Enhanced Ammonia Content in Compost Leachate Processed by Black Soldier Fly Larvae

Terrence R. Green · Radu Popa

Abstract Black soldier fly (BSF) larvae (*Hermetia illucens*), feeding on leachate from decaying vegetable and food scrap waste, increase ammonia (NH_4^+) concentration five- to sixfold relative to leachate unprocessed by larvae. NH_4^+ in larva-processed leachate reached levels as high as ~100 mM. Most of this NH_4^+ appears to have come from organic nitrogen within the frass produced by the larvae as they fed on leachate. In nitrate-enriched solutions, BSF larvae also facilitate dissimilatory nitrate reduction to ammonia. The markedly higher concentration of NH_4^+ recovered in leachates processed with BSF larvae and concomitant diversion of nutrients into insect biomass (itself a valuable feedstock) indicate that the use of BSF larvae in processing leachate of decaying organic waste could be advantageous in offsetting capital and environmental costs incurred in composting.

Keywords Compost leachate · Black soldier fly larvae · *Hermetia illucens* · Treatment · Ammonium

Introduction

Nitrogen released during microbial decay of organic matter is poorly retained in the solid phase of compost. Instead, it is released as solutes in the leachate fraction [mostly as ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and amines] and as gases (N_2, NO, N_2O, and NH_3) [8, 16, 22]. The fate of nitrogen is difficult to predict because it depends on numerous factors such as the composition of source materials, incubation conditions, microflora present, gas composition, and the evolving chemistry of the leachate. The generation of certain organic amines that are odoriferous and toxic is also undesirable. For

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these reasons, it is desirable to develop biotechnologies that maximize the conversion of organic nitrogen into NH_4^+ or NO_3^-. Both are easy to recover and can be taken up more readily by plants when introduced back to soil.

Frass, the solid waste released by phytophagous insects, introduces nutrients, including NH_4^+, to soils [14, 18, 28, 30]. We recently reported that larvae of the black soldier fly (BSF) (*Hermetia illucens*) assimilate nutrients from the leachate fraction of decaying vegetal matter, clearing it of volatile organic acids and ninhydrin-positive metabolites and reducing its chemical oxygen demand relative to that unexposed to larvae [21]. BSF larvae help process vegetal, food scrap, and manure waste and are themselves valuable animal feedstock and a lipid source for biodiesel [3, 9, 12, 13, 17, 19, 20, 24, 27]. Because BSF feeding on decaying vegetal and food scrap waste and leachate can be viewed as a specialized form of herbivory, we explored their role in converting other forms of nitrogen to NH_4^+. We discuss our results and their consequences in the context of beneficial effects BSF larvae confer in processing vegetal and food scrap waste and nitrogen management.

Materials and Methods

Standards for NH_4^+, NO_2^-, and NO_3^- were prepared in deionized H_2O using, respectively, analytical reagent grade NH_4Cl, $NaNO_2$, and $NaNO_3$ (Sigma, St. Louis, MO). All other reagents were prepared from reagent grade or better chemicals.

Leachate was prepared from vegetal feedstock made up of a mixture of grass clippings, leaves, and discarded food scraps (primarily bread, vegetables, and fruit leftovers from cafeterias and restaurants) fermented at room temperature [11]. BSF larvae (multiple generations) were raised on the same feedstock in an insect nursery maintained between 30 and 35 °C lit on a 12-h day–night cycle with natural light [26].

Approximately 200 white larvae (ranging in size between 100 and 250 mg WW per larva) were harvested by suspending and washing them in approximately 3–4 l of tap water to rid them of vegetal debris, then suspended in three separate ~200 ml wash solutions of 10 mM $NaNO_3$ made up in tap water and leachate, respectively. The larvae were transferred into duplicate sets of Hungate culture tubes (20 per tube) along with 10 ml of the same solutions they were suspended in. Controls were made up similarly using the same solutions recovered from the larvae washing steps. The tubes were closed at the top with plastic caps which allowed air and gas exchange, tilted at an angle of 20°, and placed in a 30 °C incubator for analysis over the course of 7 days of incubation in the dark.

Aliquots (100 μl) at the beginning of the experiments and thereafter at varying intervals were analyzed. All results for NH_4^+, NO_2^-, and NO_3^- are expressed as averages of triplicates ± 1SD assays of duplicate sets of experiments. The pH is reported as the average ± 1SD of duplicate measurements.

NH_4^+ was measured by a modified microtiter 96-well plate assay based upon the Nessler method [15] with readings at 415 nm on a Model FLx800 BioTek plate reader using BioTek KC4 software. Plates were set up by mixing Rochelle reagent (Na, K-tartrate tetrahydrate, 25 mg ml^{-1} made up fresh in deionized H_2O) with Nessler reagent in a ratio of 3.2:1, respectively, and mixing 20 μl of this latter working reagent with 270 μl samples and NH_4Cl standards prepared fresh (serially diluted to span a calibration concentration range of 62.5 to 1,000 μM). The concentration of unknown samples was then calculated by linear regression analysis relative to standards. H_2O was used as a "0" calibrator.

NO_2^- and NO_3^- were measured similarly on microtiter plates using sulfanilamide color reagent [10] adapted for analysis by dispensing and mixing 30 μl samples and calibration

standards into plate wells prefilled with 90 μl of 16.7 μg ml^{-1} CuSO$_4$·5H$_2$O made up in 333 μM NaOH. For NO$_2^-$, each well received an additional 60 μl of H$_2$O. For NO$_3^-$, each well received 60 μl hydrazine sulfate made up at 0.67 mg ml^{-1} in H$_2$O. The plates were then incubated with lids for 2 h at 30 °C and subsequently read at 520 nm after adding 60 μl color-developing reagent made up at 10 mg ml^{-1} sulfanilamide and 0.8 mg ml^{-1} N-(1-naphthyl)-ethylenediamine dihydrochloride in 3 M H$_3$PO$_4$ to each well. In the case of NO$_3^-$, since NO$_3^-$ converts to NO$_2^-$ with hydrazine reagent and because the color read at 520 nm measures NO$_2^-$, absorbance readings attributable to NO$_3^-$ were calculated as the difference in plate readings between readings obtained with hydrazine less the readings with H$_2$O.

The pH was determined using a Thermo Orion Model 410 pH meter. Whether or not N$_2$ was formed, it was evaluated in 18 mm culture tubes by submerging inverted Durham tubes inside the test solutions and screening for gas formation. Confirmatory follow-up analysis by gas chromatography was unnecessary because gas did not accumulate in the inverted tubes.

Results and Discussion

Larvae excreted frass from vegetal and food scrap waste they had previously ingested into the suspending solutions commencing within seconds of their transfer into particle free solutions. Figure 1a shows the evolution of NO$_3^-$, NO$_2^-$, NH$_4^+$, and pH after suspending BSF larvae in a solution of 10 mM NaNO$_3$ made up in tap water. NO$_3^-$ was depleted on the fifth day. N$_2$ was not generated over the course of these experiments based upon an absence of gas accumulation in inverted culture tubes submerged in the solution in which the larvae were confined. By the end of the seventh day, NO$_3^-$ reappeared in solution, reaching a concentration of ~2 mM (Fig. 1a).

These results show that the larvae, presented with NO$_3^-$, facilitate its reduction (i.e., denitrification). In both BSF and control samples, NO$_3^-$ reappeared and built up between fifth through the seventh day of the study. This is an indication that apart from NO$_3^-$ reduction, nitrification was also occurring. Nitrification is likely attributable to the activity of NH$_4^+$-oxidizing microorganisms released into solution, most likely from frass and using NH$_4^+$ introduced at the onset of the experiment from vegetal matter ingested by the larvae (Fig. 1b). The rise in NO$_2^-$ peaking at ~2 mM between the third and fourth day (Fig. 1a), and
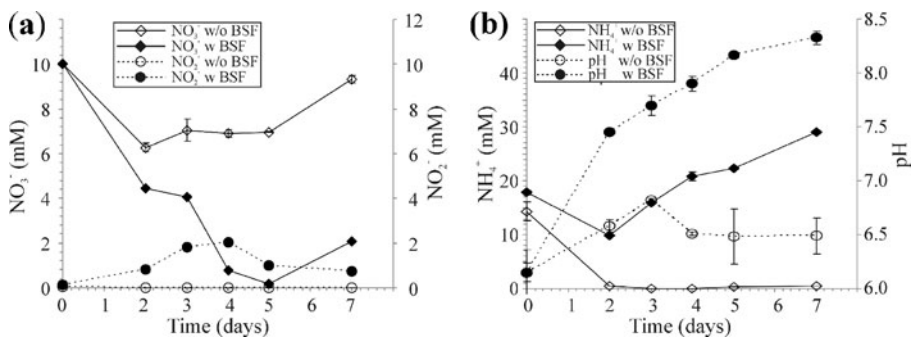

Fig. 1 The evolution of NO$_3^-$, NO$_2^-$, NH$_4^+$, and pH in a solution of 10 mM NaNO$_3$ made up in tap water ±BSF larvae. **a** Evolution of NO$_3^-$ and NO$_2^-$. **b** Evolution of NH$_4^+$ and pH. BSF larvae were first suspended in the nitrate solution, from which control solutions free of BSF larvae were drawn within 1–2 min. This time interval was sufficient for the larvae to release a small amount of NH$_4^+$ in solution along with frass. The experiments were done in duplicates, the measurements were done in triplicates, and the *error bars* represent ±1SD

its decline to ~1 mM by the seventh day, also supports data indicating that the BSF larvae facilitate denitrification.

In the controls, NO_3^- bottomed out at ~6.5 mM around the second day of incubation and returned to its initial starting level of 10 mM by the end of the seventh day, while NO_2^- was not detected (Fig. 1a). The fall in NO_3^-, and its subsequent climb back to its initial level, is likely due to the opposing activity of denitrifying and nitrifying bacteria on NO_3^- and NH_4^+, which are both present (Fig. 1a, b). There is a limited supply of reducing equivalents in the NO_3^- solution used in these experiments due to the short interval the washed larvae were temporarily retained in it. This may account for the impaired denitrification in controls relative to that seen with larvae. In the BSF experiments, additional reducing equivalents were likely provided through frass and excreta.

NH_4^+, though not present in freshly prepared stock NO_3^- solution used for washing and suspending the larvae at the onset of the study, was detected in all of the NO_3^- solutions drawn from the culture tubes (those representing controls and larvae). Its presence and concentration in the tubes is likely the result of a small amount of frass and possibly external carryover brought by the larvae in the NO_3^- solution during the setup of the experiment.

NH_4^+ started out at ~15 mM in controls, fell to zero by the second day, and did not reappear (Fig. 1b). In the larvae experiments, NH_4^+ concentration rose concomitant with the disappearance of NO_3^- (Fig. 1b). The decrease in NO_3^- (~10 mM) and shift in NH_4^+ concentration ($\Delta_{change} = [NH_4^+]_{final} - [NH_4^+]_{initial} = [28.9] - [17.8] \sim \Delta 11.1$ mM) match. This indicates that the decrease in NO_3^- (when larvae were present in the NO_3^- solution) occurred via dissimilatory nitrate reduction to ammonia (DNRA), [6, 7, 29]. DNRA pathways are commonly expressed in decaying vegetal waste, especially when partial fermentation is also occurring [6, 29] as was the case with the feedstock in which the larvae were grown on in our experiments. The pH with larvae added to the NO_3^- solution furthermore rose from a starting value of 6.3 to 8.4 over the 7-day cycle examined. The controls, on the other hand, showed a strikingly smaller pH change of only 0.2 pH units over the same interval (Fig. 1b). Enhanced accumulation of NH_4^+ in the solutions in which larvae were retained accounts for the larger shift in pH relative to the controls.

In experiments with BSF larvae suspended in tap water, a small amount of NO_3^-, starting at ~0.3 mM (originating in carryover), peaked between 0.8 and 0.9 mM by the seventh day (Fig. 2a). NO_2^- rose steadily, reaching ~0.3 mM by the seventh day. NO_3^- and NO_2^- were
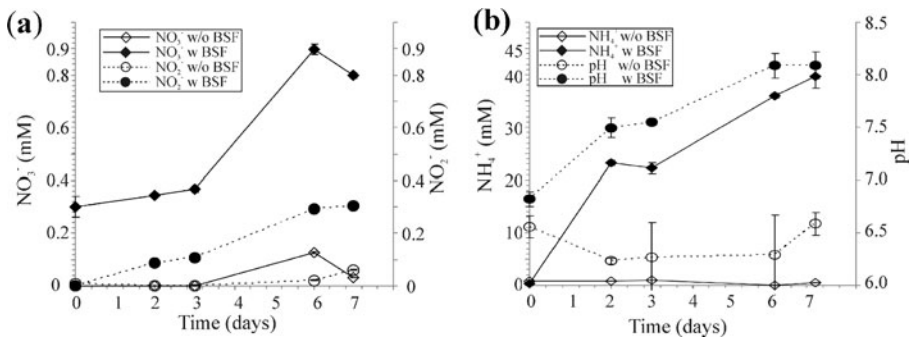

Fig. 2 The evolution of NO_3^-, NO_2^-, NH_4^+, and pH in a solution of tap water ±BSF larvae. **a** Evolution of NO_3^- and NO_2^-. **b** Evolution of NH_4^+ and pH. BSF larvae were first suspended in tap water, from which control solutions free of BSF larvae were drawn within 1–2 min. This time interval was sufficient for the larvae to release a small amount of NH_4^+ in solution (~1 mM) along with frass. The experiments were done in duplicates, the measurements were done in triplicates, and the *error bars* represent ±1SD

not detected in the corresponding controls except for a small peak of NO_3^- (~0.1 mM) on the sixth day which tapered off by the seventh day and a small rise in NO_2^- to ~0.05 mM by the seventh day.

No NH_4^+ was detected in the tap water. A small amount of NH_4^+ (just under ~1 mM) was however detected in controls as carryover from the larval wash (as discussed above in the NO_3^- experiments). Large amounts of NH_4^+ peaking near ~40 mM accumulated however in the BSF experiments (Fig. 2b). The concentration of NH_4^+ by the seventh day of the experiment with larvae suspended in solution was in excess of that of NO_3^- and NO_2^- combined by a ratio >50:1. NH_4^+, formed in the larval suspension, furthermore shifted the pH upwards, peaking at ~pH 8.2 by the seventh day (Fig. 2b) in a manner similar to that seen earlier in the NO_3^- experiments.

In leachate, NO_3^- started out at ~4.5 mM, fell to zero by the fourth day in both control and larval-treated samples, and from the fourth day through to the end of the seventh day reappeared, peaking by the seventh day at ~1 mM (Fig. 3a). NO_2^- was at ~0.1 mM, then rose slightly and held relatively constant in the range of ~0.3–0.4 mM in experiments with larvae, and in the range of 0.1–0.2 mM in controls, and tapered off by the seventh day (Fig. 3a). Except for subtle differences in NO_3^- and NO_2^- concentrations, the overall pattern of reduction/oxidation of nitrate and nitrite was similar between the samples and the controls. Based on these results, the larvae have no significant effect on the nitrite and nitrate transformations in the leachate.

However, with larvae in the leachate, NH_4^+ rose sharply by the seventh day to ~100 mM, a concentration more than 25-fold that of the combined concentrations of NO_3^- and NO_2^- detected in the leachate (Fig. 3b). Concomitant with this change in NH_4^+ concentration, the pH of the leachate shifted from ~4.4 at the start of the experiment to ~8.8 by the seventh day. The controls showed no significant changes in the concentration of NH_4^+ and a change in leachate pH by the seventh day of ~0.8 pH units above that at which it started (Fig. 3b).

On a stoichiometric basis, the magnitude of change in NH_4^+ concentration accumulating in BSF-treated leachates is unaccountable as a product of NO_3^- or NO_2^- reduction via a DNRA. Furthermore, since the experiments were conducted in the presence of O_2, because no N_2 gas was trapped, and whereas N_2 fixation occurs mainly in anaerobic conditions [1, 25, 31], the large amount of NH_4^+ produced under these experimental conditions cannot be linked with N_2 fixation.
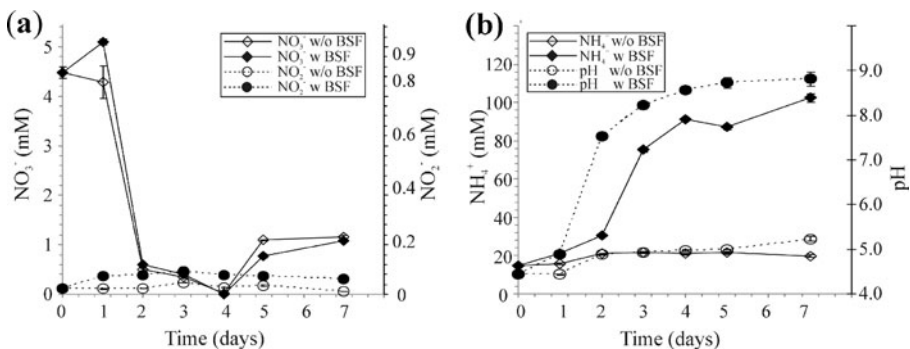

Fig. 3 The evolution of NO_3^-, NO_2^-, NH_4^+, and pH in leachate ±BSF larvae. **a** Evolution of NO_3^- and NO_2^-. **b** Evolution of NH_4^+ and pH. BSF larvae were first suspended in leachate, from which control solutions free of BSF larvae were drawn within 1–2 min. The experiments were done in duplicates, the measurements were done in triplicates, and the *error bars* represent ±1SD

Figure 4 shows a nearly perfect fit between the ΔpH shift in BSF-treated leachate and accumulation of NH_4^+ commencing on the second day of the study, when the accumulation of NH_4^+ began (Fig. 3b). Based on previously reported data [21], the consumption of organic acids in the leachate by the larvae likely accounts for the initial shift in pH from its starting value of 4.4 to ~7.4, after which NH_4^+ accumulation commenced. This accounts for the nonlinear relationship between measured NH_4^+ levels and ΔpH seen at the beginning of the experiment and the good subsequent linear fit thereafter.

Most of the NH_4^+ in the BSF-treated leachate appears to have come from organic nitrogen within the frass produced by the larvae. Most insects store N waste in the form of uric acid which precipitates in the insect's rectum (or in some instances, the proximal portion of the Malpighian tubule) [2, 32]. Several investigators studying Diptera have identified allantoin, the immediate degradation product of uric acid and a water-soluble waste product, in insect's excreta in addition to lesser albeit measureable quantities of NH_4^+ [2, 4, 5, 23]. It is difficult to establish how much of this NH_4^+ was carried over with frass or was produced by the deamination of organics from frass and leachate. Allantoin, carried into the leachate with frass, is likely getting broken down into urea, and by the action of urease, into NH_4^+. Microbes colonizing the gut of the larvae, subsequently delivered into the leachate with frass, in addition to those already present in the leachate, might also be contributing to mineralizaton of organic N.

Conclusions

Our data demonstrate that BSF larvae, while feeding on decaying vegetal and food scrap waste, markedly increase N-mineralization by elevating the concentration of NH_4^+ in the leachate fraction. The larvae furthermore facilitate recovery of NO_3^- present in the leachate fraction via DNRA. These results suggest that BSF larvae-based processing of waste could be used to offset costs incurred with N fertilization of crops. Moreover, while feeding on decaying waste, BSF larvae assimilate N and C, and other valuable nutrients into insect
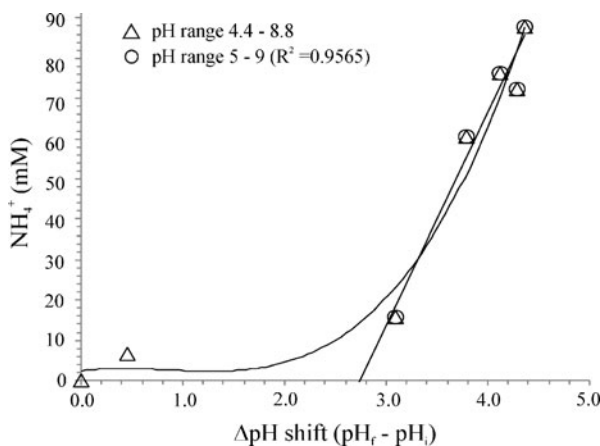

Fig. 4 The relationship between the concentration of ammonium and the change in the pH of the leachate incubated with larvae. The *curved line* is a polynomial fit through all data points in the pH range ~4.4–8.8. The *straight line* represents the linear regression of the data points in the pH range ~7.4–8.8. pH_f final pH; pH_i initial pH

biomass (a valuable animal feedstock, itself), helping in this regard to reduce the amount of C and N that otherwise would have been given up as greenhouse gases to the atmosphere.

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