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Preliminary Acute Ecotoxicological Screening of Dairy Final Effluent After Multistage Treatment in a Karst-Sensitive Setting

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Abstract

Site-specific ecotoxicological monitoring data on dairy final effluents in karst groundwater environments remain scarce. This preliminary site-specific case study combined physicochemical characterisation with a 24 h *Daphnia magna* immobilisation assay of three final-effluent samples collected after the complete treatment train in winter, spring and summer. The three sampling occasions were used as independent screening snapshots to test for an acute response at high effluent concentrations, not to estimate the seasonal variability or treatment-stage efficiency. Six effluent exposure groups (three samples × two concentrations) were tested at 80% and 100% (*v*/*v*), each in four vessels containing five organisms, together with three negative controls. Across samples, the pH was 8.1–8.4, the temperature was 11.5–26.4 °C, the dissolved oxygen was 7.89–9.75 mg/L, the TSS was 3–9 mg/L, the COD was 16.4–40.4 mg/L and the BOD_5 was 3.3–4.0 mg/L. No immobilisation occurred, and the 24 h EC_{50} was reported as >100% (*v*/*v*); the potassium dichromate EC_{50} values were 1.07–1.18 mg/L. No detectable 24 h acute immobilisation was observed in any of the three tested final-effluent samples. These findings provide a quantified site-specific final-effluent profile linked to a standardised whole-effluent effect endpoint and indicate the absence of acute immobilisation under the tested conditions.

Keywords: ecotoxicological monitoring data; dairy final effluent; karst groundwater environments; *Daphnia magna*; immobilisation assay

1. Introduction

Karst aquifers are among Slovenia's most important water resources. In 2019, groundwater supplied 97.6% of the water used for public supply, with karst sources making a substantial contribution [1,2]. Their hydrogeological structure also makes them highly vulnerable to pollution: rapid flow, a high permeability and limited natural filtration can transmit catchment pressures quickly to groundwater [3–6]. This vulnerability also has ecological implications because Slovenian karst aquifers are both water resources and habitats for specialised subterranean organisms. The olm (*Proteus anguinus*), an endemic species of the Dinaric Karst and a prominent representative of Slovenian subterranean fauna, depends on stable physicochemical conditions and is therefore often regarded as an indicator of the conservation status of subterranean karst ecosystems [4,7–10].

The conservation of the olm and its habitat is also embedded in the wider European conservation framework. The species is listed in Annexes II and IV of the Habitats Directive (92/43/EEC), and much of its habitat overlaps with Natura 2000 sites established to protect

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important habitats and species in the European Union [11]. In Slovenia, *P. anguinus* is listed in the Decree on Protected Wild Animal Species, which provides the legal basis for protecting both individuals and their habitat [12]. The IUCN classifies the species as vulnerable (VU) at the European level; the principal threats include habitat degradation, range contraction and population fragmentation [13–17].

Food processing, particularly dairy production, can exert considerable pressure on water quality. Across 18 Polish dairies, the raw wastewater COD ranged from 850 to 5032 mg/L [18,19], showing the scale of the organic load entering treatment systems. In karst environments, these loads are especially relevant because rapid transport can reduce the time available for natural attenuation.

The dairy facility examined in this study is in Laze, Municipality of Logatec, central Slovenia, within a karst-sensitive region where groundwater protection is a key management concern. Šobot et al. [20] previously evaluated the chemical treatment phase over a 30-day field investigation. Seven COD observations yielded mean concentrations of 1150.5 mg/L in raw wastewater, 850.5 mg/L after the previous chemical combination and 750.5 mg/L after the optimised combination [20]. The present study examines a different process point: the final effluent after the complete treatment train.

The treatment performance alone does not establish biological acceptability; site-specific ecotoxicological testing of the final effluent provides complementary evidence. The dataset, however, contains no discharge coordinates, tracer results, receiving-water measurements or species-occurrence data demonstrating a hydraulic pathway from the discharge to a specific *Proteus anguinus* habitat. Accordingly, the species is considered a conservation-sensitive receptor for precautionary monitoring, not a receptor demonstrated to be exposed.

The study addressed one question: whether any of the three temporally separated final-effluent samples caused detectable 24 h immobilisation of *Daphnia magna* at 80% or 100% (*v*/*v*) under the documented laboratory conditions. The quantified evidence comprises 16 physicochemical indicators, six effluent exposure groups containing 120 organisms, three negative-control groups containing 60 organisms and three reference-toxicant EC_{50} values. The unverified elements concern supporting procedural metadata rather than the recorded concentrations or organism counts.

Daphnia magna was used as a standardised test organism to assess the acute whole-effluent effects in the final-effluent samples under controlled laboratory conditions. The assay provides a well-established ecotoxicological screening endpoint relevant to the evaluation of the effluent quality in a karst-sensitive environment.

The study's scientific contribution lies in integrating a quantified final-effluent profile with a standardised acute whole-effluent endpoint at a real dairy facility in a karst-sensitive setting. The resulting evidence is deliberately bound to three sampling occasions and provides a transparent basis for designing a replicated monitoring programme; it does not support claims of the treatment-stage efficiency or habitat safety.

1.1. Chemical Treatment Phase of Wastewater Treatment

Chemical treatment is a key step in managing industrial wastewater, particularly in sectors with high organic loads such as the dairy industry [21–28]. Across 18 Polish dairies, influent wastewater contained BOD_5 concentrations of 465–3275 mg/L, a COD of 850–5032 mg/L, a TSS of 75–1109 mg/L, a total nitrogen of 16.2–168.4 mg/L and a total phosphorus of 9.0–52.5 mg/L [18]. These ranges indicate the magnitude of and variability in the organic, particulate and nutrient loads that pretreatment must manage before subsequent biological or tertiary treatment.

Chemical treatment generally combines neutralisation, coagulation and flocculation. Neutralisation establishes a pH range suitable for reagent performance; coagulation destabilises suspended and colloidal particles; and flocculation forms larger aggregates that can be separated [29–31]. In the previous site-specific study, treatment began when the equalisation tank reached 60 m^3, and jar-test samples were allowed at least 30 min for reaction and settling [20]. Under the optimised chemical combination, the mean COD was 750.5 mg/L compared with 1150.5 mg/L in raw wastewater [20].

1.2. Chemicals Used in the Optimised Treatment Process

The previous optimisation study identified a chemical-phase combination comprising a sodium hydroxide-based neutralising agent, an aluminium chloride/polyaluminium hydroxychloride coagulant and a flocculant containing sulfamic, adipic and citric acids [20,32–36]. The present study focused on the physicochemical characterisation of final-effluent samples and 24 h whole-effluent screening with *Daphnia magna*. The reagents are reported only in the upstream process context, not as separately tested exposure agents.

At the final-effluent sampling point, neither 80% nor 100% (*v*/*v*) effluent caused *Daphnia magna* immobilisation after 24 h. This result supports the absence of a detectable acute whole-effluent effect under the tested conditions. It does not demonstrate that individual treatment chemicals were absent or intrinsically non-toxic, because the residual concentrations of sodium hydroxide, aluminium species and the flocculant constituents were not quantified.

1.3. Ecotoxicity and Bioindicator Organisms

Ecotoxicity encompasses the adverse effects of chemical substances or mixtures on organisms, populations and ecosystems [37]. This concept is particularly relevant to wastewater because effluents often contain complex mixtures of organic and inorganic constituents whose combined biological effects cannot always be inferred reliably from physicochemical parameters alone [38–40]. Effect-based methods complement chemical analysis by measuring organism responses to the whole sample rather than to selected compounds in isolation [24,41].

Water fleas of the genus *Daphnia*, particularly *Daphnia magna*, are well-established bioindicators in ecotoxicology [42–44]. *D. magna* is a freshwater planktonic crustacean and primary consumer that filters algae, cyanobacteria and other small suspended particles while serving as prey for fish and predatory invertebrates [45,46]. Its transparent chitinous carapace, short life cycle, high fecundity, parthenogenetic reproduction and ease of laboratory culture make it a widely used model for acute and sublethal toxicity testing [25,47,48].

The acute immobilisation test with *D. magna* is a standardised assay for chemicals, effluents and other water samples [49–52]. The endpoint is the proportion of organisms unable to swim after 24 or 48 h and, when a concentration–response relationship is present, the EC_{50} [52]. In the present study, each treatment contained 20 organisms distributed across four vessels, the effluent was tested at 80% and 100% (*v*/*v*), and the endpoint was recorded after 24 h. The acceptance evidence included negative controls and potassium dichromate reference tests with a documented range of 0.6–2.1 mg/L [52].

1.4. Relevance of Ecotoxicological Assessment

The physicochemical line of evidence in this study comprised the pH, temperature, dissolved oxygen, TSS, settleable solids, COD, BOD_5, TOC, total nitrogen, total phosphorus, ammonium nitrogen, total chlorine, sulfate, sulfite, low-volatility lipophilic substances and AOX. The biological line of evidence was 24 h *Daphnia magna* immobilisation at 80% and

100% (*v*/*v*) final effluent. Together, these measurements relate the sample composition to a whole-effluent response without relying on individual chemical indicators alone [24,41,53].

In karst areas, this combined approach is particularly relevant [4,5]. Karst aquifers have limited natural attenuation, rapid transport pathways and a high vulnerability to pollution; even relatively small changes in water quality may therefore affect the wider subterranean ecosystem [3,20]. This concern is especially relevant to habitats of the olm (*Proteus anguinus*), which depends on stable groundwater conditions. Šobot and Bilić-Šobot [22] report that preserved olm habitats are typically characterised by temperatures of 8 to 12 °C, pH values of 6.5 to 7.5 and dissolved oxygen concentrations above 7 mg/L.

The *Daphnia magna* assay was used as a first-tier whole-effluent screen to capture the combined acute effect of measured and unmeasured sample constituents in a standardised endpoint. The objective was to determine whether the three final-effluent samples produced detectable acute immobilisation at high exposure concentrations. The assay was not designed to estimate exposure in the aquifer or species-specific effects on *Proteus anguinus*; those questions require separate hydrological and receptor-relevant evidence.

2. Materials and Methods

2.1. Study Design and Process Boundary

This preliminary site-specific case study analysed three final-effluent samples, designated A, B and C, collected after the complete treatment train in winter (February), spring (April) and summer (June), respectively. The sampling occasions were treated as independent screening snapshots used to obtain an initial effect-based observation across three operating periods, not as replicated seasonal groups.

The final-effluent samples were provided by the dairy facility operator. Although the available records did not specify whether grab or composite sampling was used, the samples enabled the site-specific characterisation of effluent quality across three operating periods.

The experimental workflow comprised five steps: (1) the provision of final-effluent samples from three time points; (2) the compilation of 16 physicochemical indicators from the corresponding records; (3) the storage of samples at −18 °C until testing; (4) 24 h *Daphnia magna* screening at 80% and 100% (*v*/*v*), with negative controls and separate potassium dichromate sensitivity tests; and (5) a descriptive comparison of the physicochemical profiles and immobilisation outcomes.

The designation "final effluent" follows the terminology used in the supplied records. The records did not identify the authorised receiving route after the sampling point. The study therefore does not assume direct discharge to the karst aquifer or entry into a particular public sewer, municipal treatment plant, surface-water recipient, or infiltration system.

The assessment focused on the final effluent collected after the completion of the treatment train, providing an effect-based evaluation of effluent quality at the final treatment endpoint.

The wider Logatec area lies within the Slovenian Dinaric karst and is characterised by fractured and karstified carbonate aquifers, rapid infiltration through fissures and point-recharge features, and conduit-dominated underground drainage. These features limit natural attenuation and may rapidly convey surface-derived loads through the groundwater system [3–6,20]. Accordingly, the term "karst-sensitive setting" is used in a regional hydrogeological sense and does not imply that this study demonstrated a hydraulic connection between the discharge point and a known *Proteus anguinus* locality.

The final-effluent sampling point followed a complete treatment train that included the upstream chemical-treatment context described by Šobot et al. [20]. That phase used Sotoplus 50% (Sotom d.o.o., Ljubljana, Slovenia), VTA Combiflock HB 52-65 (VTA Austria

GmbH, Rottenbach, Austria) and VTA F98 (VTA Austria GmbH, Rottenbach, Austria). The earlier site-specific work reported treatment at a 60 m^3 tank volume and at least 30 min of reaction and settling [20]. Figure 1 summarises the workflow evaluated in the present study.

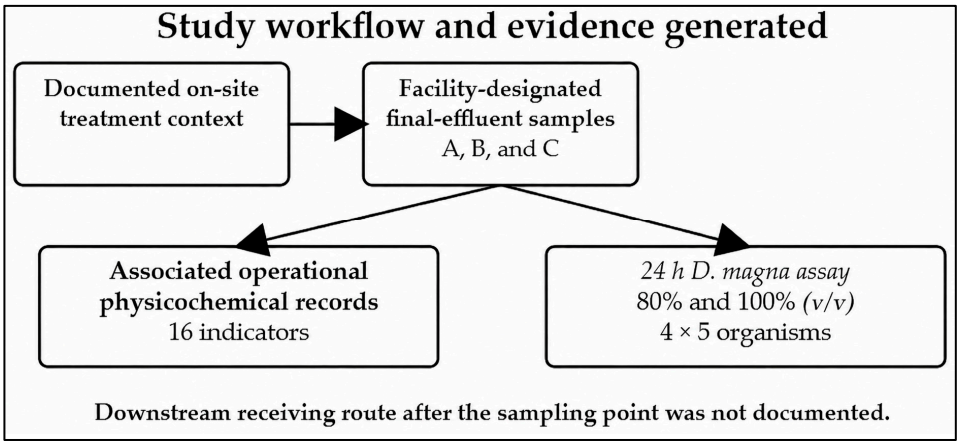


Figure 1. Study workflow and quantified evidence generated. Samples A, B and C were collected at the final-effluent point after the complete treatment train. The study combined 16 physicochemical indicators with 24 h *Daphnia magna* screening at 80% and 100% (*v*/*v*). The downstream receiving route after the facility sampling point was not documented.

2.2. Sample Collection, Preservation and Documentation

The source records stated that the three samples had been stored at −18 °C before testing. Each sample retained its temporal designation and was analysed as a separate final-effluent observation. Section 4.5 consolidates details absent from the source records rather than reconstructing them retrospectively.

The −18 °C storage note applies to the material used for the *D. magna* assay. The records did not establish whether the physicochemical values were measured on fresh aliquots before freezing, on separate contemporaneous aliquots, or after thawing. They are therefore reported as associated operational measurements rather than as the verified physicochemical conditions of the thawed exposure media. The analytical holding times for dissolved oxygen, ammonium nitrogen, and BOD5 could not be checked.

2.3. Physicochemical Characterisation and Analytical Data Status

Operational records provided 16 physicochemical indicators: the pH, temperature, dissolved oxygen, total suspended solids, settleable solids, total nitrogen, total phosphorus, total chlorine, ammonium nitrogen, sulfate, sulfite, COD, BOD_5, low-volatility lipophilic substances, TOC and AOX. The available pH and temperature series were summarised as means and ranges; all other parameters were transcribed as one reported value per sample. These records were used to describe and compare the three final-effluent profiles.

Nitrogen characterisation was based on the available measurements of the total nitrogen and ammonium nitrogen.

2.4. Daphnia magna Acute Immobilisation Screening and Documentation Audit

The laboratory sheets identified SIST EN ISO 6341:2013 [52] as the test method, *Daphnia magna* as the test organism and Spirulina powder as the culture feed. For each sample, one negative control and final-effluent concentrations of 80% and 100% (*v*/*v*) were tested. Each treatment comprised four vessels containing five mobile organisms, giving 20 organisms per treatment. Across all samples, 120 organisms were exposed to the effluent and 60 organisms were included in the negative controls.

The source sheets recorded the number of mobile organisms in each vessel after 24 h. Immobilisation was calculated as the difference between the 20 organisms introduced per treatment and the total number remaining mobile across the four vessels. The 48 h fields were blank, so the analysis was restricted to the documented 24 h endpoint. Procedural metadata not recorded on the sheets are reported in Section 4.5.

The temperatures reported in Table 1 are associated operational characterisation values, not recorded bioassay temperatures. The source sheets did not state the temperature maintained during the 24 h immobilisation test; temperature control and conformity with the relevant test condition therefore cannot be verified retrospectively.

Table 1. Basic physicochemical characteristics, solids and organic-load indicators of the final-effluent samples.

Sample	Season	pH Mean (Min–Max)	Temperature (°C) Mean (Min–Max)	DO (mg/L)	TSS (mg/L)	SetS (mg/L)	COD (mg/L)	BOD5 (mg/L)	TOC (mg/L)
A	Winter	8.1 (8.0–8.1)	11.5 (11.4–11.6)	9.75	9	0.3	29.6	3.3	13.6
B	Spring	8.2 (8.0–8.4)	15.0 (14.9–15.2)	8.94	3	0.1	40.4	4.0	20.6
C	Summer	8.4 (8.4–8.4)	26.4 (26.1–26.7)	7.89	6	0.1	16.4	4.0	10.0

Note: DO = dissolved oxygen; TSS = total suspended solids; SetS = settleable solids; COD = chemical oxygen demand; BOD5 = biochemical oxygen demand after five days; TOC = total organic carbon. The pH and temperature are shown as the mean (minimum–maximum); the remaining values are single operational measurements. The values were transcribed from associated operational records; their analytical timing relative to freezing and compliance with method-specific holding times were not documented.

The 100% treatment consisted of the supplied final-effluent material. The source sheets did not identify the diluent used to prepare the 80% treatment, and distilled water cannot be assumed. The composition of the 80% exposure medium is therefore not reproducible from the available record. No karst-water sample was used, so the assay did not simulate dilution within the aquifer.

2.5. Reference Toxicant and Verifiable Validity Elements

A separate 24 h sensitivity test with potassium dichromate ($K_2Cr_2O_7$) accompanied each assay. Reference EC_{50} values were evaluated against the documented range of 0.6–2.1 mg/L, and the negative-control performance was evaluated against the criterion of no more than 10% immobilisation after 24 h [52]. These two recorded elements were used to assess the culture sensitivity and control performance.

2.6. Data Treatment and Inferential Rules

Data were analysed descriptively at the sample and treatment levels. Physicochemical values were reported for each of the three samples, and immobilisation was reported as the n/20 and percentage for each control and effluent concentration. Because no immobilisation occurred at 80% or 100% (*v*/*v*), the 24 h EC_{50} was not estimated by a concentration–response model and was reported as >100% (*v*/*v*). No inferential seasonal analysis was undertaken because one independent sample was available at each time point.

The absence of immobilisation provided a clear descriptive outcome for all three final-effluent samples, indicating no detectable acute immobilising effect under the tested conditions.

3. Results

3.1. Physicochemical Characteristics of the Final-Effluent Samples

Tables 1 and 2 summarise the physicochemical characteristics of samples A, B and C. The mean pH ranged from 8.1 to 8.4, dissolved oxygen from 7.89 to 9.75 mg/L and the temperature from 11.5 to 26.4 °C.

Table 2. Nutrients and selected inorganic and organic indicators in the final-effluent samples.

Sample	Total N (mg/L)	Total P (mg/L)	NH4-N (mg/L)	Total Chlorine (mg/L)	Sulfate (mg/L)	Sulfite (mg/L)	Lipophilic Substances (mg/L)	AOX (mg/L)
A	3.34	0.05	1.0	0.03	6.26	0.3	6.9	0.02
B	5.69	0.05	1.0	0.03	4.67	0.3	5.5	0.02
C	3.84	0.05	1.0	0.03	5.30	0.3	5.0	0.02

Note: NH4-N = ammonium nitrogen; AOX = adsorbable organically bound halogens. "Lipophilic substances" denotes the operational parameter reported as low-volatility lipophilic substances in the source records.

The organic-load indicators varied among the samples. Sample B had the highest COD (40.4 mg/L) and TOC (20.6 mg/L), whereas sample C had the lowest COD (16.4 mg/L) and TOC (10.0 mg/L). The BOD_5 ranged from 3.3 to 4.0 mg/L; the calculated BOD_5/COD ratios were 0.11, 0.10 and 0.24 for samples A, B and C, respectively. The total suspended solids ranged from 3 to 9 mg/L and the settleable solids from 0.1 to 0.3 mg/L. Because each value represents a single sample, neither the analytical uncertainty nor the temporal variability could be quantified.

Table 2 presents the nutrient and selected inorganic and organic indicators. The total nitrogen ranged from 3.34 to 5.69 mg/L, sulfate from 4.67 to 6.26 mg/L and low-volatility lipophilic substances from 5.0 to 6.9 mg/L. Identical numerical values were reported for the total phosphorus, total chlorine, ammonium nitrogen, sulfite and AOX in all three samples. Without analytical reporting limits, these repeated values could not be interpreted as non-detects or limits of quantification.

Ammonium nitrogen was reported as 1.0 mg/L in all three samples.

3.2. Acute Immobilisation of Daphnia magna

No immobilisation occurred in any negative control or final-effluent treatment after 24 h. For samples A, B and C, all four vessels contained five mobile organisms after 24 h at both 80% and 100% (*v*/*v*), corresponding to 0/20 immobilised organisms (0%) in every treatment. Because no response occurred at either concentration, the 24 h EC_{50} could not be estimated and was reported as >100% (*v*/*v*) for each sample.

These results demonstrate the absence of detectable acute immobilising effects in all three final-effluent samples at both tested concentrations under the experimental conditions.

The potassium dichromate 24 h EC_{50} values were 1.07, 1.14 and 1.18 mg/L for samples A, B and C, respectively, and all negative controls showed 0% immobilisation. These results support the culture sensitivity and control performance for the documented validity elements, but do not establish full conformity with the standard because additional records of the test conditions were unavailable.

Across the six effluent exposure groups, 0 of 120 organisms were immobilised after 24 h; across the three negative-control groups, 0 of 60 organisms were immobilised. These aggregate counts summarise the quantifiable biological outcome while Table 3 retains the sample-level results.

Table 3. Twenty-four-hour immobilisation of *Daphnia magna* in the final-effluent screening and reference-toxicant results.

Sample	Season	Control Immobilised n/20 (%)	80% Effluent Immobilised n/20 (%)	100% Effluent Immobilised n/20 (%)	Sample 24 h EC50 (% *v*/*v*)	K2Cr2O7 24 h EC50 (mg/L)
A	Winter	0/20 (0)	0/20 (0)	0/20 (0)	>100	1.07
B	Spring	0/20 (0)	0/20 (0)	0/20 (0)	>100	1.14
C	Summer	0/20 (0)	0/20 (0)	0/20 (0)	>100	1.18

Note: Each treatment comprised four within-assay replicate vessels with five organisms per vessel. Only one independent effluent sample was available at each time point. The source documentation reports the 24 h endpoint only. The potassium dichromate reference range was 0.6 to 2.1 mg/L [52].

4. Discussion

4.1. Physicochemical Profile and Treatment Context

The three final-effluent samples showed distinct physicochemical profiles. Sample A had the highest TSS and settleable-solid concentrations; sample B had the highest COD, TOC and total nitrogen; and sample C had the highest pH and temperature and the lowest dissolved oxygen. Despite these differences, all six effluent exposure groups yielded 0% immobilisation. The supported finding is the absence of detectable 24 h immobilisation in each of the three screening snapshots.

The measured final-effluent concentrations were numerically lower than raw dairy-wastewater ranges reported elsewhere. Rodziewicz et al. [18] reported an influent BOD_5 of 465–3275 mg/L, a COD of 850–5032 mg/L and a TSS of 75–1109 mg/L, whereas the corresponding final-effluent ranges in this study were 3.3–4.0, 16.4–40.4 and 3–9 mg/L. The current COD range was also numerically lower than the mean of 750.5 mg/L reported after the optimised chemical phase in the earlier site-specific study [20]. These comparisons place the tested samples in a broader treatment context, but do not constitute paired removal estimates.

Lima and Aguiar [19] reported mean COD and BOD_5 removals of 96.2% and 97.1%, respectively, and a treated-effluent BOD_5/COD ratio of 0.30. The ratios observed here (0.10 to 0.24) were numerically lower; however, the absence of paired influent data, analytical methods and uncertainty estimates precludes a direct comparison of the plant performance or biodegradability.

The settleable solids ranged from 0.1 to 0.3 mg/L and the TSS from 3 to 9 mg/L; sample A had the highest values for both indicators. Lima and Aguiar [19] include settleable solids among the routine indicators of dairy-effluent quality, while Hamdani et al. [54] and Kushwaha et al. [55] describe removal or settling behaviour under different treatment and analytical conditions. They provide mechanistic context rather than direct numerical benchmarks. Because all three samples produced the same 0% immobilisation response, the present data cannot establish whether solids predict acute immobilisation.

Although sample B had the highest COD and TOC, it produced the same 0% immobilisation as samples A and C. Physicochemical characterisation and the bioassay yielded complementary descriptive information, but the invariant biological endpoint provides no basis for a correlation, threshold or surrogate-performance analysis. Future monitoring should therefore retain both data streams rather than treat any measured parameter as a validated or invalidated surrogate.

The final-effluent pH remained mildly alkaline across the three samples (8.1–8.4), indicating no evidence of acidification at the sampled treatment endpoint. The available total nitrogen and ammonium nitrogen measurements further contributed to the physicochemical characterisation of the final effluent.

4.2. Comparison with Other Ecotoxicological Studies

The absence of 24 h immobilisation at 100% final effluent contrasts with the findings of Albuquerque et al. [56], who examined dairy wastewater from a small facility equipped only with an oil-and-grease chamber. *Daphnia similis* was the most sensitive organism in their study, with a toxicity factor of 32 and acute effects at dairy-wastewater concentrations as low as 3.125%. The difference may reflect the treatment intensity and residual matrix composition. The comparison is not quantitative because Albuquerque et al. used *D. similis*, a broader dilution series and a 48 h acute endpoint, whereas the present screening used *D. magna*, two high effluent concentrations and a 24 h endpoint.

The findings are more closely aligned with those of Marassi et al. [57], who observed high acute toxicity in raw dairy wastewater, but no acute effect of treated microbial-fuel-cell effluent on *D. similis* during 95 days of operation. Their treatment removed 93% of the total COD and 95% of the total BOD. Although the treatment technology and test species differed, the absence of an acute daphnid response in the treated matrices is qualitatively similar. The comparison also shows why the sampling point within the treatment train matters: the present samples were collected from the final effluent after the complete treatment train, not immediately after the isolated coagulation–flocculation stage.

The potassium dichromate EC_{50} values of 1.07–1.18 mg/L and 0% control immobilisation support the culture sensitivity and control performance for the recorded assay elements.

The present findings demonstrate the absence of detectable 24 h immobilisation in all three final-effluent samples, providing direct effect-based evidence of final-effluent quality at the completed treatment endpoint.

4.3. Conservation Context and Limits of Exposure Inference

The measured effluent pH (8.1 to 8.4), temperature (11.5 to 26.4 °C) and dissolved oxygen (7.89 to 9.75 mg/L) can be compared descriptively with the published conditions for preserved olm habitats [22], but this comparison does not constitute an exposure assessment. Discharge and receiving-water flows, the initial dilution, heat exchange, pH buffering, the travel time and the groundwater conditions were not measured. The spring and summer effluent temperatures and alkaline pH should therefore be treated as variables for field verification, not as evidence that equivalent conditions occurred in the subterranean habitat.

The assay characterises a 24 h acute effect in the supplied final-effluent samples [Figure 2]. It does not establish the environmental exposure or risk to *Proteus anguinus*, and a *Daphnia magna* result cannot be converted into a species-specific toxicity estimate because the taxa differ in their physiology, life history, habitat and likely exposure duration.

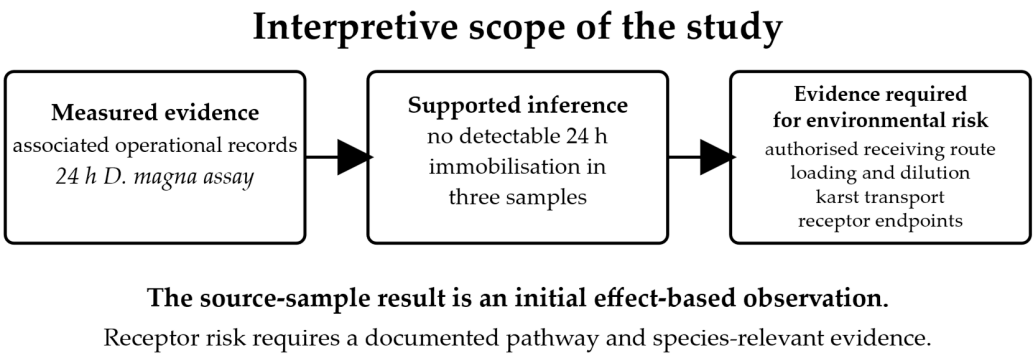


Figure 2. Interpretive scope of the study. The measured final-effluent data support a conclusion about acute *Daphnia magna* immobilisation in the three tested samples. An evaluation of habitat exposure or species-specific risk requires separate hydrological and receptor-relevant evidence. The authorised receiving route is the first unverified step between the facility sample and any environmental receptor.

Environmental interpretation would require confirmation of the authorised receiving route, discharge frequency and volume, receiving-water flow, dilution, alkalinity, recharge conditions, travel time and receptor occurrence. Because these data were unavailable, karst vulnerability was used only as regional context. Domestic wastewater, septic tanks and small municipal treatment systems were outside the study scope.

4.4. Evidence Audit and Inferential Boundaries

Table 4 summarises the study outputs, their direct interpretation and the boundaries of the current design. It separates the measurements obtained from questions that require different sampling or experimental designs.

Table 4. Quantified study outputs and scope of interpretation.

Study Component	Quantified Evidence	Direct Interpretation	Scope of Inference
Final-effluent physicochemistry	16 indicators for samples A, B and C.	Provides a site-specific descriptive profile.	One sample per time point; temporal variability was not estimated.
Acute *D. magna* response	Six effluent treatments; 120 exposed organisms; 0/120 immobilised after 24 h.	No detectable acute immobilisation at 80% or 100% (*v*/*v*).	Does not classify the final effluent as generally non-toxic.
Assay controls	Three controls: 0/60 immobilised; $K_2Cr_2O_7$ EC_{50} = 1.07–1.18 mg/L.	Supports control performance and culture sensitivity.	Full standard conformity requires additional procedural metadata.
Treatment-stage performance	Final effluent sampled after the complete treatment train.	Characterises the discharge point evaluated in this study.	No stage-specific removal or toxicity reduction can be quantified.
Temporal interpretation	One winter, one spring and one summer sample.	Three independent screening snapshots.	Not a replicated seasonal analysis.
Receptor relevance	A 24 h *D. magna* endpoint only.	First-tier whole-effluent effect indicator.	No direct inference about *P. anguinus* exposure, toxicity or safety.
Sample provenance and preservation	Facility-provided samples; storage at −18 °C recorded for the *D. magna* material.	Supports reporting of the supplied samples and the documented bioassay outcome.	Sampler identity, sampling mode, representativeness, analytical timing, and holding-time compliance were not verified.
Nitrogen speciation	Total nitrogen and ammonium nitrogen were reported; nitrite and nitrate were not.	Provides a partial nitrogen profile.	Nitrification and the distribution of oxidised nitrogen forms cannot be assessed.
Bioassay temperature and preparation of the 80% treatment	The 24 h tests used nominal concentrations of 80% and 100% (*v*/*v*); the maintained assay temperature and the 80% diluent were not recorded.	The observed organism counts and nominal test concentrations are reportable.	Exact thermal and medium conditions cannot be reconstructed, limiting reproducibility and a complete method-conformity assessment.
Receiving route after sampling	No sewer, municipal-treatment, surface-water, or infiltration route was documented.	No receiving route can be assigned from the study records.	Direct groundwater exposure or connection to a specific treatment plant cannot be inferred.

Note: The table distinguishes the quantified outputs of the present study from questions that require replicated, stage-specific, hydrological or receptor-relevant evidence.

4.5. Conceptual Operational and Monitoring Framework

The invariant 0% immobilisation endpoint across physicochemically distinct samples does not support predictive parameters or trigger thresholds. The observed values are therefore initial reference observations for future data collection, not a statistically supported baseline, operating envelope or discharge criterion.

The study's main scientific contribution is the site-specific integration of associated final-effluent physicochemical data with a standardised acute-effect endpoint. Table 5 translates this dataset into a prospective monitoring framework for testing temporal consistency, treatment-stage changes and environmental exposure in future work.

Table 5. Proposed tiered monitoring framework for prospective validation.

Tier	Trigger	Minimum Evidence	Proposed Action
1. Routine process surveillance	Scheduled monitoring and any process change.	pH, temperature, DO, conductivity, TSS/SetS, COD/TOC, N and P; discharge flow, reagent dose, solid-separation and cleaning records. Include nitrite and nitrate, identify the sampler and sampling mode, and document analytical holding times.	Review trends and obtain a fresh confirmatory sample when an unexplained deviation occurs.
2. Acute effect confirmation	Operational deviation or reproducible immobilisation.	Fresh final effluent; full dilution series; *D. magna* observations at 24 and 48 h; start- and end-of-test pH and DO; complete test metadata. Record the dilution medium used for every test concentration and link the chemistry to the actual exposure aliquot. Maintain and record the prescribed exposure temperature throughout the assay and identify the dilution medium used for every concentration.	Repeat testing, quantify the concentration–response relationship where possible, and investigate residual aluminium and other plausible causes.
3. Extended biological assessment	Repeated or unexplained acute effects, or a persistent chemical excursion.	A *D. magna* reproduction test, an algal growth-inhibition test and additional receptor-relevant endpoints.	Identify effect drivers, evaluate mitigation and verify post-correction performance.
4. Source–pathway–receptor assessment	A site-specific conservation or exposure question, particularly during low-flow conditions.	Discharge frequency, volume and mass loading; effluent and receiving-water flows; dilution, mixing and alkalinity; upstream, downstream and karst-sentinel chemistry; travel-time and hydrogeological evidence; environmental DNA or other occurrence data. Confirm the authorised receiving route and any subsequent sewer or treatment infrastructure before environmental-pathway modelling.	Determine whether a hydraulic pathway and receptor exposure exist before drawing conclusions about habitat risk.

Note: The framework is a proposal for future validation and is not a regulatory threshold system derived from the present three samples.

Acute confirmation should use fresh, preferably unfrozen samples, a full dilution series and observations after both 24 and 48 h. Any reproducible response should be accompanied by measurements of the start- and end-of-test pH and dissolved oxygen, conductivity, hardness and residual aluminium, together with a complete chain of custody and operating

metadata [52,58–67]. Extending the assessment to a *Daphnia magna* reproduction test and an algal growth-inhibition test would capture chronic and primary-producer endpoints not addressed by the present 24 h screen [63,64].

Verification in the receiving environment should focus on hydrologically critical periods and paired measurements at the discharge, within the mixing zone, at downstream points and at accessible karst sentinel locations. Operational measures such as additional equalisation, heat dissipation, pH adjustment or altered discharge timing should be considered only if the site-specific flow and mixing data show that an excursion persists beyond the source point. The current data do not establish a need for any such intervention.

4.6. Limitations and Evidence Gaps

The study included three independent environmental samples, one collected in each of three seasons, rather than replicated seasonal groups. It provides no estimate of within-season variability or seasonal trends. The biological design included only 80% and 100% effluent and a 24 h endpoint; it supports reporting $EC_{50} > 100\%$ (v/v), but not a concentration–response curve and does not exclude effects after 48 h.

Within this screening design, the consistent absence of immobilisation across all three final-effluent samples provides site-specific evidence of no detectable acute effect under the tested conditions.

The source records did not specify the exact sampling date and time, grab- or composite-sample status, container type, transport conditions, time to freezing, frozen-storage duration, thawing procedure, number of freeze–thaw cycles, post-thaw homogenisation or elapsed time before test initiation. The Daphnia records did not specify neonate age, clone or culture source, dilution-medium composition, test volume, hardness, conductivity, exposure temperature, photoperiod, feeding during exposure, random allocation, start- and end-of-test pH and dissolved oxygen, or the operational definition of immobilisation. These omissions limit the reproducibility and prevent the retrospective verification of full conformity with the standard, but they do not alter the recorded concentrations or organism counts.

Future monitoring can further strengthen the comparability between the physicochemical and bioassay datasets by documenting the timing of chemical measurements relative to sample storage and thawing, as well as the composition of the dilution medium used for the 80% exposure.

The physicochemical records also lacked analytical method identifiers, instrument details, the laboratory accreditation status, the limits of detection and quantification, the quality-control results and the measurement uncertainty. The reported values support a descriptive site-specific profile, but not an uncertainty-based compliance assessment or formal comparison of the analytical performance among studies.

Nitrogen characterisation in the present study was based on the available measurements of the total nitrogen and ammonium nitrogen.

The only biological endpoint was acute immobilisation in a standard invertebrate. It did not assess reproduction, growth, behaviour, primary-producer responses, community responses or long-term mixture effects [40,50,58,59] and cannot be extrapolated directly to *Proteus anguinus* or subterranean communities.

Without paired influent or interstage data, the removal efficiency and stage attribution cannot be calculated. The absence of receiving-water flow, mixing, tracer, groundwater-quality and species-occurrence evidence also precludes an assessment of the actual environmental exposure and risk. Comparisons with other studies remain qualitative because treatment technologies, species, exposure durations, concentrations and analytical methods differ.

The absence of discharge frequency, discharge volume, mass-loading, receiving-water alkalinity and dilution data also prevents an estimation of the dose that could enter or move through the karst aquifer.

Accordingly, the interpretation was focused on the final-effluent quality at the facility sampling point, independently of the subsequent receiving route.

These limitations affect three distinct levels of inference. Missing exposure-temperature, dilution-medium and sample-handling metadata restrict the exact replication of the bioassay; one independent sample per time point restricts temporal generalisation; and the absence of pathway and receptor data prevents environmental-risk inference. These constraints can be mitigated through the contemporaneous recording of all assay conditions, replicated testing of fresh samples and a separate source–pathway–receptor investigation.

4.7. Future Research and Validation Priorities

Future studies should collect independent replicate samples across production schedules, cleaning events, seasons and hydrological conditions, together with paired influent, interstage and final-effluent samples. Acute testing should include a full concentration series, observations after both 24 and 48 h, and the complete reporting of the organism, medium and exposure conditions, allowing for a numerical estimation of EC_{50} when an effect occurs [52,60–65].

Each sampling event should record the sampler, date and time, grab- or composite-sample status, container, transport temperature, time to analysis, freezing and thawing history, and dilution medium used in the bioassay.

If the objective is to quantify toxicity reduction, future sampling must include untreated wastewater and paired samples after the principal treatment stages.

Physicochemical analyses should be synchronised with each bioassay and reported with details of the method, instrument, accreditation status, detection and quantification limits, quality-control results and measurement uncertainty. Priority variables include the pH and dissolved oxygen at the start and end of the test, the conductivity, the hardness, the nitrates, the total and dissolved aluminium, and relevant residual treatment chemicals. The process flow, reagent dose, solid-separation performance and cleaning records should be linked to each sample.

Nitrite and nitrate should be measured together with the total and ammonium nitrogen to resolve nitrogen transformation across the treatment process.

The test battery should include a chronic *Daphnia magna* reproduction test, an algal growth-inhibition test and additional receptor-relevant endpoints. This broader assessment would test whether the absence of 24 h immobilisation also extends to longer-term or trophic-level-specific endpoints [63–67].

Any inference concerning *P. anguinus* would require validated receptor-relevant endpoints and evidence of exposure, rather than direct extrapolation from *D. magna*.

A practical validation programme could combine monthly sampling over at least 12 months with event-triggered sampling after cleaning or treatment disturbances. It should integrate final-effluent testing with discharge and receiving-water flows, upstream and downstream chemistry, and a hydrogeological investigation of potential karst transport pathways. Where conservation monitoring is justified, non-invasive environmental DNA methods could support occurrence surveys for *Proteus anguinus* [68]. This design would test, rather than assume, temporal stability, operational triggers and any source–pathway–receptor connection.

The facility's environmental permit and operator records should first be used to establish the authorised receiving route and any subsequent treatment before field-monitoring locations are selected.

A site-specific exposure assessment should quantify the discharge frequency, volume and mass loading, receiving-water flow, dilution, alkalinity, recharge conditions and travel time before evaluating the potential receptor risk.

5. Conclusions

This preliminary case study combined physicochemical characterisation with acute whole-effluent toxicity testing to evaluate three temporally separated final-effluent samples. Six exposure groups comprising 120 *Daphnia magna* individuals showed no immobilisation at either 80% or 100% (*v*/*v*), resulting in a reported 24 h EC50 value of >100% (*v*/*v*). The test validity was supported by the absence of immobilisation in control groups (0/60 organisms) and by potassium dichromate reference-toxicant EC50 values ranging from 1.07 to 1.18 mg/L.

The results indicate that no acute immobilisation response was detected under the tested conditions. However, interpretation should remain restricted to the analysed final-effluent samples and exposure period. The available physicochemical data provide useful operational context, but limitations related to documentation, sampling representativeness and endpoint selection constrain broader ecological interpretation and prevent a robust assessment of the temporal variability, treatment performance or longer-term effects.

Furthermore, the study contributes a biological line of evidence that complements the physicochemical characterisation of the examined effluent. The use of Daphnia magna enabled a standardised assessment of acute whole-effluent toxicity and provides a useful reference point for future receptor-focused and site-specific ecological investigations.

The principal contribution of this work lies in establishing a site-specific baseline dataset that integrates physicochemical measurements with a standardised biological endpoint. As an initial assessment, it provides a foundation for future validation, exposure characterisation and more comprehensive ecotoxicological investigations.

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Abbreviations

The following abbreviations are used in this manuscript:

AOX	Adsorbable organically bound halogens
BOD5	Biochemical oxygen demand after five days
COD	Chemical oxygen demand
D. magna	*Daphnia magna*
DO	Dissolved oxygen
EC50	Median effect concentration
ISO	International Organization for Standardization
K2Cr2O7	Potassium dichromate
P. anguinus	*Proteus anguinus*
SetS	Settleable solids
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
TSS	Total suspended solids
v/*v*	Volume/volume

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