Deciphering waste bound nitrogen by employing psychrophillic *Aporrectodea caliginosa* and priming of coprolites by associated heterotrophic nitrifiers under high altitude Himalayas

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Himalayan ecosystem is characterized by its fragile climate with rich repositories of biodiversity. Waste collection and disposal are becoming increasingly difficult due to topographical variations. *Aporrectodea caliginosa*, a versatile psychrophillic soil dweller, is a useful biocatalyst with potent bio-augmented capability for waste treatment at low temperatures. Microcosm experiments were conducted to elucidate the comprehensive nature of biogenic nitrogen transformation to NH$_4^+$ and NO$_3^−$ produced by coupling of earthworm-microbes. Higher biogenic recovery of NH$_4^+$-N from coprolites of garden soil (47.73 ± 1.16%) and Himalayan goat manure (86.32 ± 0.92%) with an increment of 14.12 and 47.21% respectively over their respective control (without earthworms) with a linear decline beyond 4th week of incubation was reported. NO$_3^−$-N recovery progressively sustained in garden soil and goat manure coprolites during entire incubation with highest 81.81 ± 0.45 and 87.20 ± 1.08 µg-N g$^{-1}$ dry weight recorded in 6th and 5th week of incubation respectively and peak increments as 38.58 and 53.71% relative to respective control (without earthworms). Declined NH$_4^+$-N in coprolites at low temperature (15.0 ± 2.0 °C) evidenced increased nitrification rates by taking over the process by abundant nitrifying microbes. Steady de-nitrification with progressive incubation on an average was 16.95 ± 0.46 ng-N g$^{-1}$ per week and 21.08 ± 0.87 ng-N g$^{-1}$ per week compared to 14.03 ± 0.58 ng-N g$^{-1}$ per week and 4.50 ± 0.31 ng-N g$^{-1}$ per week in respective control treatments. Simultaneous heterotrophic nitrification and aerobic denitrification (SHNAD) was found to be a prominent bioprocess at low temperature that resulted in high and stable total nitrogen and nitrate accumulation from garden soil and goat manure with relative recovery efficiency of 11.12%, 14.97% and 14.20%; 19.34%. *A. caligenosa* shows promising prospects for mass applicability in biogenic N removal from manure of Himalayan goat.

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Potential psychrophillic class of soil macrofauna are earthworms (Endogeic and Anecic species) that exploited deeper mineral soil beds, and thereby remodel and restructure nutrient pools in uppermost soil layers (herein-after: geo-engineering earthworms). *Aporrectodea caliginosa* (Endogeic) is a non-permanent horizontal burrower and lives in the uppermost soil horizons[2][3], ingesting enormous load of soil, rich in organic matter, leaving the casting as a key input into the soil and contributing biogeochemical cycles. Such earthworms have a direct impact on soil nutrient status and microbial population (GAPs i.e., gut associated processes) or an indirect impact (CAPs i.e. coprolites associated processes)4,5. They have the capacity to transform as well as restructure nitrogen (N) pools6,7 and geo-engineering by earthworms may have considerable effects on arctic soils1. Earthworms may influence the entire soil food web6,9 with significant influence on soil structure through horizontal and vertical drillosphere burrows and castings10 escalating the distribution and community composition of the temperate soil micro flora[11][12].

In temperate ecosystems earthworm cast production can range from 36 to 250 tons ha−1 year−114. Earthworms represent biocatalysts of class oligochaeta that stimulate N mineralization15–17 and can bring about augmented nitrification18,19 or denitrification owing to their gut microbial community20,21. Earthworms stimulate microbial processes for recovery of N from organic compounds22. Moreover, microorganisms thrive in earthworm galleries because of transit through the worm gut and presence of lavish mucus and casts23,24. Biodegradable or perishable organic wastes could be converted into stable humus-like substances by way of vermicomposting25 as an end product which is a good organic fertilizer containing abundant humic acids and beneficial microbes27. Vermicomposts have been observed to encompass elevated levels of NH4+, NO3−, Mg, P and K relative to surrounding soil12,26,27. The nitrogen transformation is an important component of waste conversion during composting and vermicomposting processes20,21 and processes like nitrification and denitrification are the key activities contributing to nitrogen cycle, playing an important role in nutrient supply, (NH4+−N), immobilization and greenhouse gas (GHG) emissions for various ecosystems20,21. Most of the reported heterotrophic nitrifying-aerobic bacteria are mesophilic bacteria, adapted to temperatures ranging from 15 to 37 °C34,35. Temperate microbes have obvious physiological advantages36 and unique ability to perform nitrification and denitrification synchronously37 and improve the overall nitrogen removing efficacy at lower temperatures. Guraz valley, a fragile cold ecosystem, located in high Himalayas with prominent features of cold habitats. Moreover, the positive priming through the endogeic geophagous earthworm’s influence is expected to the Guraz valley, with the goal as (i) determine the involvement of coprolite associated microorganisms in the activity of urease, nitrifier communities, and nitrification rate in the soil. Transition in NH4+ source of nitrogen pollution in different forms viz; NH3 and N2O to air and N-NO3− to ground and surface water resources39,40. Changes in transformation/emission rates are affected by the soil temperature, because it affects the activity of urease, nitrifier communities, and nitrification rate in the soil. Transition in NH4+−N, NO3−N and total nitrogen in the interim of vermicomposting had been reported, however, divergence and succession of N-transformation process through involving functional psychrophillic microbes and earthworms were seldom reported. Therefore, the information on cold tolerant earthworms and associated nitrifying and denitrifying microbial species can furnish novel insights into the N transformation during the vermicomposting process in cold habitats. Moreover, the positive priming through the endogeic geophagous earthworms influence is expected to foster the recycling of nutrients, especially organic carbon, nitrogen and phosphorus41,42. The present study, which is perhaps the first of its kind under cold habitat of Guraz valley in Kashmir region, will pledge cognition and contribute to sound understanding of mineral N dynamics using psychrophillic *A. caligenosa* indigenous to the Guraz valley, with the goal as (i) determine the involvement of coprolite associated microorganisms in biocconversion of N, (ii) to enumerate the changes in N-NH4+, N-NO3− and (iii) specifically, recycle and evaluate the impact of locally available garden soil and Himalayan goat manure on physico-chemical flux’s in coprolites resulting in minimizing nitrogen pollution in temperate ecosystems.

**Results and discussion**

**Ammonium and nitrate dynamics.** The analysis performed on coprolites of *Aporrectodea caliginosa* evidenced that ammonium (NH4+−N) concentration after one week of incubation were significantly affected by the source of food (Fig. 1). As evident from Fig. 1 the concentration of NH4+−N in all the earthworm released coprolites increased steadily up to the 4th week of the vermicomposting process, however, coprolites of goat manure (GM) shows continuous increase in NH4+−N content up to the 5th week. The NH4+−N concentration in GM coprolites was significantly (p < 0.05) higher than GM(control) concentration up to 5th week of incubation. The NH4+−N concentration in GM coprolites was significantly (p < 0.05) higher than GM(control) concentration up to 5th week of incubation, after which the NH4+−N concentration began to fall steadily until the 6th week of incubation. Further, all treatments showed decline in the NH4+−N concentration after 4th week continued up to the 6th week of incubation. It was observed that after 6 weeks of vermicomposting the NH4+−N concentration in the coprolites was in the order; GM(control) (24 µg NH4+−N g−1 DW) > GS(Control) (35 µg NH4+−N g−1 DW) > GS (38 µgNH4+−N g−1 DW) > GM (80 µg NH4+−N g−1 DW). Our results reflect that the gross transformation rate of N-NH4+ responded differently based on substrate type and feeding preference of *A. caligenosa*. GM aids gut associated microbes and was found to accelerate the mineralization of N to NH4+ the elevated NH4+−N levels in coprolites might be due to various nitrogen-containing compounds released by earthworm metabolism through mucu proteins and urine. Previous studies also suggest that adult earthworms enhance ammonification43,44 by breakdown of large volumes of organic residues with the aid of gut associated bacteria thereby increases biogenic NH4+31,45. The steady increase of NH4+−N concentration in GM and GS coprolites is related to the initial carbon and nitrogen content of the substrate. However, subsequent decrease with incubation time was reflected by the conversion of part of NH4+−N
to NO$_3^-$-N, influenced by the dominance of nitrifying chemolithotrops at low temperatures (16.5–2 °C) (Fig. 1). Earlier research also suggests that ammonification is a temperature sensitive microbial mediated mineralization process$^{46-48}$ and an increase in temperature up to 25 °C will significantly increase the transformation rate of N$^{49}$. The trend for nitrate (NO$_3^-$ N) concentration varied between 8.37 and 81.88 µg NO$_3^-$ N g$^{-1}$DW in coprolites influenced by different treatments. The NO$_3^-$ N concentration of coprolites from earthworms fed on GM and GS was found higher. NO$_3^-$-N concentration showed a linear increase from the 1st to 6th week of vermicomposting from coprolites of both GM and GS with the highest values recorded as 90.2 µg NO$_3^-$ N g$^{-1}$ DW and 81.81 µg NO$_3^-$ N g$^{-1}$ DW respectively. The usefulness of earthworm’s gut microbes inflates the process of nitrification compared to the situation without earthworms$^{43,50}$. The NO$_3^-$ N removal from the GM was exceptionally high and ranged between 22.03 to 90.20 µg-NO$_3^-$ g$^{-1}$DW with an average value of 64.24 µg-NO$_3^-$ g$^{-1}$ DW during the entire incubation period (Fig. 2). Similarly, for GS NO$_3^-$ N recovery ranges from 8.37 to 81.81 µgNO$_3^-$ g$^{-1}$ DW. Further, our findings reveal a linear increase in NO$_3^-$-N concentration from GM coprolites along the dura-
tion of incubation, which is assumed to be correlated with a higher concentration of NH$_4$$^+$-N in coprolites, which is a primary source for the nitrification process. Our results also suggest that continuous escalation in NO$_3^-N$ could be due to the involvement of heterotrophic bacteria and fungi in coprolites (Table 1) preferring a moderate temperature between 15 and 25 °C which also shows that heterotrophic nitrification process exceeds autotrophic nitrification. Previous research findings suggest that earthworm gut and castings tend to have more active heterotrophic microorganisms that positively influence the amounts of extractable NO$_3$-N in upper layer of soil$^{31-33}$ moreover, increased NO$_3$-N concentration in coprolites is a good indicator of the nitrification process and abundance of nitrifiers$^{23,25,54-56}$. It is also reported that the heterotrophic nitrification has an optimum temperature requirement of 15 °C$^{57}$ while, it exceeds over autotrophic nitrification if the temperature is increased to 35 °C$^{49}$. Further, we confirm that the heterotrophic nitrification is an ecosystem dependent process which is directly dependent on substrate type, microbial diversity and abundance. The samples of study material yielded a large number of indigenous heterotrophic nitrifiers, which were determined to have high nitrogen removal abilities. Temperature stimuli, NH$_4$$^+$-N and NO$_3$-N content in substrates influenced the overall performance of heterotrophic nitrification process. Our previous research in the cold arid Ladakh and Kashmir valley backs up this argument. The influence of low temperature on heterotrophic nitrification at the experimental site (Guraz valley) is consistent with the previous findings, which demonstrated that an optimal temperature, substrate type and ecology conditions favor heterotrophic nitrification process$^{15,58,59}$. Low concentration of NO$_3$-N is related to low microbial dominance (nitrifying bacteria) in coprolites from control treatments of both the substrates suggesting the loss of potency in coprolites which inhibited the nitrification process. Lower functional redundancy in the nitrification process is due to the lack of earthworms in the control treatments which could not either enhance biogeochemical stability of organic matter or stimulate microbial activity (Table 1). Thus, the results clearly elucidated an amicable relationship between earthworm and heterotrophs which favors the heterotrophic nitrification. Previous studies support the increased mineralization of nutrients in earthworm coprolites relative to surrounding garden soil (without earthworm), which is associated with enrichment in liable compounds due to various factors such as, increased activity of nitrifying microbes$^{40}$, digestion of earthworm could influence gut microbiome$^{41}$ and earthworm-microbe association produces enzymes that are reported to increase NO$_3$-N and NH$_4$$^+$-N content in coprolites by 31 and 14% respectively$^{42-45}$.

### Denitrification/ N$_2$O emission during incubation.

The interaction between earthworms and denitrifiers directly affect the nitrogen dynamics via nitrous oxide (N$_2$O) fluxes. Our study reveals that denitrification predominantly turned out to be limited by low temperature and C supply. N$_2$O emission rates from mesocosm surface coprolites (soil fed earthworms) ranged from 5.90 ± 0.20 ng-N g$^{-1}$ (1st week) to 16.6 ± 0.48 ng-N g$^{-1}$ (6th week) with an average of 16.95 ± 0.22 ng-N g$^{-1}$ (Fig. 3). However, the emission rates of N$_2$O from the control treatments, on the hand were relatively low ranging from 4.05 ± 0.29 ng-N g$^{-1}$ (1st week) to 14.60 ± 0.22 ng-N g$^{-1}$ (6th week) with an average of 14.0 ng-N g$^{-1}$ per week. N$_2$O emissions showed a steady increase along with the incubation period except from the 6th week onwards, there was a downward trend, with an average increase of 17.03% in emission from coprolites of GS compared to GS (control). Earlier research has shown that A. caliginous is capable to emit significant amounts of N$_2$O emissions from the soil through different activities$^{46-48}$. Earthworms have the potential to dramatically regulate the physico-chemical properties of their habitats and therupon affecting the production of GHG$^{49}$ however, denitrification is affected by a variety of environmental factors including availability of dissolved oxygen (DO), carbon (C), pH, temperature, denitrifying bacterial population and congregations of NO$_3$-, NO$_2$- and S$^{2-}$.$^{31-33,70}$.

| Microbial isolates | Garden soil (control) | Goat Manure (control) | Goat Manure (inoculated with earthworms) | Garden soil (inoculated with earthworms) |
|--------------------|-----------------------|-----------------------|------------------------------------------|------------------------------------------|
| **Bacteria**       |                       |                       |                                          |                                          |
| Nitrosomonas sp    | +                     | –                     | +                                       |                                          |
| Bacillus sp        | +                     | +                     | –                                        | +                                        |
| Citrobacter sp     | –                     | –                     | +                                        |                                          |
| Nitrosospira sp    | +                     | –                     | +                                        |                                          |
| Klebsiella sp      | +                     | –                     | –                                        |                                          |
| Pseudomonas sp     | +                     | –                     | +                                        |                                          |
| Proteus sp         | –                     | +                     | +                                        |                                          |
| Serratia sp        | –                     | –                     | +                                        |                                          |
| Staphylococcus sp  | –                     | –                     | +                                        |                                          |
| **Fungi**          |                       |                       |                                          |                                          |
| Aspergillus sp     | +                     | –                     | –                                        | –                                        |
| Fusarium sp        | +                     | –                     | –                                        | +                                        |
| Penicillium sp     | –                     | –                     | +                                        |                                          |
| Saccharomyces sp   | –                     | +                     | +                                        |                                          |

Table 1. Distributions of microbial isolates in the different cast-types.
Figure 3. Temporal variation in denitrification rates (ng-N g\(^{-1}\) per week) from coprolites of *A. caligonosa* by food source (garden soil).

Figure 4. Temporal variation in denitrification rates (ng-N g\(^{-1}\) per week) in casts from goat manure (GM) with and without *A. caliginosa*. 
Under mesocosm condition, incubation of earthworm with GM stimulates the denitrification process, which showed a favorable relationship with \( N_2O \) emission from manure coprolites ranged from 7.26 ± 1.32 ng-N g\(^{-1}\) (1st week) to 20.19 ± 1.03 ng-N g\(^{-1}\) (6th week) (Fig. 4). On an average \( N_2O \) emission from coprolites of GM was 78.65% higher compared to GM(control), suggesting that \textit{A. caliginosa} is playing a critical role in conversion of \( NO_3^- \) to \( N_2O \). It might be attributed to the higher initial availability of carbon in goat manure (Fig. 5), which constituted 70% of the total mass in this substrate, led to faster degradation and enhanced mineralization of N to \( NO_3^- \). Limited reports are available about the mechanism involved in the combined relationship between the nitrifying and denitrifying microbes that help towards better understanding of the N-cycle in terrestrial ecosystem\(^{71,72}\). Bioconversion of cow dung, duck manure, kitchen waste by earthworms is reported to induce \( N_2O \) emissions\(^{54,73}\); however, emission rates are significantly lower than thermophiles composting\(^{74,75}\). Vermicomposting under high moisture conditions were reported to decrease \( N_2O \) and \( CH_4 \) emissions by 25–36 and 22–26% compared to thermal composting\(^{74}\). Increased \( N_2O \) emission in GM coprolite must be owing to the activities of denitrifying bacteria, as evidenced by significant \((p < 0.05)\) difference in \( N_2O \) emission between samples from GM and GM(control) treatments. The research also found a link between nitrification and denitrification (Figs. 4, 8), and demonstrate how a heterogeneous microbial population and function may coexist. Previous research also found that potential denitrification rates were positively correlated with coexistence of aerobic and anaerobic microbes (denitrifiers)\(^{76–80}\). In our study, low \( N_2O \) accumulation was found in headspace of bottles with no \( C_2H_2 \) or with low concentration for both control treatments of GS(control) and GM(control). However, significant \((p < 0.05)\) difference in \( N_2O \) emission was evidenced between the two control treatments GS(control) and GM(control). On an average, \( N_2O \) produced from the coprolites fed on GM were 19.57% higher compared to castings obtained from GS.

**Physico-chemical analysis of worm casts.** Coprolites produced from GS and GM were rich in nutrients and \textit{A. caliginosa} had a substantial impact on the analyzed parameters. Significantly \((p < 0.05)\) higher pH was observed in coprolites from GS and GM treatments relative to the GS(control) and GM(control) (Fig. 5, 6). The final pH values of both the coprolites from GS and GM were slightly acidic to alkaline attributed to gut microbial activity, indicating that these coprolites could be useful to remediate soil reaction. Vermicomposting of fruit and vegetable wastes\(^{81,82}\); seaweeds, sugarcane trash, coir pith amended with cow dung\(^{83}\); and flowering plants (\textit{Lantana camara})\(^{84}\) was also reported to produce vermicompost with a pH close to neutral. Analyses of variance for total organic carbon indicated significant \((p < 0.05)\) differences between the coprolites from GS (18.24%) and GM (27.18%) with their controls GS(control) (29.42%) and GM(control) (32.02%) respectively. A linear decline in total organic carbon from GS and GM coprolites is attributable to utilization of carbon by microbes during the entire process (Fig. 7). Previous reports have also mentioned the loss of 19–67% of carbon during the process of vermicomposting\(^{85,86}\), where dehydrogenase activity plays a key role in the hydrolysis of cellobiose and other

![Figure 5. Physico-chemical characteristics of manure (initial) and coprolites of Goat manure (GM) with and without \textit{A. caliginosa} (mean ± SD). Corresponding means followed with same letters are not significantly different at \(P < 0.05\).](https://www.nature.com/scientificreports/)
disaccharides during vermicomposting process\textsuperscript{82}. It has been also reported that the chief mechanism for the carbon loss from the substrates could be attributed to the respiration of earthworms and microorganisms during the decomposition and transformation of substrates\textsuperscript{87,88}. The Fig. 7 depicts a significant ($p < 0.05$) increase in NO$_3^-$ by 15 and 39\% in coprolites of GS and GM respectively, when compared to GS\textsubscript{(control)} and GM\textsubscript{(control)}. Increased NO$_3^-$ content in coprolite is attributed to the significant influence of gut associated nitrifying microbes in the production of NH$_4^+$ which is a primary substrate for NO$_3^-$ yield, in addition, earthworm mucus and nitrogen-rich excretory secretions also contributed to NO$_3^-$ content. The NH$_4^+$ concentration is also correlated with the initial N content of waste substrates, which was 1.85 ± 0.04 and 2.14 ± 0.05\% in GS\textsubscript{(control)} and GM\textsubscript{(control)}, respectively (Fig. 7). Previous research have noted that earthworms may ameliorate the castings as a consequence of N transformation from wastes by associated microbes through bio-waste mineralization and gut N-fixation\textsuperscript{21,89–91}. Total N concentration in the coprolites significantly ($p < 0.05$) increased and could be interpreted due to the factors such as: initial N content of the substrate; bioconversion efficiency; possible death of a few baby worms; secretions of mucus, addition of nitrogenous substances during the entire process. Gut and skin of earthworm can secrete nitrogenous compounds which is also one of the reasons for enriched N content in the end product of the process\textsuperscript{86,92}. At the end of vermicomposting process, the total phosphorus (TP) recovery was found 6.15\% in GM and 8.19\% GS from the respective substrates (Fig. 7). Increased P concentration in the GM castings could be due to secretion of various organic acids by related microbes and decomposition of substrates by earthworm, as corroborated with previous studies\textsuperscript{93,94}. Table 1 shows that the bacterial population in both coprolites from GS and GM increased that includes Aerobacter sp., Bacillus sp., Citrobacter sp., Escherichia sp., Klebsiella sp., Pseudomonas sp., Proteus sp., Serratia sp. and Staphylococcus sp. The coprolites of GS and GM demonstrated significantly ($p < 0.05$) higher bacterial density than GM\textsubscript{(control)} and GS\textsubscript{(control)}. Similarly, higher fungal density was also observed in earthworm inoculated coprolites of GS and GM. The fungal species included Aspergillus sp., Fusarium sp., Penicillium sp. and Saccharomyces sp. The presence of more bacteria and fungi in earthworm amended coprolites indicate that earthworms could favor and complement the microbial communities during conversion of substrates. This is in accordance with earlier results of using mill waste\textsuperscript{95,96}, forest litter waste\textsuperscript{97}, sewage sludge and rice straw\textsuperscript{31} as substrates in vermicomposting which favors the microbial population. It has also been reported that presence of the earthworms in vermicomposting enhances the beneficial microflora and suppresses harmful pathogenic microbes\textsuperscript{98}, that later enhance plant growth via the production of plant growth promoting compounds. Thus, the higher population of microbes in the earthworm castings could be due to the abundance of beneficial microbes in the earthworm gut. This concept was also supported by the findings of previous research\textsuperscript{99,100}. The potential availability of nutrients in coprolites
was revealed by regression analysis between the indices of physico-chemical properties of GM (Table 2). The Fig. 8 shows that the selected parameters were appropriate to determine the stabilization of coprolites in the current investigation. With a cumulative variance 93.10% and high rate of loading from first and second principal component, parameters MC, TN, TP, K, and Zn are strongly correlated with Fe, CN ratio, TOC in the first main component which reflects mineralization and breakdown potential of GM (goat manure). Nitrates and pH are negatively correlated with the second component. It can be inferred that *A. caligenosa* may have good impact on stabilization of coprolites with positive correlation to initial nutrient status of GM. It implies that coprolites produced from GM are superior to GS as a soil fertilizer. The loading of the variables on the components are presented in Table 3. Which indicates score data of extracted eigenvectors with a corresponding loading of 56.90 and 36.20% for principal component-1 and principal component-2 these loadings in have been computed from the correlation coefficients between these variables.

| Parameter      | Equation              | $R^2$ |
|-----------------|-----------------------|-------|
| pH              | $Y = -0.0081X^2 - 0.6860X + 2.56$ | 0.64  |
| Moisture content| $Y = -0.0487X^2 - 3.4389X + 34.11$  | 0.43  |
| Total organic carbon | $Y = 0.0457X^2 - 1.9482X + 42.28$ | 0.85  |
| Total phosphate | $Y = -5.6127X^2 - 6.9485X + 1.44$  | 0.62  |
| Nitrate         | $Y = -0.6396X^2 - 0.3569X + 0.122$ | 0.91  |
| Total nitrogen  | $Y = 3.778X^2 - 14.975X + 16.90$  | 0.89  |

Table 2. Regression between indices of physico-chemical characteristics of goat manure (GM).
Nitrification potential. Replicated sampling analysis at intervals revealed an increase in potential nitrification rates, demonstrating a continuous nitrification process in coprolites in all the treatments except control (Fig. 9). The nitrification sampling in triplicates was done at three different stages of vermicomposting; nitrification-I (2nd week), nitrification-II (4th week) and nitrification-III (6th week) to estimate \( \text{NO}_3^- \) levels. Nitrification rates increased over ammonification, as performed by the involvement of both nitrifying bacteria and fungi (Table 1). The mean potential nitrification rates of GS and GM coprolites at three stages of the nitrification process were 4.63, 43.46, 51.21 \( \mu \text{N g}^{-1} \text{DW} \) and 6.48, 130.43 135.71 \( \mu \text{N g}^{-1} \text{DW} \) respectively that were significantly \( (p<0.05) \) different from the potential nitrification rates of the respective GS(control) & GM(control) (Fig. 9). Abundances of nitrifying bacteria and nitrifying fungi increased nitrification during the entire incubation period, apart from that nitrification potential by earthworm, it also depends on the initial N content of organic substances used by earthworms as a source of food. The study evidenced that unlike other microbial processes, nitrification progressed with a steady increase in \( \text{NO}_3^- \) concentration at all the three stages of nitrification process. In contrast, the net rate of nitrification was comparatively low in the GS(control) & GM(control). Previous studies indicate that earthworm castings have strong associated activity for nitrification\textsuperscript{101–103}. Our findings,

![Figure 8. Principal component analysis of physico-chemical parameters in GS and GM coprolites compared to controls. The uninoculated treatments with *A. caliginosa* are referred to as the control.](image-url)

| Parameters | Coefficients of PC-1 | Coefficients of PC-2 |
|------------|-----------------------|-----------------------|
| pH         | −0.39427              | 0.02769               |
| % MC       | 0.34199                | −0.01981              |
| TOC%       | 0.05875                | −0.52019              |
| TP %       | 0.41406                | 0.07825               |
| % Nitrate  | −0.16718              | 0.47157               |
| % TN       | 0.40588                | 0.07231               |
| K %        | 0.40687                | 0.07334               |
| Fe %       | 0.1531                | 0.47115               |
| Zn %       | 0.41179                | −0.04123              |
| CN ratio   | 0.03815                | −0.51521              |

Table 3. Score data of extracted eigenvectors of principal component-1 (56.90% loading) and principal component-2 (36.20% loading).
support the hypothesis that earthworm activities with consumption of N-rich food material could increase the nitrification process in coprolites which is also supported by earlier research\textsuperscript{44,91,104}. 

**Figure 9.** Effect of different substrates (garden soil and goat manure) on nitrification potential (three successive nitrification stages are designated as nitrification-I, nitrification-II, and nitrification-III) of coprolites at variable dates of incubation with and without \textit{A. caliginosa}.

**Figure 10.** Study area map of Guraz valley in Kashmir region-India (Microsoft power point 2013; https://world mapblank.org/wordpress/wp-content/uploads/2021/08/Location-of-India-on-the-World-map.pdf).
Materials and methods

Site description and cold tolerant earthworms. Geophagous endogeic earthworms *Aporrectodea caliginosa* (*A. caliginosa*) were obtained from the Mountain Agricultural Research and Extension Station—Izmaq, Guraz valley (74.73° E, 34.64° N; altitude 2580 masl) of District Bandipora, Jammu and Kashmir—India (Fig. 10). The annual mean temperature and precipitation at experimental site are 15.6 °C and 2200 mm, respectively.

Experiment. The present study used *A. caliginosa*, which is found in the garden and coniferous forest soils of Guraz valley103. Garden soil (GS) from a field planted with maize + beans and goat manure (GM) from Bakewal (Himalayan shepherds with rearing Himalayan goats) were used as substrate treatments in triplicates with corresponding control as designated in Table 4. Earthworms were placed in 12-mesocosms (polyvinylchloride-PVC) with a diameter of 550 cm² filled with substrates separately (Fig. 11). The activity of nitrogen dynamics from the earthworm coprolites was sampled three times in a week. Mesocosms were filled with the same feeding material and inoculated with ten (10) non-clitellated young worms for 42–45 days. Surface coprolites samples were shifted to petri dishes with moistened filter paper for further analysis. The mesocosms were placed under ambient light, with an average air temperature and relative humidity of 16.8 ± 1.5 °C and 67 ± 4% respectively, to ensure similar microclimatic conditions during the course of the experiment.

Measurements. Surface coprolites of *A. caliginosa* were collected three times a week from each mesocosm, oven dried at 40 °C for 36 h and weighed for further examination. In the first week, a few dead earthworms were found on substrate surface and promptly replaced with new living specimens. Coprolites were analyzed for ammonium (NH₄⁺) and nitrate (NO₃⁻) concentration by extracting with 2 M KCl with KCl: coprolites ratio of 4:1. The mixture obtained was mechanically shaken for 3 h at 24 °C followed by filtration. NH₄⁺ was determined by the indophenol blue method (IBM) (Keeney and Nelson 1982) using an automated unit (Sysmex BX4000) while NO₃⁻ by the cadmium reduction method (CRM)106. Triplicate surface coprolites (3 cm diameter) from each mesocosm were collected and evaluated for NH₄⁺ and NO₃⁻ at each sampling time, and the results were reported on dry weight basis. At three distinct stages of experiment, the nitrification potential of coprolites from both types of substrates was determined using the method107 as: Coprolites samples (2–4 g fresh weight) from both substrates and control were put into flasks with 25 ml (NH₄⁺ & SO₄²⁻) solution. Foam plugged flasks were shaken mechanically at 25 °C for 2 h. A mixture sample of 10 ml was taken for N₂O analysis. Flasks were then exposed to aerobic conditions for two days followed by filtration and the amount of N₂O was measured. The difference between the initial and final readings of N₂O was recorded as the nitrification potential. Nitrification rates were expressed on a dry weight basis. During the experiment, denitrification rates were determined three time (2nd, 4th and 6th weeks) using surface coprolites taken from each mesocosm. All castings were removed from mesocosm surface two days before coprolite sampling, so all coprolite samples evaluated for denitrification in the experiment were ≤ 2 days old. The acetylene block method108 was used to determine denitrification rate as: Coprolite sample (2 g fresh weight) of *A. caliginosa* from each mesocosm surface were placed in 10 ml test tubes fixed with headspace, and 1.0 ml of hydrogen and carbon was added to the headspace of each test tube to inhibit the reduction of N₂O to N₂. Coprolite samples were incubated for 24 h at 20 ± 2 °C. The acetylene block method was used to determine the initial and final readings of N₂O. Modified automated method109, was used to determine the diffusional N₂O.

Coprolite microbiome. For differential analysis of the micro flora, coprolites samples were sub-sampled. In addition, earthworms were put under starvation for two days in petri dishes with 1% sterile agar. This much time was sufficient to get the transit microbes come over the agar. Coprolite samples were dissolved into 10 ml of sterile 0.85% NaCl and stirred vigorously for 20 min using the method110 described as: Suspension was diluted with headspace, and 1.0 ml of hydrogen and carbon was added to the headspace of each test tube to inhibit the reduction of N₂O to N₂. Coprolite samples were dissolved into 10 ml of sterile 0.85% NaCl and stirred vigorously for 20 min using the method110 described as: Suspension was diluted

![Table 4. Treatment combinations of garden soil and goat manure.](https://doi.org/10.1038/s41598-022-12972-1)
Sodium were quantified by the procedure of John (1970) through flame photometer-128 (Systronics) after digesting samples in a diacid suspension (HClO₄: HNO₃ in the ratio of 4:1). pH and electric conductivity (EC) were determined in double-distilled water blend each with a concentration ratio of 1:10 (w/v) plying digital meter (COM-100) and Eqip-tronics (EQ-614A) respectively. Nitrogen (N) was determined by the Micro-Kjeldhal method as described by Bremner and Mulvancy (1982) using digestion extract (H₂SO₄ + K₂SO₄: CuSO₄: SeO₂ in the ratio of 10:4:1). Phosphorus (P) content was determined by nitro-vanadomolybdate method, potassium (K) by using photometry and micronutrients (Zn and Fe) by atomic-absorption spectrometry (AAS) after digestion of both coprolite samples from GS and GM with HNO₃:HClO₄ by the method113. Diacid mixture digested samples were analyzed for transition metal elements using an atomic absorption spectrophotometer (Electronic Corporation of India).

Statistical analysis. Analysis of variance (ANOVA) was used to compare the results between the control and treatment groups followed by post hoc analysis. A significance level of $P < 0.05$ was used to determine significance in the treatment means using R-software. Regression analysis was carried out using an equation ($y = b_2x^2 - b_1x + a$) and to workout maximum responses in different study parameters were determined from the formula ($x = -b_1/2b_2$). In the experiment, the mean differentiation of chemical parameters of nitrogen dynamics was done using student’s t-test. Parameters such as pH, moisture content (MC), total organic carbon (TOC), total phosphorus (TP), nitrates, total nitrogen (TN), potassium (K), iron (Fe), zinc (Zn), and the carbon and nitrogen ratio (CN ratio) were utilized to determine correlation matrices affecting coprolites’ quality and stability. The findings were plotted and tabulated using principal component analysis.

Conclusion

The study confirms that GM coprolites contains more nitrogen (NH₄⁺-N and NO₃⁻-N) than the GS, which is readily logical when the selective feeding habits of earthworms are considered. At low temperature, simultaneous heterotrophic nitrification and aerobic denitrification (SHNAD) was found to be stable processes and main N transformation mechanism in both substrates. A. caliginosa promotes microorganism growth, which would otherwise be severely limited due to harsh winter and low ambient temperature. The study highlights the importance of the SHNAD as a pilot scale process showing positive interaction with A. caliginosa contributing in physico-chemical parameters of its coprolites and thus has substantial potential for N removal from wastes at low temperatures. Interaction between psychrophillic earthworms and microbial genera need to be further

Figure 11. Graphical abstract: Microcosm design depicting set up for recovery of biogenic nitrogen with simultaneous heterotrophic nitrification and aerobic denitrification (SHNAD) and determination of nitrification potential of garden soil and Himalayan goat manure employing Aporrectodea caliginosa.
investigated to provide insight evidences of co-occurrence pattern of both, which could help to minimize NH$_3$ emission by effectively reducing N$_2$O.

**Data availability**

The data sets generated are available as supplementary file.

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The authors declare no competing interests.

**Additional information**
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