Effects of Antibiotics on soil microbial activities and microbial biomass

Sakshi Srivastava [1] , Prerna Dixit [1], *Monisa Anwer
*Bansal Institute of Engineering and Technology
CytoGene Research and Development Lab

Abstract:

In recent years several releases from the industries of pharmaceuticals have been reported in soils nearby because of which several of the soil microbial biomass activities have been found to be effected. These activities are generally responsible for plant growths. Dehydrogenase activities, Ferrous (III) activities and substrate induced respiration. The traces of antibiotics in soil has also been found to kill microorganisms which are usually present in the soil for this antibiotic susceptibility pattern of microorganism was tested. In a while of research Effects of Sulfonamide and Tetracycline Antibiotics on Soil Microbial Activity and Microbial Biomass were determined. Effects of sulfonamide and tetracycline on the obtained microbial cultures of soil were found diminishing for Dehydrogenase activities of soil was decreased to 80 % on the fifth day. The value obtained was 12.34 formazan microgram/mland substrate induced inhibition (45.9% at 100 ppm of antibiotics). Antibiotic susceptibility was found only for microorganisms against both the antibiotics tetracycline was suitable to kill nearly 17 mm of ZOI for isolate SP03.

Keywords: Dehydrogenase activity, Antibiotic susceptibility,Substrate Induced respiration, Antibiotics

Introduction:

Pharmaceutical antibiotics are widely used for the medical treatment of microbial infective diseases. Consequently, heaps of antibiotics area unit annually administered to humans and animals, particularly to farm animals **(Thiele-Bruhn, 2003)**. Most pharmaceutical antibiotics area unit designed to be quickly excreted from the treated body, either dateless or as metabolites, a number of that area unit still. Bioactive **(Zuccato *et al.*, 2001).** Thus, sewerage sludge and manure, used as fertilizer for agricultural land, area unit typically contaminated with antibiotics **(Thiele-Bruhn, 2003).** Residues of pharmaceutical antibiotics have already been discovered in soils in concentrations of up to 330 gm for tetracyclines and eleven weight unit gone for sulfonamides **(Hamscher *et al.*, 2002).** Additionally, numerous antibiotics occur naturally in soils as a result of association may be a natural defense, with various soil microorganisms manufacturing antibiotics via their secondary metabolism **(Thomashow *et al.*, 1997).** However, the concentrations and kinds of pharmaceutical antibiotics free into soil disagree wide from the natural background. Hence, the unwanted effects of those antibiotics on soil microorganisms and microbial turnover processes in the soils area unit suspected and need investigation.

Till now , a very wide range of Bioassays have been found exiting which can be used to determine effects of different chemicals from soil microorganism **(Hart and Brookes, 1996; Welp and Brummer, 1999).** However while reviewing the literatures no much data was obtained on the effect of pharmaceutical formulations on soil microbial activities and its microflora the knowledge is somehow scarce. It is well known and established that microorganisms are usually killed by antibiotics **(Nye *et al.,* 2014).** In the work reported by Schmitt, the contamination induced in the soil by release of Sulfonamide and Tetracycline resulted in small changes in physiological profiles of people living out there **(Schmitt *et al.*, 2004).**In several other works microbial resistance induced by antibiotics have been also reported.

The complete goal of this study was to interrogate and research the outcomes of dual pharmaceutical antibiotics on the primordial microorganisms of surface soil samples from the agricultural soils nearby Pharmaceutical lands. The chosen compounds belong to two antibiotic structural classes that are widely for the treatment of cattle of domestic farms. In particular our objectives were to assess the appropriateness generally used to evaluate the toxic level of chemicals as well as to indicate effects of these formulations, particularly Dehydrogenase activity, Fe (III) reduction power, Substrate induced respiration and also to determine Antibiotic- susceptibility pattern of microorganisms isolated from the nearby agricultural lands of Pharmaceutical Industry.

2. Materials and Methodology

2.1 Soil Sample

Above surface of soil samples from the nearby soils of Pharmaceutical Industries sampling depth chosen for the 2-10 cm also the soil from nearby area was chosen which was in the grassland the turf of grasses were removed which was nearly of 15 cm. The chosen soil samples were characterized by the methods given by Giradiet al. (2011)also the properties are mentioned in the table 1. The chosen soil samples before any kind of analysis was sieved through filters and were homogenized.

2.2 Sample preparation and incubation:

To determine microbial activities to test soil, soil moisture was adjusted to near about 50 % for the capacity to hold water this was done using Ultra- pure water. For the applications of the antibiotics in the soil samples 100 ppm solutions of both the antibiotics viz. Tetracycline and Sulfonamide were placed in the sand in order to give soil the equal mix of the antibiotics which actually helped to further study the application so that further responses could be studied and dealt for the scenario in which soil microbial studies have to be further brought on pages. The solutions of antibiotics were made in methanol. Methanol from the solutions was further dried in order to prevent any toxicity from it.

2.3 Microbial activities of Soil.

Microbial soil activities firstly included DHA that meant Dehydrogenase activity this included pre incubation (providing specific temperature and pH) of dry soil with moisture. However it also became the causal of increase in DHA levels. This phenomena of performing Dehydrogenase activity was later studied by Stevenson, 1956; another soil microbial activity included was Fe (III) reduction pattern.

Similar clear dose response curves obtained with the Fe (III) reduction test were reported for organic pollutants such as pesticides, and chlorinated and aromatic solvents by **Zelles *et al.* (1986)** and **Welp and Brummer (1999),** who suggested that the Fe(III) reduction test is a suitable routine method to test dose related toxic effects of organic pollutants on soil microorganisms. We assume that this holds true for pharmaceutical antibiotics as well. Substrate Induced respiration were checked through respiration apparatus. Residue samples were incubated with appropriate amendments at 22.5 20.5 C in I3 ml Erlenmeyer flasks attached to a continuous flow-through respiration system modified and adapted from **Gullberg *et al.*, (2011).** CO:-free air was pumped over the samples at constant, preset Row rates and rapircd CO, trapped in 50 ml of alkali solution (NaOH). Preliminary experiments demonstrated that flow rates of 50 ml min’ and 0.04 N NaOH traps maximized CO,-trapping efficiency (97-98%) under standard conditions. The CO2 content of base solutions was determined by titration with standardized HCI (0.04 s) and all samples were corrected for CO2 content sample blanks.

2.4 Enumeration of Microbial content on Nutrient Agar Media:

Enumeration of microorganism was performed on Nutrient Agar Medium (N.A.M) Hi Media. The soil samples were spread after serial dilution on the Agar plates containing 25 ml of medium. The samples were spread over the plate with the help of a glass spreader. Keeping the plates incubator at around 25 ^{0}C in the BOD incubator, resulted in the growth of colonies. The colonies were streaked onto the fresh medium (N.A.) and were further purified.

2.5Antimicrobial susceptibility pattern

To analyze the antimicrobial effect of microorganismsthe media plates were spread over the agar plate of Mueller Hinton (Hi Media). The isolates were spread over the plate of MH Agar. Wells were pricked with the backside of the 20 microliter tip. The wells were filled with different concentrations of Sulfonamide and Tetracycline; different concentrations constituted 20 -100 microgram/ ml. The clear zone around the well demarcated the no microbial growth could have been supported in the presence of antibiotics.

3. Results and Discussions:

3.1 Effect on Antibiotics on Soil microbial activity

Both Sulfonamide and Tetracycline had significant effects on the Soil dehydrogenase activities. The parameters were found deviating from the values of Control, In the light of the type and applied dosage of the medicines. The chosen three samples showed significant difference in the values of soil microbial activities despite of being same in physiochemical parameters.

Table 1: Physiochemical analysis of soil

Soil Type	pH $CaCl_2$	$CaCO_3$ (%)	OC (%)	Fe	Mn	Al	pH of soil	N Total	CECpota (cmolc kg^{-1})^{A}
Alluvial	7.3	1.3	1.82	2.11	3.21	0.78	7.4	0.135	13.5
Cambisol	7.1	2.6	1.74	2.23	3.28	0.72	7.5	0.69	12.36
Control Alluvial soil	7	1.5	1.5	2.76	3.12	0.71	7.5	0.56	12.69

3.1.1 Dehydrogenase activity of soil

In while of biochemical procedures, bacteria and other microorganism extricate out enzymes such as dehydrogenase as well as phosphatase. One of the very crucial index of soil fertility is its enzymatic activity shown by proliferating microorganism. The enzymatic properties are generally rate limiting. It is a rate limiting step in decomposition as well as nutrient cycle. The microorganisms of soil play a key role in cycling of nutrients. The microorganisms of soil play key role in cycling of nutrients and its activity on mineralization. It also supports transformation of organic matter of soil ecosystem and their capacity to degrade organic matter. Nutrients thus released form the pattern of growth. The dehydrogenase activity was calculated as in terms of Formazan Microgram /gm and Standard deviations were also calculated.

Table 2: Dehydrogenase activity of Soil when at different concentrations the soil was treated with different concentrations of antibiotics Tetracycline and Sulfonamide

Antibiotics	**Concentrations**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Tetracycline	100 ppm	14.04±0.24	12± 0.27	11± 0.27	12 ±0.23	11±0.56
	200 ppm	13 ±0.34	12 ±0.45	12±0.21	13 ±0.43	12±0.24
	300 ppm	10±0.21	10±0.31	10±0.23	10±0.32	11±0.21
	400 ppm	10±0.24	11 ±0.32	11±0.24	11±0.23	10±0.24
	500 ppm	11±0.21	10±0.23	9±0.23	9±0.23	9±0.23
Sulfonamide	100 ppm	11.04±0.24	11± 0.27	10± 0.27	10 ±0.23	10±0.56
	200 ppm	12 ±0.34	11 ±0.45	11±0.21	11 ±0.43	10±0.24
	300 ppm	9±0.21	9.8±0.31	9.9±0.23	9.6±0.32	9.2±0.21
	400 ppm	11±0.24	11 ±0.32	10±0.24	9.4±0.23	9.8±0.24
	500 ppm	10±0.21	9.8±0.23	9.8±0.23	9.7±0.23	9.8±0.23

From the above mentioned data it could be easily said that presence of Tetracycline and Sulfonamide could be a vigorous parameter to be unveiled. The Dehydrogenase activity was found to be delineating and further showed no shift. A constant decrement was their fate.

Hampering in enzyme activity such as Dehydrogenase activity in soil represents the adverse effects on microorganism of soil and decrement in itsindex could probably state that microflora is not at its good (**Unger *et al*., 2013; Liu e*t al*., 2014**). Here in our research work both the samples 1 and 2 showed Continous decrement in dehydrogenase activity with the increasing concentration of antibiotics both Tetracycline and Sulfonamide. Tetracycline in Figure 1 represents that sample 1 showed decrement in the Dehydrogenase activity but sample 2 somewhat got stucked to decrement at the concentration of around 100 ppm. In the case of control the Dehydrogenase activity was calculated every-time when the activity of sample calculated it temporarily showed decrement but all along the procedure it remain in its optimum values

Substrate induced Inhibition

Substrate induced inhibition though decreased relatively in soil sample 1, soil sample 2 and Control with the increment in dosage of antibiotics. Soil microbial biomass affects different physical properties of Soil and their nutrient cycles **(Lynch and Bragg, 1985)** Soil ecosystem are also effected by restrictions to microbes as reported by **(Coleman and Crossley, 1996)** has been reported. In order to develop crop management practices that promotes the fertility and productivity of soils, which are needed to examine the ascendancy, because these two groups separately influence **(Hendrix *et al*., 1986; Beare *et al*., 1990).**

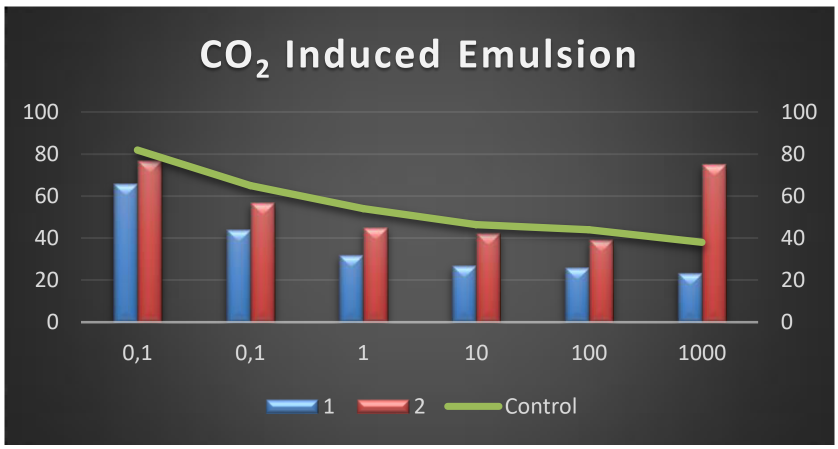

Figure 1: Substrate induced inhibition in sample 1, Sample 2 and Control against them Tetracycline

In the case of Sulfonamide the value remained same at all the concentrations, it could be inferred that action of Sulfonamide on soil and it microbial activity hasn't been so prevailing as for Tetracycline, to prove this antimicrobial susceptibility pattern the isolates driven out combinely from the two samples were checked.

Enumeration of culture:

A known volume of a soil suspension is filtered through a 0.2 µm pore size filter. The microbes on the filter are stained with a fluorescent dye and counted by using an epifluorescence microscope. At least 20 fields each containing 20–50 cells are counted and the total count is calculated from the area observed and the volume of suspension filtered.

A fixed amount (mainly in between 2-3 gm) was filtered through micro-membrane of pore size 0.2 micrometers. The microbes on the filter were collected by swab and spread over 100 mm petri plate. The colonies then observed were counted on digital colony counter. The CFU value turned out to be $3 * 10^{-2}$ in case of sample 1, $4.2 * 10^{-4}$ in case of sample 2. The third sample of control showed high CFU values majorly $5 * 10^{-2}$, since it wasn't contaminated by antibiotics. The cultures were streaked on Agar plate. The pure cultures obtained were further stained to observe the differential microbiology. 10 isolates were chosen these were mainly picked from the control soil.

Colony morphology and staining details are enlisted below

3.1.2 Antimicrobial activity by Agar Well Diffusion Assay

Antimicrobial activities were assessed by the procedure of Agar Well Diffusion assay using Mueller Hinton Agar. 5 isolates chosen on the basis of staining. The isolates were tried atleast to look different in their morphology. Therefore the chosen isolates were tested and following outcomes of Table 3 was obtained.

Table 3: The below mentioned table explains the Zone of Inhibition around the well in which the antibiotic tetracycline was poured.

Tetracycline					
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
SP01	12± 0.23	10±0.21	11±0.23	14±0.23	15±0.23
SP02	13±0.32	14±0.32	14±0.43	12±0.32	17±0.32
SP03	13 ±0.32	12±0.23	13±0.32	14±0.34	14±0.24
SP04	12±0.23	13±0.34	14±0.43	14±0.23	15±0.23
SP05	13±0.23	14±0.23	15±0.42	16±0.23	16±0.23

For the first case when tetracycline was used as antibiotic the isolate sp01 showed maximum ZOI of 15 mm on a very high concentration of 500 ppm, while the isolate SP02 showed maximum ZOI the minimally effected of all 3was Isolate SP03 all other showed subsequently similar antibiotic susceptibility.

Table 4: The below mentioned table explains the Zone of Inhibition around the well in which the antibiotic Sulfonamide was poured.

Sulfonamide					
	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
SP01	11± 0.23	11±0.21	12±0.23	13±0.23	14±0.23
SP02	12±0.32	13±0.32	13±0.43	13±0.32	13.5±0.32
SP03	13 ±0.32	13.6±0.23	13.9±0.32	14±0.34	14.2±0.24
SP04	13±0.23	13.5±0.34	13.23±0.43	13.29±0.23	14±0.23
SP05	13.24±0.23	13.65±0.23	14.21±0.42	15±0.23	15.24±0.23

*ZOI: Zone of Inhibition, PPM: Parts per million

For the second scenario when Sulfonamide was chosen as antibiotic the zone of inhibitions were maximum for the isolate SP05 and minimum for the isolate of SP02.

By relating our data to a field situation, it is reasonable to hypothesise that, although extractable concentrations of antibiotics in field soils are generally small **(Hamscher *et al.*, 2002)**, initial concentrations immediately after addition can be high enough to affect soil microorganisms. Moreover, an increase in the effective concentration due to bioaccumulation should also be taken into account **(Dojmi di Delupis *et al.*, 1992).**

Conclusion

Pollutions of environment caused by antibiotics is expected to welcome more attention in the close future since the consumption of antibiotic is still on its increment around the world. The above mentioned research shows that adverse impacts of antibiotic contaminations have affectedthe flora of nearby area by decreasing soil fertility. Soil fertility decrement could not be a process of one day. It has been due to effect on microbial biomass activity and soil microbes.

The contamination of soil by antibiotic may lead to low fertility of soil and become responsible for the adverse effect on microorganisms contained by them.

Reference

- **Thiele-Bruhn, S., Seibicke, T., Schulten, H.-R., Leinweber, P., (2004).** Sorption of sulfonamide pharmaceutical antibiotics on whole soils and particle-size fractions. J. Environ. Qual. 33, 1331–1342.
- **Zuccato, E., Bagnati, R., Fioretti, F., Natangelo, M., Calamari, D., Fanelli, R., (2001).** Environmental loads and detection of pharmaceuticals in Italy. In: Ku¨mmerer, K. (Ed.), Pharmaceuticals in the Environment—Sources, Fate, Effects and Risks. Springer-Verlag, Berlin, Germany, pp. 19–27.
- **Hamscher, G., Sczesny, S., Ho¨per, H., Nau, H., (2002).** Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. Anal. Chem. 74, 1509–1518.
- **Ho¨per, H., Kues, J., Nau, H., Hamscher, G., 2002.** Eintrag and Verbleib von Tierarzneimittelwirkstoffen in Bo¨den. Bodenschutz 4, 141–148.
- **Thomashow, L.S., Bonsall, R.F., Weller, D.M., 1997**. Antibiotic production by soil and rhizosphere microbes in situ. In: Hurst, C.J., Knudson, G.R., McInerney, M.J., Stetzenbach, L.D., Walter, M.V. (Eds.), Manual of Environmental Microbiology. ASM Press, Washington, DC, pp. 493– 499.
- **Stevenson, L.L., 1956.** Some observations on the microbial activity in remoistened air-dried soils. Plant Soil, 8: 170-182.
- **Zelles, L., Scheunert, I., Korte, F., 1986.** Determination of the effect of pentachlorophenol on the bioactivity of soils by the iron-reduction test. Chemosphere 15, 309–315.
- **Welp, G., Bru¨mmer, G.W., 1999.** Effects of organic pollutants on soil microbial activity: the influence of sorption, solubility, and speciation. Ecotoxicol. Environ. Saf. 43, 83–90.
- **Chenp W. and Coleman D. C. (1989)** A simple method for measuring CO, in a continuous air-flow system: modifications to the substrate-induced respiration technique. Soil Biology and Biochemistry.
- **Lynch, J.M. and Bragg, E. (1985)**. Microorganisms and soil aggregate stability. Adv. Soil Sci. 2: 133-161.
- **Paul, E.A. and Clark, F.E. (1989).** Soil Microbiology and Biochemistry. Academic Press. 1-273.

- **Hendrixs,P.F., Parmelee, R.W., Crossley, D.A.Jr., Coleman, D.C., Odum, E.P. and Groffman, P.M.(1986)** Detritus food webs in conventional and no-tillage agroecosystems. Bioscience 36 : 374-380.
- **Beare, M.H., Neely, C.L., Coleman, D.C. and Hargrove, W.L. (1990)** A substrate induced respiration (SIR) method for measurement of fungal and bacterial biomass on plant residues. Soil Biol. Biochem. 22 : 585-594.
- **Unger, I. M., Goyne, K. W., Kennedy, A. C., Kremer, R. J., McLain, J. E. T., and Williams, C. F. (2013).** Antibiotic effects on microbial community characteristics in soils under conservation management practices. *Soil Sci. Soc. Am. J*. 77, 100–112. doi: 10.2136/sssaj2012.0099.
- **Liu, B., Li, Y., Zhang, X., Wang, J., and Gao, M. (2014**). Combined effects of chlortetracycline and dissolved organic matter extracted from pig manure on the functional diversity of soil microbial community. *Soil Biol. Biochem*. 74, 148–155. doi: 10.1016/j.soilbio.2014.03.005.
- **Ma, T., Pan, X., Chen, L., Liu, W., Christie, P., Luo, Y., et al. (2016).** Effects of different concentrations and application frequencies of oxytetracycline on soil enzyme activities and microbial community diversity. *Eur. J. Soil Biol*. 76, 53–60. doi: 10.1016/j.ejsobi.2016.07.004.
- **Dojmi di Delupis, G., Macri, A., Civitareale, C., Migliore, L., 1992.** Antibiotics of zootechnical use: Effects of high and low dose contamination on Daphnia magna. Aquatic Toxicol. 22, 53–60.
- **Hart, M.R. and P.C. Brookes**. Soil microbial biomass and mineralization of soil organic matter after 19 years of cumulative field applications of pesticides. Soil Biol. Biochem. 28: 1641-1649. 1996.
- **Mark Nye, Nigel Hoilett, Cliff Ramsier, Peter Renz, Richard P. Dick.** Microbial Community Structure in Soils Amended With Glyphosate-tolerant Soybean Residue. *Applied Ecology and Environmental Sciences*. 2014; 2(3):74-81. doi: 10.12691/aees-2-3-1.
- **Schmitt, H., van Beelen, P., Tolls, J., & Van Leeuwen, C. L. (2004**). Pollution-induced community tolerance of soil microbial communities caused by the antibiotic sulfachloropyridazine. Environmental science & technology, 38(4), 1148-1153.
- **Girardi, C., Greve, J., Lamshöft, M., Fetzer, I., Miltner, A., Schäffer, A., et al. (2011).** Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. *J. Hazard. Mater*. 198, 22–30.

- **Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D., et al. (2011).** Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7:e1002158. doi: 10.1371/journal.ppat.1002158.
- **Coleman, David & Crossley, Dac & Hendrix, P.F.. (2004).** Fundamentals of Soil Ecology: Second Edition. Fundamentals of Soil Ecology: Second Edition. 1-386.