilution compared with of other locations A, B and C 12.5%, 38.75% and 35%, respectively, at the same dilution 10%. That means sewage is becoming toxic after dumping which might increase the potential of contamination. It is also unclear why the toxicity of sewage is increasing here, although this water was early dumped and had long period of time. Also soil structure, soil type (clay) and high level of underground water of Al-Rummah valley might play a critical role in increasing toxicity as water does not absorb quickly in clay soil that can hold more water. Therefore, weak absorption of clay soil can increase widespread of sewage resulting in exposure animals, birds and reptiles to sewage. Moreover, high level of underground water can increase the potential of water pollution by sewage in this area. Thus, it is very obvious that the contamination potential by sewage is very high. In case of rain, however, the environmental fate of sewages is unknown as they will be flown with rain to other locations which might be farming lands. Furthermore, it is unclear that how much dilution it might be, which is depending on the volume of rainfall. For example, say the dilution will be 10% after rainfall, thus it would expect at least to have about 38% mortality in *C.elegans* regardless other microorganisms and earthworm. Therefore, soil fertility and sustainability can be disturbed by sewage as well as ecosystem. Also it results in decreasing the availability and activity of free-living organism in soil.

In Saudi Arabia for example, there are no lakes or rivers as the only water source for drinking and farming is underground water. Therefore, it must be careful to avoid contamination of water source. Therefore, sewage must not be dumped without being treated.

On the other hand, the main use of carbamate and organophosphate nematicides is to impair movement and paralyze nematodes [16]. For example, 1000 ppm oxamyl had 0% mortality on *C.elegans* but most treated worms are completely paralyzed [1]. By comparing the above statement with the effect of sewage on *C.elegans*, it is clear that mortality produced by sewage on *C.elegans* cannot be probably obtained from any nematicides at any concentration normally used in agriculture. *C.elegans* can also tolerate high concentrations of many chemical compounds including heavy metals such as copper [3]. Furthermore, *C.elegans* does not seem to be directly affected by the pH and salinity, as the sensitivity of *C.elegans* to both pH (range of 3.1 to 11.9 for 24 h and 3.2 to 11.8 for 96 hours) and salinity (up to 15.49 g/l NaCl (15,490 ppm)) in aquatic media [11] had no significant effects on lethality and was tolerated by *C.elegans*.

Conclusions

It would be expected to report many issues nearby these locations such as number of dead birds and reptiles, unhealthy animals and contamination of underground water. In the current study, the toxicity of sewage has been determined by using the simple model *C.elegans*. This study can be considered as a base study for further investigations in the environmental impacts and toxicity of sewage to animals, reptiles, birds and other free-living organisms. This includes the water pollution of underground water mainly sources of drinking water. The results obtained in this study can also at least give initial and serious warning about dumping sewage. In addition, it would be very important to assess the potential of heavy metal pollution in dumped area as it was reported that the addition of sewage can contribute to both mobility and heavy metal potential in soil such as Cd. The provided evidence of toxicity of sewage must be seriously considered and try to avoid conventional treatment. It is also highlighted that there is a need to revise treatment requirements and regulations which are necessary to keep our environment safe and clean.

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