Evaluation of Effects of Municipal Sludge Leachates on Water Quality

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Abstract: Biosolids made from municipal sludge are an attractive solution instead of chemical fertilization. Nevertheless, their effects on the ecosystem should always be considered. In the present study, anaerobically digested sludge was subjected to two leaching methods (EN 12457-2 and NEN 7341) and the main physicochemical parameters were measured in the leachates. The aquatic organisms Daphnia magna and Vibrio fischeri were exposed to the leachates in order to test for adverse effects. Mixtures of biosolid/solid, simulating the high dose of 80 tn/ha, were also created, and the same parameters were measured for EN 12457-2 leachates. The results show a strong seasonal variation for the results for the municipal sludge, even though the sludge did not originate from a touristic area. The biosolid/solid mixtures did not produce toxic responses to the organism tested. Nevertheless, the parameters nitrites and nitrates in the leachates were increased in relation to control and they continued to increase even at Day 40 post-application. This increase was soil-type-dependent. The biosolids in question could be used for field fertilization, however measures should be taken against underground water nitrate pollution.

Keywords: activated sludge; biosolids; ecotoxicity; risk assessment; wastewater treatment

1. Introduction
Since the beginning of the last century, the management and disposal of solid and liquid municipal waste has become an important issue. Over time, this problem has been exacerbated as a result of increasing urbanization and industrialization [1]. Therefore, large amounts of municipal sludge are produced in wastewater treatment plants (WWTP), where various physicochemical and biological processes take place in order to efficiently treat wastewater [2]. This process produces an increased amount of sludge that has regularly been disposed in sanitary landfills. Data for EU-27 show that large quantities have been produced in 2006, reaching, in total, 9,866,728 tn of dried solids (DS), while the average sludge production was 20 kg/per capita for the same year. Greece presented a modest value of 11 kg/per capita, however there was a clear increase when compared with values of 1999 [3]. According to the requisites of [4], the number and the capacity of WWTP is bound to be increasing in Greece.

Furthermore, new legislative and scientific directives propose the reuse of the sludge in order to assist energy and nutrient recirculation in the biosphere [5]. As such, since 2006, most EU countries have ceased depositing sludge in landfills; some of them (Sweden, Luxemburg, Holland) have completely stopped this practice, while others have reduced it to 1–2% of produced sludge. However, more than 90% of sludge which is produced in Greece ends up in landfills [3]. This is clearly a non-sustainable solution, therefore the National Solid Waste Management Plan currently in place...
dictates that only 5% of biodegradable waste can be landfilled and the remaining 95% should be managed in alternative ways [6].

In this context, it has been shown that properly treated domestic sludge in the form of biosolids may improve soil characteristics, and it may also enrich both soil matter and plant biomass with valuable micronutrients, such as N and P [7,8]. Large quantities of municipal sludge are used in EU as an efficient, non-chemical means of fertilization; data from 2006 show that countries such as Spain, France, UK, Ireland and Slovakia use approximately 62–70% of their annually produced sludge as a fertilizer in agriculture [3]. This use is compatible with the principals of solid waste management, where the reuse of resources and of energy is prioritized and landfiling is the last solution.

Nevertheless, one should always consider the hazard posed by the presence of pollutants within the biosolids that may be transferred to the environment via land spreading, and especially when there is a long-term application of treated sewage sludge that may lead to a potential accumulation in soil [9]. This danger has been deemed as significant; as such, the Directive 86/278/EEC [10] has posed concentration limits for the metals of toxicological concern in the sludge and in the soil that receives it. Nowadays, the multitude of classic and of emerging environmental pollutants necessitate an update of the directive: polyaromatic hydrocarbons (PAHs) [11,12], polychlorinated biphenyls (PCBs) [11,12], various pharmaceuticals [13] have been detected in municipal sludge. Therefore, the updated sludge directive may also include limits for PCBs, PAHs, flame-retardants, pharmaceuticals and personal care products [14].

Biosolids may also affect groundwater and surface water and cause water quality deterioration. This is mostly due to the leaching of ingredients initially bound in the biosolid which then, due to climatic conditions, microbial activity, physicochemical changes or other interactions, end up in runoff or drainage. These ingredients may then pollute nearby water sources or infiltrate into groundwater. For example, it has been shown that Zn, an important trace element, is indeed released from heavily sludge-amended soil, especially at low pH values [15]. Sludge spreading in typical degraded soils (containing sand or fly ash) led to the vertical mobilization of Ni and Cd deeper than 0.8 m in soil [16]. Nitrogen from biosolids can also leach into groundwater. Groundwater is an important resource that may satisfy human consumption and plant irrigation needs [17], but it is under constant deterioration in the Mediterranean region [18–20]. The leaching of NO$_3^-$ to groundwater is one of the most important factors limiting the long-term viability of biosolid application to lands [21]. Elevated nitrate levels cause adverse health effects for the consumers, including neoplasias [22], methemoglobinemia [23] and cardiovascular problems [24]. The NO$_3^-$ contamination potential of soil, when amended with biosolid, is strongly affected by the biosolid type. Liquid digested and lagooned liquid undigested biosolids seem to have the greatest accumulation due to their large content of NH$_4^+$-N, while air-dried digested biosolids have the lowest [25].

Therefore, biosolid application in agricultural land can be beneficial and profitable when it is properly treated and used. Nevertheless, the risks should always be considered, and it is imperative that biosolids are fully characterized and processed in order not to cause harm instead of benefit when released to the environment. An effective way of assessing the environmental consequences of biosolids, when used for land application, is the use of ecotoxicity tests. Ecotoxicity tests are routinely performed in combination with chemical analyses [26], since the former can highlight the potential harmful effects on organisms.

The objectives of the present study were to characterize a number of treated biosolids produced from a WWTP in Attica (Greece) regarding their ecotoxicological potential throughout the year as well as monitoring the characteristics of mixtures of soil/treated biosolids, using practical and low-cost methods to assess their environmental impact. Therefore, samples of dehydrated and anaerobically digested sludge were subjected to two methods of leaching (EN 12457-2 and NEN 7341) and main physicochemical parameters were measured in the leachates. The aquatic organisms *Daphnia magna* and *Vibrio fischeri* were exposed to the leachates in order to simulate the environmental impact of these leachates, if they reach superficial water bodies. Additionally, mixtures of soil/biosolids were
made and placed under controlled conditions in containers for different time intervals. Those mixtures were subjected to the EN 12457-2 one-stage batch-leaching test at a liquid to solid ratio of 10 L/kg, main physicochemical parameters were measured in the leachates and the same organisms were exposed to them. The results may act as a valuable enrichment in sludge toxicity databases and may aid the characterization of sludges regarding whether they can be used for agricultural purposes.

2. Materials and Methods

2.1. Sampling and Creation of Mixtures

Four samples of dehydrated and anaerobically digested sludge were obtained from a WWTP in Attica, which receives municipal sewage and septic sewage in four different periods: spring on 18/5/2018 (SP, rain event 0.4 mm, 22–28 °C), summer on 7/9/2018 (SU, no rain event, 24–30 °C), autumn on 23/10/2019 (AU, no rain event, 16–22 °C) and winter on 29/11/2019 (WI, no rain event, 19–22 °C). The sample dates were chosen according to the WWTP convenience and when no extreme weather conditions prevailed. This research was part of a preliminary analysis of the WWTP in order to test for overt toxicity of the treated sludge. In this WWTP, septic sewage is received and primarily treated in a separate system. Municipal wastewater is prescreened in 25 and 8 mm screens and its waste is collected and landfilled. This is followed by grit removal in a 385 m$^3$ tank; primary sedimentation follows, and the influent is then treated in an activated sludge system of a total volume of 21,000 m$^3$ divided into five subsections, aerated by 15 slow vertical surface aerators. Secondary (final) sedimentation is performed in two sedimentation tanks, 4570 m$^3$ volume each. The effluent is disinfected with sodium hypochlorite and further clarified with sand filters. The WWTP has a total capacity of 24,000 m$^3$/day of septic sewage and 20,000 m$^3$/day of municipal wastewater corresponding to a total organic load of 30,470 kg/day BOD (500,000 inhabitants). In the WWTP, primary and secondary sludge is anaerobically digested in two main digesters of 7900 m$^3$ each, at 35 °C for approximately 28 days. The sludge is then pumped and dehydrated to 28% DS through four gravity belt thickeners and through the addition of polyelectrolytes at 4 kg/tn DS. The whole process is performed in a closed space equipped with deodorizer machinery. The samples (2 kg each) were taken from the final conveyor belt that carries the treated sludge to truck transfer and bagged into double-walled clear plastic bags. The bags were closed with plasticized wire, put in sealed carton boxes and transported to the Laboratory of Water Resources Engineering and Management, Division of Hydraulics and Environmental Engineering, Department of Civil Engineering of the Aristotle University of Thessaloniki. There, they were stored, wrapped with dark plastic bags and maintained in a cold room until use. Moreover, a fraction of the SP sample was mixed with two types of soils (clay or sandy) at a soil to wet sludge ratio of 50 w/w (corresponding to 80 tn/ha) and placed in plastic containers for different time intervals (1, 15, 30 and 45 days). Finally, the containers were placed in a chamber at 20–25 °C, with adequate luminescence (12 h) and were impregnated with water at regular intervals to simulate field conditions.

2.2. Leaching Tests and Parameters Tested

The sludge samples were subjected to two methods of leaching: the static one-step method EN 12457-2 [27] and the availability test NEN 7341 [28]. All the physicochemical and ecotoxicological parameters tested (toxicity to water organisms Daphnia magna and Vibrio fischeri) were measured in the leachates. For the EN 12457-2 method, sludge moisture was calculated for all samples, in order to achieve a liquid to solid ratio of 10 L/kg dry matter. The sludge samples were mixed with an appropriate amount of distilled water (L/S = 10) in 1 L polypropylene flasks. The flasks were then rotated for 24 ± 0.5 h at a speed of 10 rpm (Rotax 6.8 rotary shaker, VELP Scientifica, Usmate Velate, Italy). Subsequently, sludge mixtures were centrifuged at 4000 rpm for 10 min (Rotofix 32 A, Hettich, Tuttingen, Germany) and filtered through 0.45 μm pore filter. For the NEN 7341 leaching method, the sample was oven-dried for 24 h, crushed and diluted with deionized water (L/S = 50) in 1 L polypropylene bottles. HNO$\text{}_3$ (0.2 N) was added under constant stirring (3 h) to pH 7 ± 0.5.
The mixture was then centrifugated and filtered as before, the leachate was stored and the remaining solid was carefully collected and resubmitted to dilution with deionized water (L/S = 50). HNO₃ (1 N) was added under constant stirring (3 h) to pH 4 ± 0.5. The second leachate was also centrifugated and filtered, and finally both leachates were mixed and stored (final L/S = 100).

The mixture samples (sludge/soil) were subjected to the EN 12457-2 one-stage batch-leaching test at a liquid to solid ratio of 10 L/kg. The main physicochemical and biochemical parameters pH, electric conductivity, nitrates, nitrites, TOC and toxicity to the aforementioned organisms were measured in the leachates.

pH and EC were measured with a pH-meter BASIC 20+ (Crimson, Barcelona, Spain) and an electrical conductivity meter WTW inoLab Cond 720 (Xylem Inc., New York, USA) respectively. For the measurement of nitrates, the LCK 339 method of the company HACH LANGE was followed [29], using known dilutions of a standard solution of nitrates (detection limits: 1–60 mg/L). Nitrites were measured according to [30] at 542 nm with the UV-1800 UV-Vis SHIMADZU spectrometer (SHIMADZU CORPORATION, Kyoto, Japan) using known dilutions of a standard solution of nitrites (detection limits: 0.005–0.5 mg/L). TOC was measured according to the catalytic oxidation method [31] with combustion at 680 °C on a TOC-5000A organic carbon analyzer (SHIMADZU CORPORATION, Kyoto, Japan) using known dilutions of a standard solution of glucose (1, 5, 10 and 100 mg/L) for a four-point calibration curve. The precision and accuracy of the methods is periodically checked in the laboratory using known value samples in triplicates. The organisms *Daphnia magna* and *Vibrio fischeri* that were used for the ecotoxicity tests were exposed to the leachates according to ISO Guidelines [32] and the protocol of [33], respectively.

For the *Daphnia magna* immobilization test, the measurement of the toxicity was based on the immobilization of the organisms after exposure to the leachates for a period of 24 h. When it was possible, the parameter EC50 (based on the immobilization of the individuals) was calculated, through Probit Analysis (Microsoft Office 2013, Microsoft, Redmond, WA, USA) utilizing dilutions 0%, 6.25%, 12.5%, 25%, 50%, 100% of the initial leachate. Whenever the undiluted leachate could not cause at least 50% immobilization of the neonates, the parameter % immobilization was calculated instead. For the *Vibrio fischeri* inhibition test, the inhibition of the bioluminescence in relation to control sample (without leachate addition) was calculated through the MicrotoxOmni 4.1 software (SDI, Newark, USA), after 5, 15 and 30 min of exposure. For this test, the measurement protocols used are the 81.9% Screening Test, and when it was possible, the parameter IC50 was calculated through 81.9% Basic Test. The highest sample concentration tested was 82% due to the necessary salinity adjustment.

For both *D. magna* and *V. fischeri*, the parameter Toxic Unit (TU) was calculated according to the formula of [34] for all available leachates. When the parameters EC50 and IC50 could be calculated, they were transformed into toxic units (TUs) according to Equation (1). Otherwise, if the effect in the undiluted leachate was below 50%, the TUs were calculated proportionally (e.g., 20% effect is equivalent to 0.4 TU, and 50% effect is equivalent to 1 TU).

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TU = \left( \frac{1}{EC50} \right) \times 100
\]  

(1)

2.3. Statistical Analyses

The differences between seasons for each ecotoxicological parameter (*D. magna* immobilization, 5-, 15- and 30-min *V. fischeri* inhibition test) were assessed through repeated-measure ANOVA. Whenever significant differences were found (p < 0.05), ANOVA was followed by post-hoc HSD tests. Time differences between the biosolid/soil mixtures were assessed though two-way repeated measures ANOVA, for all the parameters with more than three repeats (TOC, nitrates, nitrites, *D. magna* immobilization, 5-, 15- and 30-min *V. fischeri* inhibition test). When an interaction between time duration and soil type was noted, the database was split and post-hoc HSD tests were performed in order to test for significant differences between timepoints. When no interaction was noted, only the effect of time duration was tested through post-hoc HSD tests on the database. All statistical analyses were performed using SPSS25 (IBM, Armonk, NY, USA).
3. Results

3.1. Results for the Sludge Samples

Table 1 shows the physicochemical parameters on the leachates of the four sludges tested for both leaching tests. As can be seen on Table 1, the sludge sample of spring showed the highest moisture content and EC, while pH was the lowest, for the EN 12457-2 leaching test. On the contrary, for the same leaching test, the summer sample showed the highest pH and the lowest EC. Figure 1a–d shows the EC50 values for *D. magna* and % inhibition values for *V. fischeri* for each season for both leaching tests. Table 2 shows the TUs calculated according to [34] for the four sludges and for both leaching tests and organisms tested. As can be noticed in Figure 1a–d and Table 2, the spring sample showed the highest toxicity on *D. magna*, while the autumn sample showed the lowest toxicity for both leaching tests. Actually, there was no EC50 available for *D. magna* for autumn for the EN 12457-2 leaching test; this means that the undiluted leachate did not produce toxicity in at least 50% of the organisms. This is also shown in Table 2, where the corresponding TU is only 0.7. On the contrary, *V. fischeri* showed slightly toxic results for the spring sample, and the autumn sample showed the highest toxicity for the EN 12457-2 leaching test and the second highest for the NEN 7341 leaching test.

### Table 1. Physicochemical parameters on the leachates of the four sludges tested.

| Sample | Moisture (%) | pH  | EC (mS/cm) |
|--------|--------------|-----|------------|
| SP     | 77.1         | 7.35 | 2.91       |
| SU     | 67.1         | 8.18 | 1.49       |
| AU     | 66.7         | 7.83 | 2.42       |
| WI     | 67.3         | 7.85 | 1.84       |

1 EN 12457-2 leaching test. 2 NEN 7341 leaching test.

### Table 2. Toxic units (TUs) calculated according to [34] for the four sludges and for both leaching tests and organisms tested.

| Leaching Test | Organism Tested | SP | SU | AU | WI |
|---------------|-----------------|----|----|----|----|
| EN 12457-2    | *D. magna*      | 4  | 2.1| 0.7| 1.5|
|               | *V. fischeri 5 min* | 0.3| 0.8| 1.3| 0.8|
|               | *V. fischeri 15 min* | 0.2| 0.8| 1.5| 0.9|
|               | *V. fischeri 30 min* | 0.3| 0.8| 1.5| 0.9|
| NEN 7341      | *D. magna*      | 2  | 1.4| 1.5| 1.8|
|               | *V. fischeri 5 min* | 0.8| 1  | 2.6| 3.7|
|               | *V. fischeri 15 min* | 0.7| 0.9| 2.6| 4.2|
|               | *V. fischeri 30 min* | 0.7| 0.8| 2.9| 4.6|

Figure 1. Cont.
3.2. Results for the Biosolid–Soil Mixtures

Different values are noted in relation to time elapsed for the measured parameters (Table 3). As shown in the line time charts (Figure 2a–g), the time elapsed since the biosolid application affected most of the parameters tested. For the TOC parameter, repeated measures ANOVA was conducted to explore the impact of soil type and the time elapsed since the biosolid application. The interaction effect between soil type and time duration was not statistically significant, (F = 3.18, p = 0.149). There was a statistically significant main effect of the time elapsed, F = 8337, p < 0.001. Post-hoc comparisons...
showed that samples on Day 1 were not significantly different from Day 0, but, except for Day 45, but they were different from samples on Day 15 or on Day 30. For nitrates, the interaction effect between soil type and time elapsed was highly significant, \(F = 64.059, p = 0.001\). There was also a statistically significant main effect of the time elapsed, \(F = 5875, p < 0.001\) with nitrates increasing and staying high on Day 45. For nitrites, the interaction effect between soil type and time elapsed was again highly significant, \(F = 39.483, p < 0.001\), while the time elapsed significantly increased nitrite leaching \(F = 213.368, p < 0.001\). On the contrary, both control and biosolid/soil samples were not acutely toxic for the tested organisms *D. magna* and *V. fischeri*. There were differences between timepoints especially for *V. fischeri* and for *D. magna* \(F = 947.43, p < 0.001\) and \(F = 14.23, p = 0.02\) respectively. Furthermore, there was no apparent difference in response between clay and sandy soil for these two tests.

Table 3. Mixtures of biosolids with sandy soil and clay soil, respectively.

| Treatment * | pH  | Electric Conductivity (μS/cm) | TOC (mg/L) | Nitrates (mg/L) | Nitrites (mg/L) | D. magna Immobilization (% in Relation to Blank) | V. fischeri Inhibition (% in Relation to Blank) |
|-------------|-----|-------------------------------|------------|-----------------|---------------|-----------------------------------------------|-----------------------------------------------|
| SA-C        | 7.92| 98.3                          | 6.93 ± 0.10| 3.19 ± 0.09     | 0.44 ± 0.01   | 3.33 ± 1.67                                   | 10.19 ± 0.96                                 |
| SA-1        | 7.87| 160.4                         | 7.06 ± 0.17| 2.47 ± 0.13     | 0.39 ± 0.01   | 3.33 ± 1.67                                   | −6.18 ± 0.37                                 |
| SA-15       | 7.94| 152.5                         | 9.72 ± 0.28| 4 ± 0.31        | 0.59 ± 0.01   | 8.33 ± 4.41                                   | −25.99 ± 4.19                                |
| SA-30       | 7.89| 166.2                         | 9.94 ± 0.07| 4.96 ± 0.16     | 0.83 ± 0.01   | 8.33 ± 4.41                                   | −7.41 ± 2.55                                 |
| SA-45       | 7.93| 169.6                         | 6.21 ± 0.07| 5.36 ± 0.07     | 0.63 ± 0.01   | 3.33 ± 1.67                                   | −19.88 ± 1.01                                |
| CL-C        | 8.15| 138.1                         | 6.18 ± 0.06| 3.72 ± 0.08     | 0.05 ± 0.01   | 8.33 ± 4.41                                   | −4.38 ± 0.18                                 |
| CL-1        | 7.91| 170.8                         | 7.07 ± 0.06| 4.03 ± 0.03     | 0.13 ± 0.01   | 3.33 ± 1.67                                   | −10.91 ± 1.94                                |
| CL-15       | 7.82| 200                           | 8.36 ± 0.53| 4.32 ± 0.04     | 2.18 ± 0.03   | 6.67 ± 1.67                                   | −15.78 ± 1.12                                |
| CL-30       | 7.84| 226                           | 9.02 ± 0.04| 5.5 ± 0.17      | 2.41 ± 0.02   | 3.33 ± 1.67                                   | −7.77 ± 0.53                                 |
| CL-45       | 7.9 | 230                           | 7.69 ± 0.12| 7.06 ± 0.36     | 2.44 ± 0.01   | 6.67 ± 1.67                                   | −10.28 ± 1.05                                |

* SA and CL means sandy and clay soil, respectively, C means sample without biosolid, and the numbers 1, 15, 30 and 45 indicate how many days have passed since biosolids have been incorporated. Data show mean value ± SEM. Data for *V. fischeri* shown are data for 5 min.

Figure 2. Cont.
Figure 2. Cont.
4. Discussion

According to [35], the sewage sludge produced in WWTPs has increased from 6.5 million t DM (dry matter) up to 9.5 million t DM in the last two decades. Consequently, the problems of sewage sludge management are increasing. Since the disposal of this by-product in landfills should be reduced according to directive 2008/98/EC, the land application of treated sludge in the form of biosolids as soil amendments is a sustainable solution. However, the presence of metals and other persistent pollutants may affect the applicability of land application of biosolids. The risk posed by these pollutants can be quantified through ecotoxicity tests, in combination with chemical analyses. Since the toxicants may have a synergistic effect, a potential effect cannot be predicted by the chemical analysis alone [36].

In the present study, biosolids (dehydrated and anaerobically digested sludge) were subjected to two methods of leaching tests and the organisms D. magna and V. fischeri were exposed to the leachates in order to evaluate their toxicity. Furthermore, biosolids were mixed with soil, placed under controlled conditions in containers for different time intervals and subjected to the EN 12457-2 one-stage batch-leaching test. The main physicochemical parameters were measured in the leachates and the same organisms were exposed to them. In this way, a simulation of an actual field spreading (80 tn/ha) was performed.

No leaching method is ideal for simulating all environmental conditions for all metals and for all kinds of organic waste; however, a few leaching methods may be sufficiently characteristic for specific (pH-related) environmental conditions. Therefore, two different leaching methods were chosen:
As can be seen in Table 2, almost all leachates showed slight acute toxicity (0.4 < TU < 1) or acute toxicity (1 < TU < 10) on the two organisms tested. On the contrary, only the spring sample for EN 12457-2 leaching method and for *V. fischeri* showed no acute toxicity (TU < 0.4). More specifically, for *D. magna*, which is characterized as one of the most effective bioassays to detect the toxicity of sludge eluates [37], almost all leachates showed acute toxicity. This was also confirmed in the study of [38], where the average toxicity against *Daphnia pulex* was 3.85–4.35 and 3.2–3.6 TU, respectively, for sludge press filtrates and for sludge water extracts. For *V. fischeri*, an organism that has also been reported to be highly susceptible and sensitive towards the wastewater toxicity [38], most of the leachates showed slight acute toxicity. However, autumn sample for EN 12457-2 leaching test and autumn and winter sample for NEN 7341 leaching test showed acute toxicity in *Vibrio*.

The toxicity levels of both organisms and leaching tests showed a large variation among the seasons. Biosolids are complex materials and, even in well managed treatment systems, it is common for the influent characteristics of a WWTP to change rapidly and unexpectedly [39,40]. Two of the main reasons that strongly affect the biosolid quality could be the weather conditions and the seasonal variation in the residents facilitated by the WWTP in question [41–43]. However, in our study, the samples were taken on days with little or no rain and there were no extreme temperatures for these days. Furthermore, the population of the area remains roughly the same, except for August each year, where a sharp decrease in the wastewater input is noted. The mixing of the sludge with the sludge produced from the septic tank liquid treatment may worsen the characteristics of the former and produce more toxic-treated sludge. In any case, the treatment of septic tank liquid is a common service in the WWTP in question and it has been successfully practiced for a long period of time. Higher sampling frequencies may make a significant contribution to the detailed recording of the quality of the treated sludge and can be a useful database for the evolution of the sludge quality characteristics over time.

In the mixtures of biosolids and soil that were created, no acute toxicity against the tested organisms was observed. None of the leachates were able to cause toxicity 50% or above; as such, no EC50 or IC50 could be calculated. Again, *V. fischeri* was sturdier than *D. magna* since most of the leachates (from the EN 12457-2 method, which was the only one performed on the biosolid/solid mixtures) caused an increase in bioluminescence and not inhibition. This may be due to the ion-rich mixture of biosolids, which usually contain large amounts of K, Ca and Mg [44]. These elements, in the form of their cations, may enhance sulfur-dependent luminescence in various ways in *V. fischeri* [45]. As such, any possible toxicity was counterbalanced by this enhancement. The general lack of toxicity is in contrast with other field applications of biosolids; [46] noticed significant toxicity on *D. magna* (48 h test) and on plants when treated sludge was utilized, at 23 tn/ha (water elutriates). The authors of [47] noted toxicity to *V. fischeri*, dependent on the type of soil and time elapsed after incorporation, at 90 tn/ha (EN 12457-2 leachate). On the contrary, [48] did not observe significant toxicity (water elutriates, 24 h test, 22 tn/ha) for *D. magna* after biosolid application, on Day 40. However, there was up to 20% decrease in *D. magna* survival, in the worst-case scenario (runoff or drainage) on Day 1. In relation to these results, the examined biosolids, even at 80 tn/ha, seem to be an innocuous solution. Regarding physicochemical parameters, pH reduction was only observed in the clay soil. Generally, studies have shown that pH reduction is expected right after incorporation of treated sludge [49]. On the other hand, biosolid incorporation significantly affected the electrical conductivity and nitrates and nitrite concentrations in the leachates. This was also found in the research study of [50]. Mainly in clay, but also in sandy soil, nitrate and nitrite accumulation increased significantly as time elapsed since biosolid incorporation, as also confirmed in the studies of [21,25]. In the former study, two of the main factors controlling the rate and extent of NO₃⁻ were biosolid type and time duration since biosolid incorporation. Moreover, in that study, the anaerobically digested sludge was the biosolid that showed the greatest NO₃⁻ accumulation potential due to its large content of NH₄⁺-N.
In the latter study, similar biosolid application rates accumulated NO$_3^-$-N up to three times as much in clay than in sandy soil.

Although, nitrite concentration in soils is usually low (below 0.1 mg NO$_2^-$-N kg$^{-1}$), sometimes it can exceed 50 mg NO$_2^-$-N kg$^{-1}$, mainly in cases of animal waste application and urea fertilizer application [51]. Excessive nitrates and nitrites in soil can lead to groundwater contamination, which has been recognized as a main reason for failing drinking water standards for decades [52]. The application of a slow-release organic fertilizer, such as this sludge-based biosolid, also caused nitrate and nitrite leaching, especially in clay soil. It would have been interesting to compare this leaching in relation to N-normalized doses of chemical fertilizers on the same type of soil. In general, the ecotoxic risk due to land application of biosolids at these rates, could be considered low; however, the contamination of underground water with nitrates/nitrites could not be excluded.

5. Conclusions

In the present study, anaerobically digested and dehydrated sludge was obtained from a WWTP that approximately receives 23,353 m$^3$/day of municipal wastewater for autumn, winter, spring and summer dates. The sludge was subjected to two leaching methods (EN 12457-2 and NEN 7341), and the main physicochemical parameters were measured in the leachates. The adverse effects of the leachates on the water organisms *Daphnia magna* and *Vibrio fischeri* were also measured. The results showed that almost all leachates showed acute toxicity for *D. magna*, while only some EN 12457-2 or NEN 7341 leachates showed acute toxicity on *Vibrio*. When the quite toxic (for *D. magna*) spring sample was mixed with soil (either clay or sandy soil) at the high dose of 80 tn/ha, some interesting results were noted; the toxicity of these leachates towards the organisms was insignificant, and there was no effect of the type of soil on this toxicity. pH was reduced, as was expected after biosolid incorporation, and this was prominent in the clay soil. An increase in nitrates was noted as time elapsed, which was more prominent in the clay soil. An increase was also noted for nitrites which was very prominent in the clay soil while in the sandy soil it was modest. Therefore, some but not all physicochemical parameters were affected by the type of receiving soil. The examined sludge was assessed as harmless for water organisms in realistic scenarios, however, it was not of negligible risk on the grounds of nitrate pollution. Organic soil amendments, such as treated sludge, are an appealing fertilizing solution which transforms waste into a valuable product. Nevertheless, a detailed risk assessment encompassing multiple parameters should be performed before field application in order to protect water resources.

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