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Ecotoxicological effects of pelagic *Sargassum* on *Eisenia andrei* earthworms: influence of washing and implications for vermicomposting

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Abstract

The massive accumulation of pelagic *Sargassum* in coastal regions has created an urgent need for sustainable management strategies. Vermicomposting is a promising alternative; however, the high salinity, variable chemical composition, and potentially toxic elements of *Sargassum* may limit its suitability as a substrate for earthworms. This study evaluated the ecotoxicological responses of *Eisenia andrei* to washed and unwashed pelagic *Sargassum* incorporated at different proportions, using survival, growth, and reproduction as biological endpoints. Standardized earthworm reproduction testing was used with modifications to accommodate the *Sargassum*-based substrates.

The results show that unwashed Sargassum causes acute toxicity, resulting in 100% mortality within 15 days at concentrations of 50% or higher due to its high electrical conductivity (EC). However, washing successfully eliminated acute mortality, although sublethal effects remained significant. Earthworm reproduction decreased drastically, from nearly 50 juveniles in the control group to fewer than 10 at higher concentrations of sargassum, resulting in total inhibition. Correlation analyses revealed strong negative associations between juvenile production and EC, Na, Cl, As, and the C/N ratio. These findings suggest that washing alone is not enough to make the substrate suitable and show the need for integrated pretreatment strategies to make sargassum-based vermicomposting safer and more viable.

Keywords

Seaweed; ecotoxicology; heavy metals; earthworm; organic waste management; salinity

Declarations

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Competing interest

The authors declare that they have no competing interests.

Code availability

Not applicable

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Mayela Martínez-Cano: Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – original draft. **Ángel Isauro Ortiz-Ceballos:** Conceptualization, Supervision, Methodology, Project administration, Writing – review & editing. **José Antonio García-Pérez:** Conceptualization, Writing – review & editing. **Sergio Martínez-Hernández:** Methodology, Conceptualization, Writing – review & editing. **Enrique Salgado-Hernández:** Investigation, Methodology, Writing – review & editing. **Ana Elena Dorantes-Acosta:** Conceptualization, Writing – review & editing. **Huemantzin Balan Ortiz-Oliveros:** Conceptualization, Resources, Methodology, Writing – review & editing, Supervision

1. Introduction

Since 2011, the Caribbean and the tropical Atlantic have experienced recurrent massive strandings of holopelagic *Sargassum*, primarily *S. natans* and *S. fluitans* (Debue et al. 2025). Massive accumulations and strandings can cause ecological impacts, including mortality of coastal flora and fauna and disruption of coastal ecosystems (Rodríguez-Martínez et al. 2025). The decomposition of stranded *Sargassum* can also pose risks to human health through the release of gases such as hydrogen sulfide, ammonia, and other air pollutants (Lutin et al. 2026). These events have socioeconomic consequences for coastal communities, particularly those dependent on tourism (Mohan and Strobl 2024). In July 2025, the total *Sargassum* amount in the Atlantic Ocean, including the Caribbean Sea and Gulf of Mexico, was estimated at approximately 38 million metric tons (University of South Florida Optical Oceanography Lab 2025).

Various approaches have been proposed for the treatment and valorization of pelagic *Sargassum*, including composting and vermicomposting. Among these approaches, vermicomposting has emerged as a promising strategy for converting organic residues into stabilized products with potential agricultural value. During vermicomposting, earthworms and associated microorganisms contribute to the decomposition and stabilization of organic residues, producing vermicompost with potential value as a soil amendment (Windarto et al. 2026). Previous studies have demonstrated the feasibility of using *Sargassum* in vermicomposting systems, with reports of successful vermicast production using *Eudrilus eugeniae* and *Eisenia foetida* after substrate pretreatment (Fantonalgo and Salubre 2019; Méndez-Covarrubias et al. 2023). However, these findings do not necessarily indicate that untreated or minimally treated *Sargassum* is suitable for earthworms, as recent evidence has shown avoidance and biomass loss in *E. fetida* exposed to high proportions of pelagic *Sargassum* (Martínez-Cano et al. 2024).

In addition to its potential for valorization, the chemical composition of pelagic *Sargassum* may limit its suitability as an agricultural amendment. During decomposition, this macroalgae can release As and other potentially toxic elements into leachates, raising concerns about their environmental mobility (Olguin-Maciel et al. 2022). Its elevated salt content further restricts direct application to soil. Pretreatment through washing has been shown to remove soluble salts and reduce biomass salinity (Olivier et al. 2022). Nevertheless, it remains uncertain whether such measures are sufficient to mitigate the long-term effects of *Sargassum* exposure on composting earthworms. Together, these findings indicated the need to assess the ecotoxicological effects of pelagic *Sargassum* on composting earthworms under standardized and prolonged exposure conditions.

Eisenia fetida and *E. andrei* are commonly used in standardized earthworms ecotoxicity tests, in which adult survival, biomass change, and reproductive performance provide sensitive endpoints for detecting sublethal effects (OECD 222 2016; ISO 11268-2 2023). Evaluating these endpoints is particularly relevant when assessing whether pretreatment of *Sargassum* can mitigate its potential adverse effects on earthworms. This study aims to evaluate the chronic ecotoxicological effects of pelagic *Sargassum* on *E. andrei* and assess its suitability for vermicomposting applications. Specifically, we evaluated earthworm survival, biomass change, and reproductive performance in mixtures of *Sargassum* and coffee pulp at different proportions, with and without washing pretreatment. In addition, the elemental composition of the *Sargassum* biomass was characterized to support the interpretation of the observed biological responses.

2. Materials and Methods

2.1 Earthworms

Eisenia andrei earthworms were obtained from an outdoor vermicomposting facility located in Teocelo, Veracruz, Mexico. The worms were kept in composting municipal solid waste under uncontrolled environmental conditions at this facility. Before the beginning of the experiment, earthworms were fed coffee pulp for a period of 10 months. For the trials, 250 adult clitellate individuals were selected from the culture. Taxonomic identity of the population was confirmed through DNA barcoding (COI gene) using a representative subset of individuals, as described in Martínez-Cano et al. (2026).

2.2 Pelagic *Sargassum*

Two independent samplings of pelagic *Sargassum* were conducted in Playa del Carmen, Quintana Roo, Mexico, exactly one year apart (May 2024 and May 2025). Approximately 10 kg of fresh biomass was manually collected during each sampling event. The material was briefly rinsed with seawater at the site to remove large external impurities such as sand, plastics, and shells. Samples were transported in polyethylene bags and stored frozen until processing.

The *Sargassum* biomass obtained in 2024 was preserved without further treatment, while the biomass from 2025 was washed with fresh water (10 L per kg of fresh biomass) with manual stirring for 5 minutes, followed by sieve draining. This procedure was adapted from Olivier et al. (2022). Finally, both fractions were air-dried at room temperature and stored for subsequent analysis and experimentation.

2.3 Coffee pulp

The coffee pulp utilized in this study was obtained from the vermicomposting company "Lombricompostaje Olmos", located in the municipality of San Marcos, Veracruz, Mexico. The material was stored in the laboratory until its use in the experimental setup.

2.4 Characterization of biomass

The N and C contents in coffee pulp and *Sargassum* were determined using a TruSpec Micro CHNS/O elemental analyzer (LECO Corporation, St. Joseph, MI, USA), based on high-temperature combustion and infrared/thermal conductivity detection. The instrument was calibrated using certified soil standards supplied by LECO. pH was measured using a bench-top pH meter (pH/mV/Temp PH700, Oakton Instruments, Vernon Hills, IL, USA) calibrated with standard buffer solutions at pH 4, 7, and 10. Electrical conductivity (EC) was measured using a Corning Pinnacle 54[1] conductivity meter (Corning Inc., Corning, NY, USA), calibrated with an 80 dS m^{-1} standard solution. All measurements were performed in duplicate.

Elemental composition (Na, Mg, Al, Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Cu, Zn, As, Br, Rb, Sr, Ni, Pb, and I) was determined by energy-dispersive X-ray fluorescence (EDXRF) using a Rigaku NEX CG X-ray fluorescence spectrometer (Rigaku Corporation, Japan). The instrument was equipped with a Pd-anode X-ray tube (maximum power 50 W, maximum voltage 50 kV) and four secondary targets arranged in a Cartesian optical geometry. A Si-Li Flash® Drift detector with a resolution of <150 eV at Mn Kα was employed. Measurements were carried out under vacuum using a 20 mm diaphragm without spectral optimization. Samples were analyzed in triplicate under the following tube conditions: 50 kV and 1 mA (200 s) with an Al secondary target; 50 kV and 1 mA (100 s) with Mo and Cu secondary targets; and 25 kV and 2 mA (100 s) with an RX9 secondary target. Elemental concentrations were calculated using the Fundamental Parameters (FP) method implemented in FPR-SQX FP® software.

2.5 Chronic ecotoxicological tests

Clitellate earthworms (*E. andrei*) were exposed to *Sargassum*-based substrates to perform chronic ecotoxicological assays based on standardized earthworm reproduction tests (OECD 222 2016; ISO 11268-2 2023), with modifications adapted to the experimental conditions of this study. The washed *Sargassum* experiment followed an eight-week exposure period, whereas the unwashed *Sargassum* experiment was terminated after 15 days because of severe toxicity.

Two independent experiments were conducted using pelagic *Sargassum*: i) unwashed *Sargassum* (2024 collection), and ii) washed *Sargassum* (2025 collection). Each experiment was performed separately using freshly collected biomass. The experimental substrates were formulated on a dry

weight basis to evaluate the effect of increasing proportions of *Sargassum* on earthworm performance, using coffee pulp as a complementary organic substrate. Five treatments were established, each with five replicates: (1) 100% coffee pulp (control); (2) 25% *Sargassum* + 75% coffee pulp; (3) 50% *Sargassum* + 50% coffee pulp; (4) 75% *Sargassum* + 25% coffee pulp; and (5) 100% *Sargassum*. This gradient allowed the assessment of dose-dependent effects of *Sargassum* incorporation. The moisture content was adjusted to 75% of the total water-holding capacity, determined by saturating the substrate with water, allowing for free drainage, and calculating the retained water as a percentage of the dry weight. All mixtures were pre-conditioned for 7 days to allow homogenization of the substrate before the introduction of organisms.

The experimental units consisted of 1 L plastic containers with perforated lids to allow aeration. All containers were maintained at room temperature under natural light–dark cycles. Humidity was kept constant by periodic adjustments with tap water. For each replicate, 10 adult clitellate individuals were introduced. In the unwashed *Sargassum* experiment, survival and biomass were assessed after 15 days of exposure. The experiment was terminated at this time due to complete mortality at *Sargassum* proportion ≥50 and observation of severe organism deterioration, including loss of clitellum. Consequently, biomass analysis was only assessed on treatments with surviving individuals (0% and 25%). The washed *Sargassum* experiment was conducted for the full eight-week period. Adult biomass was evaluated at 15 and 30 days. After the first four weeks, adults were removed, and the experiment continued for an additional four weeks to allow for cocoon hatching and juvenile development. At the end of the experiment, juvenile production and individual biomass were quantified. Survival remained at 100% across all treatments throughout the experimental period.

2.6 Statistical Analysis

2.6.1 Adult survival

In the unwashed *Sargassum* experiment, the survival of the number of adult individuals (alive versus dead, per replicate) was analyzed after 15 days of exposure. This analysis was performed using a generalized linear model (GLM) with a binomial distribution and logit link function. Exact binomial 95% confidence intervals were calculated for descriptive visualization.

In the washed *Sargassum* experiment, survival was 100% across all treatments during the experimental period; therefore, no statistical analysis was performed for this variable.

2.6.2 Adult biomass

Due to differences in data structure between experiments, different analytical approaches were applied. Biomass change was used in the unwashed *Sargassum* experiment to account for mortality and initial variability, whereas absolute biomass was analyzed in the washed experiment to evaluate temporal dynamics under controlled conditions. For the unwashed *Sargassum* experiment, biomass change was calculated as the difference between day 15 and initial biomass (day 0) to account for initial variability and mortality at higher concentrations. Due to the absence of surviving individuals in some treatments, statistical analyses were restricted to treatments with survival (0% and 25%). Differences between treatments were evaluated using Welch's two-sample t-test.

For the washed *Sargassum* experiment, biomass was analyzed using a linear mixed-effects model (LMM) including *Sargassum* concentration (0–100%), time (0, 15, and 30 days), and their interaction as fixed factors, and replicate as a random factor. Heterogeneity of variances was accounted for using a variance structure. In both cases, normality and homogeneity of variance were assessed using the Shapiro–Wilk and Levene's tests, respectively. Post hoc comparisons for the washed *Sargassum* experiment were conducted within each treatment using Dunnett's test, considering day 0 as the reference.

2.6.3 Juvenile production and biomass

Juvenile production and biomass were assessed only in the washed *Sargassum* experiment, as no reproduction was observed in the unwashed treatments. Juvenile counts were analyzed using a generalized linear model (GLM) with a Poisson distribution and a log link function. Model fit was evaluated by examining dispersion through the ratio of residual deviance to degrees of freedom. When significant effects were found, pairwise comparisons were performed using Tukey-adjusted contrasts.

Spearman's rank correlation analysis was conducted to assess the relationships between reproduction and selected physicochemical variables, including EC, Na, Cl, As, C/N ratio, and pH. This non-parametric approach was chosen due to the non-normal distribution of the data and the presence of tied values. Variables were selected based on their relevance to salinity-driven stress (EC, Na, Cl), potential toxicity of As, and substrate quality (C/N ratio and pH). Correlation analyses were performed using individual replicate data from washed *Sargassum* treatments. Additionally, generalized linear models (GLMs) with a Poisson distribution and log-link function were used to evaluate the effect of physicochemical variables on reproduction (number of juveniles). EC was included as the main predictor variable, and a second model incorporating both EC and As

concentration was fitted to assess the potential contribution of trace elements. Model performance was compared using an analysis of deviance with a Chi-square test.

Individual juvenile biomass did not meet the normality assumptions (Shapiro–Wilk test), and therefore differences among treatments were evaluated using the nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison test with a Bonferroni adjustment. As no juveniles were produced in the 100% treatment, individual biomass could not be calculated, and this treatment was excluded from the analysis. All statistical analyses were performed in R version 4.2.0 (R Core Team 2022). Statistical significance was set at $\alpha = 0.05$.

3. Results

3.1 Chemical Composition of Unwashed Sargassum Mixtures (2024 Collection)

The elemental composition of substrates containing increasing proportions of unwashed *Sargassum* (2024 collection) is presented in Table 1. Increasing *Sargassum* incorporation was accompanied by higher concentrations of salinity-associated elements, particularly Na and Cl. Calcium (Ca) reached its highest concentration in the 75% treatment (190,333 ± 1,155 mg kg^{-1}), whereas Na and Cl peaked in the 100% treatment at 81,150 ± 1,385 mg kg^{-1} and 73,633 ± 2,255 mg kg^{-1}, respectively. This pattern was accompanied by a progressive increase in electrical conductivity (EC), rising from 4.76 dS m^{-1} in the control to 29.10 dS m^{-1} in the 100% treatment.

Strontium (Sr) increased with higher *Sargassum* incorporation, rising from 205 ± 2 mg kg^{-1} in the control to 2,783 ± 44 mg kg^{-1} in the 100% treatment, mirroring the pattern observed for Ca. Arsenic (As) also increased progressively, reaching 120 ± 2 mg kg^{-1} in the 100% treatment. Conversely, concentrations of Fe, Si, and Rb decreased across the gradient, while Pb, Ni, Cu, and Zn remained stable.

Regarding C/N dynamics, nitrogen content decreased more substantially (≈70%) than carbon (≈34%) across the treatment gradient. Consequently, the C/N ratio increased from 12.46 in the control to 16.23, 21.19, and 27.62 in the 50%, 75%, and 100% treatments, respectively. Substrate pH values ranged between 7.47 and 8.98 across all treatments.

Table 1. Elemental composition (mg kg^{-1} dry weight) in treatments with unwashed pelagic *Sargassum* (0%-100%) in the May 2024 collection.

Element	0%	25%	50%	75%	100%
Ca	16,233 ± 58	34,900 ± 100	125,667 ± 577	190,333 ± 1,155	117,667 ± 3,670

Na	ND	12,300 ± 361	24,500 ± 600	34,300 ± 1217	81,150 ± 1,385
K	81,700 ± 173	69,400 ± 200	47,367 ± 153	35,667 ± 208	45,583 ± 1,023
Cl	2,407 ± 6	7,947 ± 12	17,600 ±100	27,867 ± 231	73,633 ± 2,255
Mg	3,480 ± 46	5,067 ± 29	12,467 ± 155	16,967 ± 231	15,067 ± 891
S	4,750 ± 26	6,117 ± 32	8,463 ± 23	8,973 ± 29	14,517 ± 98
Br	14± 1	85 ± 1	202 ± 2	256 ± 3	474 ± 3
I	ND	ND	58 ± 1	59 ± 1	133 ± 3
Sr	205 ± 2	604 ± 2	1,610 ±6	2,327 ±6	2,783 ± 44
Si	6,587 ± 40	3,763 ± 12	2,710 ± 46	2,870 ± 10	1,952 ± 136
Al	3,180 ± 30	2,200 ± 10	1,587 ± 12	1,737 ± 60	1,938 ± 46
Fe	1,747 ± 21	1,030 ± 20	572 ± 9	386 ± 17	159 ± 10
P	2,640 ± 17	2,100 ± 21	2,287 ± 6	2,187 ± 6	1,243 ± 82
Rb	199 ± 1	155 ± 1	86 ± 1	51 ± 1	27 ± 3
Mn	157 ± 6	125 ± 6	97 ± 5	84 ±4	34 ± 2
Ti	153 ± 6	80± 4	50 ± 3	56 ± 3	25 ± 1
As	ND	18 ± 1	67 ± 1	99 ± 4	120 ± 2
Zn	18 ± 1	15 ± 1	14 ± 1	15 ± 1	42 ± 1
Cu	33 ± 1	28 ± 1	21 ± 2	19 ± 1	13 ± 1
Ni	NA	NA	NA	NA	9 ± 1
Pb	NA	NA	NA	NA	10 ± 1
C (%)	42	40.40	38.80	35.40	27.90
N (%)	3.37	2.82	2.39	1.67	1.01
C/N	12.46	14.32	16.23	21.19	27.62
pH	8.98	8.31	8.19	7.76	7.47
EC (dS m^{-1})	4.76	9.32	13.51	20.30	29.10

Values represent the mean ± standard deviation (n=3); ND: Not detected; NA: Not analyzed.

3.2 Chemical Composition of Washed Sargassum Mixtures (2025 Collection)

The washing pretreatment significantly modified the physicochemical composition of the *Sargassum* biomass (Table 2). In the 2025 unwashed biomass (100% UW), Na and Cl concentrations were 66,800 ± 4,468 mg kg^{-1} and 71,267 ± 577 mg kg^{-1}, respectively. Washing

reduced these levels to 28,633 ± 58 mg kg^{-1} for Na and 13,900 ± 150 mg kg^{-1} for Cl. Consequently, electrical conductivity (EC) decreased by approximately 73%, from 33.70 dS m^{-1} in 100% UW to 9.07 dS m^{-1} in 100% washed *Sargassum*. In contrast, Ca and Mg concentrations exhibited minor differences between washed and unwashed biomass. Potassium (K) decreased progressively with increasing *Sargassum* incorporation, dropping from 51,700 ± 173 mg kg^{-1} in the control to 18,733 ± 115 mg kg^{-1} in the 100% washed treatment.

Arsenic (As) concentration decreased by 41% following washing, from 118 ± 2 mg kg^{-1} in the unwashed biomass to 70 ± 1 mg kg^{-1} in the washed biomass. Bromine (Br) and iodine (I) levels also declined after washing. Conversely, concentrations of Al, Si, Mn, and Rb decreased steadily as the *Sargassum* proportion increased. Regarding C and N content, the C/N ratio increased alongside *Sargassum* incorporation (Table 2). The C/N ratios for the 25–75% washed treatments ranged between 13.89 and 19.79, reaching 31.94 in the 100% washed treatment. Substrate pH shifted from 7.94 in the 100% UW treatment to 7.39 in the 100% washed treatment, with all washed treatments remaining near neutral.

Table 2. Elemental composition (mg kg^{-1} dry weight) in treatments with washed pelagic *Sargassum* (0%-100%) in the May 2025 collection.

Element	**0%**	**25%**	**50%**	**75%**	**100%**	**100% UW**
Ca	17,533 ± 115	32,867 ± 58	29,167 ± 115	118,000 ± 120	94, 667 ± 58	96,200 ± 781
Na	ND	ND	13,633 ± 513	18,567 ± 231	28,633 ± 58	66,800 ± 4,468
K	51,700 ± 173	45,833 ± 115	42,233 ± 115	21,600 ± 100	18,733 ± 115	48,900 ± 529
Cl	1,400 ± 85	3,880 ± 26	4,740 ± 17	7,300 ± 46	13,900 ± 150	71,267 ± 577
Mg	5310 ± 46	7,807 ± 35	7,760 ± 72	20,767 ± 115	21,300 ± 100	18,667± 153
S	5,833 ± 16	6,533 ± 59	8,007 ± 12	9,033 ± 76	1,603 ± 58	9,483 ± 112
Br	9 ± 1	40 ± 1	85 ± 1	199 ± 1	264 ± 1	418 ± 3
I	ND	16 ± 1	23 ± 3	63 ± 3	106 ± 2	164 ± 4
Sr	167 ± 1	327 ± 1	659 ± 1	1,183 ± 6	1,977 ± 6	1,330 ± 17

Si	18,433 ± 58	20,833 ± 153	12,467 ± 58	7,643 ± 51	2,217 ± 15	1,320 ± 10
Al	13,500 ± 155	15,533 ± 208	9,740 ± 69	5,063 ± 25	1,537 ± 12	2,087 ± 153
Fe	4,387 ± 12	5,707 ± 6	2,703 ± 15	1,673 ± 15	180 ± 4	79 ± 3
P	3,130 ± 10	3,037 ± 25	1,957 ± 15	2,443 ± 26	1,583 ± 26	1,473 ± 26
Rb	138 ± 2	127 ± 1	107 ± 1	56 ± 1	21 ± 1	25 ± 1
Mn	278 ± 5	266 ± 5	188 ± 3	130 ± 2	54 ± 2	43 ± 2
Ti	617 ± 22	860 ± 24	394 ± 22	257 ± 7	37 ± 4	ND
As	ND	8 ± 1	16 ± 1	49 ± 1	70 ± 1	118 ± 2
Zn	39 ± 1	39 ± 1	27 ± 1	26 ± 1	17 ± 1	9 ± 1
Cu	42 ± 1	41 ± 1	30 ± 1	36 ± 1	28 ± 1	13 ± 1
Ni	5 ± 1	7 ± 1	4 ± 1	9 ± 1	9 ± 1	8 ± 1
Pb	ND	5 ± 1	4 ± 1	ND	5 ± 1	ND
Cr	11 ± 1	15 ± 1	ND	ND	ND	ND
Ba	66 ± 2	78 ± 2	55 ± 1	ND	ND	ND
C (%)	42	41	40	39	34.50	28.20
N (%)	3.37	2.95	2.35	1.97	1.08	1.15
C/N	12.46	13.89	17.02	19.79	31.94	24.52
pH	8.98	8.45	8.2	7.86	7.39	7.94
EC (dS m^{-1})	4.76	5.9	6.5	7.05	9.07	33.70

Values represent the mean ± standard deviation (n=3); ND: Not detected;

3.3 Earthworm Response to Unwashed Pelagic Sargassum

Adult survival of Eisenia andrei declined significantly with increasing proportions of unwashed Sargassum in the substrate (binomial GLM, $\chi^2(4) = 273.48$, $p < 0.0001$). Survival was 100% in the control (0% treatment) and decreased to 94% in the 25% treatment. At incorporation rates of 50%, 75%, and 100% Sargassum, complete adult mortality (100%) occurred prior to day 15 (Fig. 1).

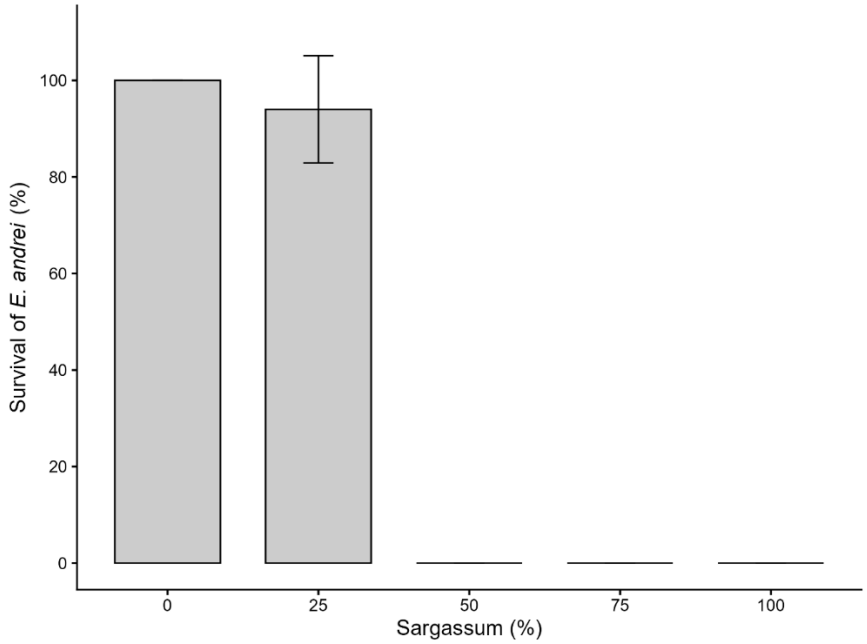

Fig. 1. Survival (%) of adult *Eisenia andrei* after 15 days of exposure to increasing proportions of unwashed *Sargassum* spp.. Values represent mean survival and error bars indicate 95% confidence intervals (n = 5). Differences among treatments were significant (binomial GLM, $\chi^2 = 273.48$, $p < 0.0001$).

Biomass changes were evaluated exclusively for treatments where earthworms survived (0% and 25%). A significant difference in biomass change was observed between these two groups (Welch's *t*-test, $t = 8.87$, $df = 4.45$, $p < 0.001$). Organisms in the control treatment exhibited an average biomass gain of +0.97 g (≈ 25%). In contrast, earthworms exposed to 25% unwashed *Sargassum* experienced an average biomass loss of −1.51 g (≈ 40%). Morphological and behavioral alterations were recorded in surviving organisms in the 25% treatment. These included clitellum reabsorption, body rigidity, reduced mobility, body fragmentation, and distinct shifts in body coloration (Fig. 2). Due to the acute mortality and severe physiological decline observed across treatments, this experimental phase was terminated on day 15.



Fig. 2 Comparative morphology of *Eisenia andrei* at 15 days of exposure to (a) Control organism exposed to 0% *Sargassum* spp., showing a prominent clitellum (swollen glandular band); and (b) Organism exposed to 25% unwashed *Sargassum* spp., showing absence of a clitellum, alterations in skin coloration, and change in body condition.

3.4 Earthworm Response to Washed Pelagic Sargassum

Treatments containing washed *Sargassum* exhibited 0% mortality throughout the 30-day experimental period. Adult biomass was significantly influenced by both *Sargassum* concentration and exposure time (linear mixed-effects model; treatment: $F_{4,53} = 22.82$, $p < 0.001$; time: $F_{2,53} = 33.01$, $p < 0.001$; treatment × time interaction: $F_{8,53} = 3.19$, $p = 0.005$) (Fig. 3).

Post hoc comparisons relative to day 0 revealed distinct temporal biomass trajectories across treatments. The control (0%) exhibited a sustained increase in biomass from day 15 onward ($p < 0.0001$). In the 25% treatment, biomass gain was delayed, becoming statistically significant only by day 30 (day 15: $p = 0.179$; day 30: $p = 0.012$). The 50% and 75% treatments showed significant increases in biomass beginning on day 15 (day 15: $p = 0.023$; day 30: $p = 0.008$). In contrast, the 100% treatment displayed no significant changes in biomass throughout the 30-day period (day 15: $p = 0.499$; day 30: $p = 0.162$).

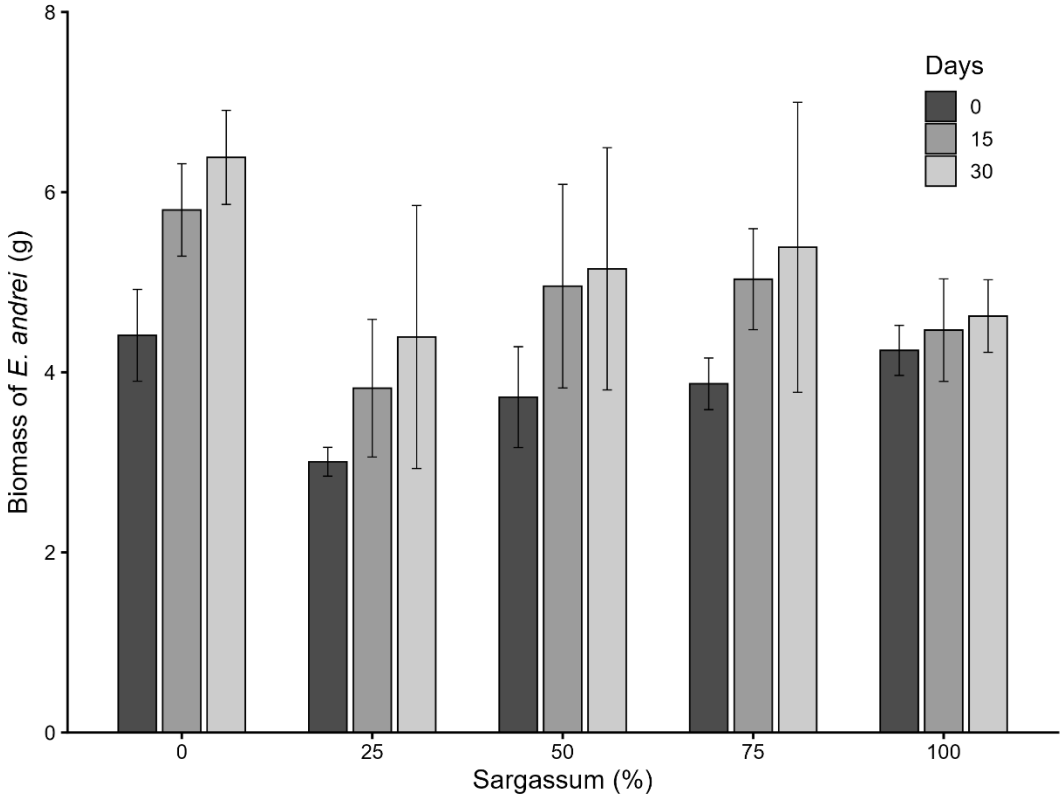

Fig. 3 Biomass (g) of adult *Eisenia andrei* exposed to different proportions of washed *Sargassum* spp. (0, 25, 50, 75, and 100%) over time (0, 15, and 30 days). Bars represent mean biomass per treatment (n = 5 replicates), and error bars indicate 95% confidence intervals.

Reproductive output decreased significantly with increasing *Sargassum* proportion (Poisson GLM, $\chi^2(4) = 461.99$, $p < 0.0001$) (Fig. 4). Juvenile production declined from an average of approximately 50 individuals in the control group to fewer than 10 in treatments containing ≥ 50% *Sargassum*, with complete reproductive inhibition (0 juveniles recovered) observed in the 100% washed *Sargassum* treatment. Spearman correlation analysis (Fig. S1) revealed strong negative correlations between juvenile production and electrical conductivity (EC; $\rho = -0.98$, $p < 0.001$), as well as Na, Cl, As, and the C/N ratio ($\rho \approx -0.95$ to -0.98, $p < 0.001$). In contrast, substrate pH showed no significant relationship with reproduction ($\rho = -0.04$, $p = 0.86$). The Poisson generalized linear model confirmed EC as a significant predictor of reproduction ($z = -16.98$, $p < 0.0001$), whereas adding As did not significantly improve model fit ($\chi^2 = 1.00$, $p = 0.317$). Individual juvenile biomass varied significantly among treatments (Kruskal-Wallis $\chi^2(3) = 14.41$, $p = 0.0024$), with juveniles from the 75% treatment exhibiting higher individual biomass than those from the intermediate treatments (25% and 50%) (Fig. 6).

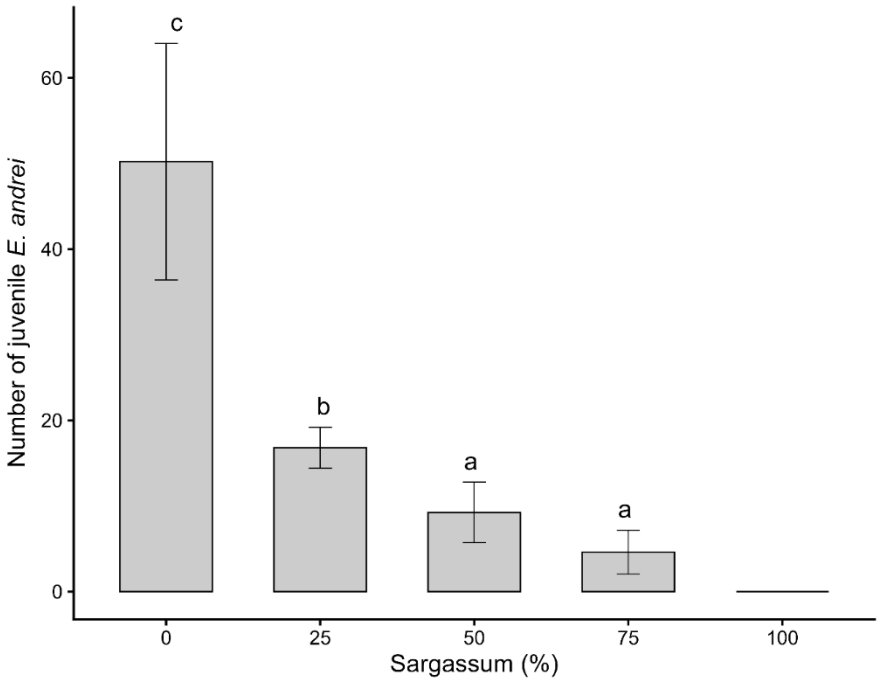

Fig. 4 Juvenile production of *Eisenia andrei* after 8 weeks in treatments with increasing proportions of washed *Sargassum* spp.. Bars show mean juvenile number per replicate (n = 5) ± 95% confidence intervals. Different letters indicate significant differences among treatments ($p < 0.05$; Poisson GLM, Tukey-adjusted comparisons).

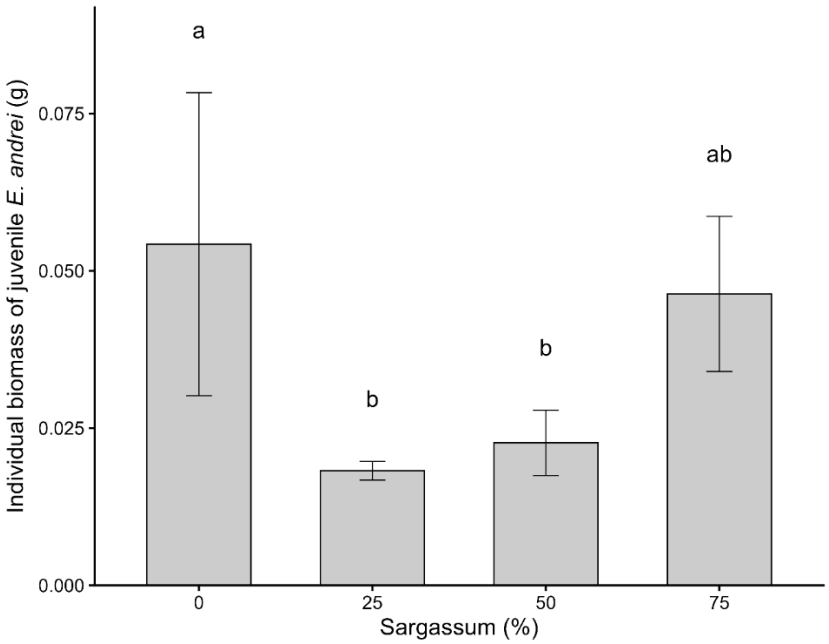

Fig. 6 Individual juvenile biomass of *Eisenia andrei* after 8 weeks in treatments with increasing proportions of washed *Sargassum* spp.. Bars show mean biomass (n = 5) ± 95% confidence intervals. Different letters indicate significant differences among treatments ($p < 0.05$; Kruskal-Wallis/Dunn-Bonferroni). The 100% *Sargassum* treatment was excluded because no juveniles were present.

4. Discussion

4.1 Chemical Constraints and Salinity Dynamics in Unwashed Sargassum

The chemical composition of unwashed *Sargassum* mixtures highlights severe osmotic and elemental constraints for vermicomposting. The marked accumulation of Na, Cl, and elevated EC (exceeding 16 dS m^{-1} in high-incorporation treatments) places the unwashed substrate in a high-salinity classification (NOM-021-RECNAT-2000 2002). Earthworms are highly sensitive to saline conditions; previous studies have reported adverse effects of NaCl exposure on the survival, growth, and reproduction of *Eisenia fetida*, with a 28-day LC_{50} of approximately 5,436 mg kg^{-1} NaCl (Owojori et al. 2008). In the present study, the 100% treatment contained 81,150 Na and 73,633 mg kg^{-1} Cl. Although these elements were quantified separately and cannot be directly equated with NaCl concentrations reported in toxicity tests, their high concentrations, together with the elevated EC (29.10 dS m^{-1}), indicate severe salinity-related constrains. These conditions likely contributed substantially to the complete mortality observed in *Eisenia andrei* at *Sargassum* proportions ≥50%, although contributions from other chemical properties of the substrate cannot be excluded.

The co-occurrence of elevated Sr alongside Ca is consistent with their chemical similarity and shared geochemical behavior in biological systems (Nitzsche et al. 2022). Furthermore, the maximum As concentration observed (120 ± 2 mg kg^{-1}) falls within the upper range documented for pelagic *Sargassum* (60–130 mg kg^{-1}; (Gobert et al. 2025), confirming that trace element levels in this batch align with reported biomass variability. The stable levels of Pb, Ni, Cu, and Zn were also within typical ranges for *Sargassum* (Liranzo-Gómez et al. 2023), whereas the decline in Fe, Si, and Rb reflects a predictable dilution effect caused by increasing biomass incorporation.

Although C/N ratios in the 50% (16.23) and 75% (21.19) treatments fell within optimal ranges previously reported for *Eisenia* vermicomposting—such as 18:1 for *E. andrei* (Dume et al. 2023) and 13.76 for *E. fetida* (Muthuveni et al. 2024)—C/N optimization alone is insufficient to ensure

371 substrate viability. Severe salinity and toxic elemental loads override favorable C/N ratios. Finally,
372 although pH values (7.47–8.98) remained within acceptable boundaries for vermicomposting
373 (Katiyar et al. 2023) and can influence nutrient availability and microbial dynamics (Barrow and
374 Hartemink 2023), pH was not a limiting factor for earthworm survival compared to extreme salinity.

375 **4.2 Impact of Washing Pretreatment on Salinity, Trace Elements, and Substrate Viability**

376 The baseline elemental composition of unwashed 2025 *Sargassum* was comparable to the 2024
377 collection, confirming a consistent saline profile across sampling years. The washing pretreatment
378 proved highly effective at removing soluble salts, as demonstrated by the ~73% reduction in EC and
379 substantial losses of Na and Cl. This aligns with prior studies demonstrating that aqueous washing
380 removes soluble inorganic fractions and reduces ash content in species such as *S. fluitans* and *S.*
381 *muticum* (Olivier et al. 2022). However, despite this marked reduction, the EC of the 100% washed
382 substrate (9.07 dS m^{-1}) remained within the highly saline category (NOM-021-RECNAT-2000
383 2002), indicating that residual salinity continues to represent a key constraint for *Eisenia andrei*.

384 The resistance of Ca and Mg to washing reflects their integration into less readily mobilized
385 structural or matrix-bound fractions, distinct from highly soluble Na and Cl ions (Nitzsche et al.
386 2022). Conversely, the declining trend of K across treatments highlights coffee pulp—the co-
387 substrate used in the control—as the main contributor of K in the mixtures. The 41% reduction in As
388 concentration confirms the leaching of water-soluble arsenic species, consistent with reported
389 aqueous extraction efficiencies in marine macroalgae (Ostrowski et al. 2024; Zhao et al. 2025). The
390 persistent As fraction (70 mg kg^{-1}) suggests the presence of organic arsenicals (e.g., arsenosugars)
391 or matrix-bound forms that resist simple aqueous washing (Yu et al. 2024). Although arsenic
392 speciation was not evaluated here, these findings confirm that washing partially mitigates trace
393 element loads but cannot eliminate them entirely. Reductions in Br and I further confirm the
394 removal of soluble halogen fractions, whereas decreases in Al, Si, Mn, and Rb reflect a
395 straightforward dilution effect.

396 While C/N ratios in the 25–75% washed treatments (13.89–19.79) fall within ranges reported as
397 suitable for *Eisenia* vermicomposting, the parameter alone does not guarantee substrate safety when
398 residual EC remains elevated. The slight pH drop toward near-neutral conditions (7.39) after
399 washing provides a favorable environment for microbial activity and nutrient uptake (Katiyar et al.
400 2023; Barrow and Hartemink 2023). Nevertheless, because single-step washing leaves residual EC

at stress-inducing levels, optimization strategies—such as multi-stage washing cycles, leaching, or co-substrate blending—must be integrated to achieve full substrate suitability.

4.3 Acute Toxicity, Sublethal Stress, and Substrate Constraints of Unwashed Biomass

The acute mortality observed at ≥ 50% unwashed *Sargassum* incorporation underscores severe ecotoxicological limitations. In standardized testing, mortality serves as a fundamental endpoint for evaluating acute substrate toxicity (ISO 11268-1 2012). The rapid death recorded at 50–100% incorporation coincides with extreme electrical conductivity (EC) values (13.51, 20.30, and 29.10 dS m^{-1}, respectively). Previous studies on *Eisenia fetida* report total mortality at EC levels between 8 and 16 dS m^{-1} after 14 days (Yan et al. 2024). Conversely, *E. fetida* exposed to arsenic (As) concentrations up to 100 mg kg^{-1} exhibited no mortality (Ma et al. 2026). Given that As concentrations in our 100% treatment reached 120 ± 2 mg kg^{-1} (partially overlapping non-lethal ranges), high substrate EC emerges as the primary driver of acute toxicity, though potential additive interactions with trace elements cannot be ruled out. High ionic strength can also alter metal bioavailability and bioaccumulation, potentially amplifying toxicity in soil fauna (Karimi et al. 2020).

The severe response observed here contrasts with (Martínez-Cano et al. 2024), who reported low mortality in *E. fetida* exposed to high *Sargassum* proportions. While interspecific differences in sensitivity between *E. fetida* and *E. andrei* may exist (ISO 11268-1 2012; OECD 222 2016; ISO 11268-2 2023), these discrepancies are more likely driven by temporal and geographic variability in the biomass itself. Macro- and micro-element profiles in pelagic *Sargassum* fluctuate considerably depending on sampling site and season, particularly regarding major ions (K, Mg, Ca) and heavy metals (Pb, Cd, Ni, Zn) (Tonon et al. 2022; Bauta et al. 2024). Consequently, the ecotoxicity of pelagic *Sargassum* depends strongly on the specific chemical profile of the harvested batch.

At sublethal levels (25% incorporation), earthworms suffered marked physiological stress, evidenced by a 40% biomass loss, clitellum reabsorption, body fragmentation, and reduced mobility. Similar biomass loss (~50%) and physical fragmentation have been described in oligochaetes under high-salinity conditions (8–16 dS m^{-1}) or elevated arsenic exposure (>80 mg kg^{-1}) (Rodriguez et al. 2006; Wang et al. 2019; Yan et al. 2024; Ma et al. 2026). Overall, these findings confirm that unwashed pelagic *Sargassum* poses severe osmotic and chemical barriers to earthworm survival and growth. Direct application of untreated biomass into vermicomposting systems or agricultural soils

poses significant risks to beneficial soil fauna, underscoring the absolute necessity for effective pretreatment and desalinization protocols.

4.4 Sublethal Dynamics, Reproductive Impairment, and Pretreatment Optimization

The absence of adult mortality in washed *Sargassum* treatments confirms that washing effectively eliminates acute lethality by alleviating extreme osmotic stress. However, sublethal responses—particularly regarding growth trajectories and reproduction—revealed persistent physiological constraints. The significant treatment-time interaction for adult biomass reflects variable stress intensities over time. While moderate treatments (50–75%) achieved biomass gains similar to those reported in other seaweed-based vermicomposting studies using pretreated or blended algal biomass (Fantonalgo and Salubre 2019; Ananthavalli et al. 2019; Méndez-Covarrubias et al. 2023), the 100% treatment completely suppressed growth. This stagnation aligns with findings showing that moderate salinity (4–8 dS m^{-1}) permits slow growth, whereas higher ranges (8–16 dS m^{-1}) progressively inhibit earthworm development (Yan et al. 2024). A similar lag phase at 25% indicates an initial metabolic cost associated with adjusting to residual salinity before partial recovery occurs (Spurgeon and Hopkin 1996).

Reproduction was the most sensitive endpoint. Complete inhibition at 100% and sharp declines at ≥ 50% demonstrate that reproductive output remains severely impaired even when adult survival is 100% (OECD 222 2016; ISO 11268-2 2023; Zhang et al. 2026). The strong negative correlations with EC, combined with the GLM validation, identify residual salinity as the principal driver of reproductive decline. The lack of a significant improvement after adding As to the EC-based model suggests that EC explained reproductive variation more effectively under the conditions tested, although a contribution of As cannot be ruled out. Variations in individual juvenile biomass (with higher values in the 75% treatment) highlight differential developmental performance under stress, echoing previous evidence that early life stages exhibit heightened sensitivity to environmental contaminants and stressors (Spurgeon and Hopkin 1996; Podolak 2025).

Ultimately, while single-step washing makes vermicomposting feasible by preventing mortality, it is insufficient to fully mitigate residual EC at high proportions. Further process optimization—such as multi-stage washing cycles to progressively lower EC (Olivier et al. 2022) or integrating a pre-composting stage with free-living microorganisms to degrade toxic compounds—represents a promising pathway for safe algal management (Patón et al. 2023). However, larger-scale applications must carefully weigh water requirements, saline effluent generation, arsenic mobility

into leachates (Thouin et al. 2025), and the final safety of the vermicast for agricultural use (Abdool-Ghany et al. 2023). Exploring salt-tolerant earthworm species or blending with low-EC organic amendments offers additional avenues to overcome these limitations.

Conclusions

The results of this study demonstrated that the use of pelagic *Sargassum* as a substrate for vermicomposting is limited by its physicochemical properties, especially the salinity. Unwashed *Sargassum* presents high concentrations of salinity-associated elements and elevated electrical conductivity, resulting in acute toxicity and complete mortality of *E. andrei* at proportions ≥50%. Washing significantly reduced lethal effects; however, residual salinity remained at levels capable of inducing sublethal stress, as evidenced by reduced growth and complete inhibition of reproduction at high *Sargassum* proportions. Therefore, washing is an effective pretreatment for reducing toxicity; however, it is not sufficient to ensure that the substrate is suitable for vermicomposting with *E. andrei*. It is recommended that *Sargassum* undergo preliminary physicochemical characterization and that integrated treatment strategies be implemented. For example, washing and pre-composting can be used to reduce salinity and improve substrate quality. Finally, although adding As did not significantly improve the EC-based model of juvenile production, its presence in the substrate highlights the need to monitor As concentrations in both vermicompost and leachates to ensure environmental safety and suitability for future agricultural applications.

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