NITROGEN TRANSFORMATIONS IN FLOODED AGROECOSYSTEMS: A CASE STUDY WITH TARO (*COLOCASSIA ESCULENTA*)

Jonathan L. Deenik[a], **C. Ryan Penton**[b], **Brian L. Popp**[c], **Gregory L. Bruland**[d], **Pia Engstrom**[e], **Jacqueline Mueller**[f], and **James Tiedje**[b]

[a]Department of Tropical Plant and Soil Sciences Univ. of Hawaii at Manoa, Honolulu, HI
[b]Center for Microbial Ecology, Michigan State Univ., Lansing, MI
[c]Department of Geology and Geophysics, Univ. of Hawaii at Manoa, Honolulu, HI [d]Department of Natural Resources and Environmental Management, Univ. of Hawaii at Manoa, Honolulu, HI
[e]Sven Lovén Center for Marine Sciences, 450 34 Fiskebäckskil, Sweden
[f]School of Ocean and Earth Science and Technology, Univ. of Hawaii at Manoa, Honolulu, HI

ABSTRACT

Wetland agriculture covers an estimated 170 million ha and contributes significantly to global food supply. Nitrogen fertilizers are subject to numerous potential transformation pathways in flooded systems. The present research was focused on improving our understanding of N transformations in a flooded agricultural system by addressing the following two broad objectives: 1) determine whether the presence of anammox bacteria and its activity contribute significantly to N losses, and 2) evaluate the contribution of the coupling of nitrification and denitrification in the rhizosphere to gaseous N losses. Quantitative PCR was performed for anammox *16S rRNA* genes. Anammox activity was measured using a slurry-based isotope pairing technique (IPT) and a a whole core $^{15}NH_4^+$ perfusion technique with porewater equilibrators for the extraction of $^{14+15}N\text{-}NO_3^-$, NH_4^+, and N_2 was used to quantify rhizosphere coupling of nitrification and denitrification. Although significant numbers of anammox *16S rRNA* genes were detected (~10^5 copies g^{-1} wet sediment), only negligible anammox activity was measured using the slurry IPT. The whole core $^{15}NH_4^+$ experiment demonstrated that diurnal O_2 transport into the sub-surface stimulated nitrification-denitrification in the extensive root rhizosphere accounting for the majority of NH_4^+ loss.

INTRODUCTION

The presence of aerobic and anaerobic zones and their interface in flooded agricultural systems plays a critical role in controlling microbially mediated transformations of dissolved mineral N species to gaseous N. For example, losses of fertilizer NH_4^+-N applied to the aerobic surface waters proceeds through the following steps: (1) NH_4^+ diffusion into the aerobic soil layer, (2) conversion of the NH_4^+ to NO_2^- and NO_3^- through nitrification (3) diffusion of NO_3^- into the anaerobic layer, and (4) denitrification[1,2]. The presence of aerenchyma and lacunar systems in wetland plants, conduits for O_2 transport through the plant into anoxic sediment layers, creates a second oxic-anoxic interface in the rhizosphere where the coupling of nitrification and denitrification can lead to the loss of fertilizer N[3]. The discovery of anaerobic ammonium oxidation (anammox) activity in a broad range of environments offers yet another potential pathway for fertilizer N loss in flooded agricultural environments[4,5,6,7,8.9]. The anammox metabolic pathway involves the anaerobic autotrophic oxidation of ammonium coupled with

nitrite reduction to N_2 gas. The presence of ammonium and nitrate in the oxic surface layer coupled with nitrate reduction to nitrite in anoxic adjacent layers is a prerequisite condition that provides a consistent supply of nitrite for anammox bacteria inhabiting anoxic sediments[10,11,12].

The present research was focused on improving our understanding of N transformations in a flooded agricultural system by addressing the following two broad objectives: 1) determine whether the presence of anammox bacteria and its activity contribute significantly to N losses, and 2) evaluate the contribution of the coupling of nitrification and denitrification in the rhizosphere to gaseous N losses.

METHODS

Our research was conducted using wetland taro (*Colocasia esculenta*) agroecosystems in the Hawaiian Islands as a model system for flooded agriculture. To address objective 1, soil samples for anammox enumeration were collected from the 0-5 cm depth samples from four taro fields under alternative fertilizer management and a river sediment to represent a natural ecosystem. Anammox bacteria belonging to both freshwater and marine groups were targeted using primers and the methods outlined in Penton et al.[4]. Additionally, to get a broad view, analysis targeting functional genes specific to N cycling was performed and included *nosZ* and *nirS* (denitrification), *amoA* (proteobacterial ammonia oxidation), and the *16S rRNA* gene for the total microbial community. Measurements for anammox activities and potential denitrification were performed using a modified slurry isotope pairing technique (IPT)[7]. Three separate incubations were performed. The first was a control ($^{15}NH_4^+$), used to assess the removal of all oxygen and the lack of oxidants in the incubations. The second ($^{15}NH_4^+ + {}^{14}NO_3^-$) was used to determine if NH_4^+ was limiting to anammox activity. The third ($^{15}NO_3^-$) was used for establishing potential anammox and denitrification activity. Methods for the characterization of the microbial community and the IPT experiments are outlined in detail in Penton et al.[13].

In pursuit of objective 2, we developed a whole core $^{15}NH_4^+$ perfusion technique with porewater equilibrators for the extraction of $^{14+15}N\text{-}NO_3^-$, NH_4^+, and N_2, which enabled the quantification of rhizosphere mediated coupling of nitrification and denitrification in wetland sediments vegetated with growing taro plants. In brief, taro plants were grown for 13 weeks in 6 separate PVC cores (h=50 cm, d=24.7 cm) placed in a commercial taro field, followed by excavation and split placement in controlled environment growth chambers under two simulated wind treatments (+wind, -wind) along with 3 non-vegetated control cores. All cores were labeled with $^{15}NH_4^+$ to an estimated 95% of total NH_4^+ concentration (14.9±0.4 mmols $^{15}NH_4^+$ per core), corresponding to an equivalent of ~23 kg N ha^{-1} by slow perfusion using a peristalstic pump. Surface and porewater samples were collected immediately prior and following introduction of $^{15}NH_4^+$ and at 2, 3, 6, 8, and 11 days after perfusion. Samples were withdrawn from 7 depths for measurement of dissolved $^{29+30}N_2$, $^{15}NH_4^+$, total NH_4^+ and NO_3^-, and $^{15}NO_3^-$ assays using a Hamilton gas-tight syringe. At the same sampling occasions, gas from the aerenchyma space midway up the stem was also withdrawn using a Hamilton gas-tight syringe for measurement of $^{29+30}N_2$. Additionally, $^{15}NO_3^-$ and $^{15}NH_4$ in porewater were analyzed at each sampling interval, and at the conclusion of the 11-day incubation the intact soil core including the taro plant was extruded. Soils were analyzed at 2 cm intervals for *nirS* (cd3aF-R3cd), *nosZ* (nosZ1F-1R), *amoA* (amoA1F1-A2R), archaeal *amoA* N cycle functional genes and exchangeable NH_4^+-N. Above and below-ground components were separated and dried at 70°C to constant weight in preparation for N isotope analysis. Oxygen flux from individual taro roots was measured on a separate set of 1-month old taro plants using an O_2 microelectrode mounted to a micro-

manipulator with continuously logged output. Details on all analytical procedures and N balance equations are presented in Penton et al.[13].

RESULTS AND DISCUSSION

Samples collected for anammox enumeration showed the presence of anammox bacteria belonging to both the "marine" (primer set 541F-616R) and "freshwater" (primer set 818F-1066R) clades. Anammox *16S rRNA* gene abundance varied by less than one order of magnitude across the sampling sites. *Candidatus* 'Scalindua' type anammox abundances were on the low range of those reported in marine sediments[14] but within the range of abundances of *amoA* and *nirS* from our sites. The total anammox population derived with these primer sets was generally an order of magnitude lower than those found in fertilized paddy soils with broader range primer sets[9]. Though the size of the total anammox population indicated that the anammox process would be likely to occur, potential anammox-derived $^{29}N_2$ accumulation was absent in all samples with the exception of one of the taro sediments, which showed an anammox contribution of only 6% total N_2 production. On the other hand, IPT derived anaerobic denitrification rates for the taro sediments ranged from a low of 11.7 µmol N m^{-2} h^{-1}) to 571 µmol N m^{-2} h^{-1}, which were on the high range compared with undisturbed and disturbed habitats reported in the literature, and indicated denitrification as a significant potential loss pathway.

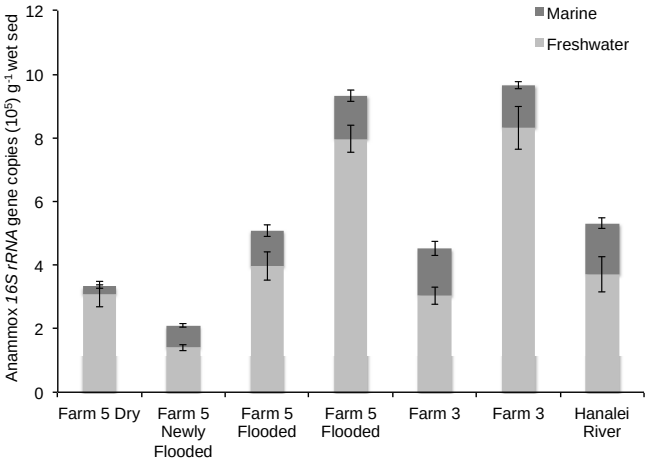

Fig. 1. Anammox 16S rRNA gene copies (x106) g-1 wet sediment for the "marine" anammox (primer set 541F-616R; dark grey) and the "freshwater" anammox (primer set 818F-1066R; light grey) from farm 3, 5, and the Hanalei River.

The whole core taro experiment combined with rhizosphere O_2 measurements established that the coupling of nitrification and denitrification in the rhizosphere was a significant pathway for N loss. Root O_2 flux was significant from the root tips ~7.5 h after the light cycle began under the wind treatment (Fig. 2). Likewise, we observed a light effect on nitrification-denitrification activity with $^{29+30}N_2$ appearing in the aerenchyma gas 6.25 to 6.75 h after initiation of the light cycle. Total $^{29+30}N_2$ production rates

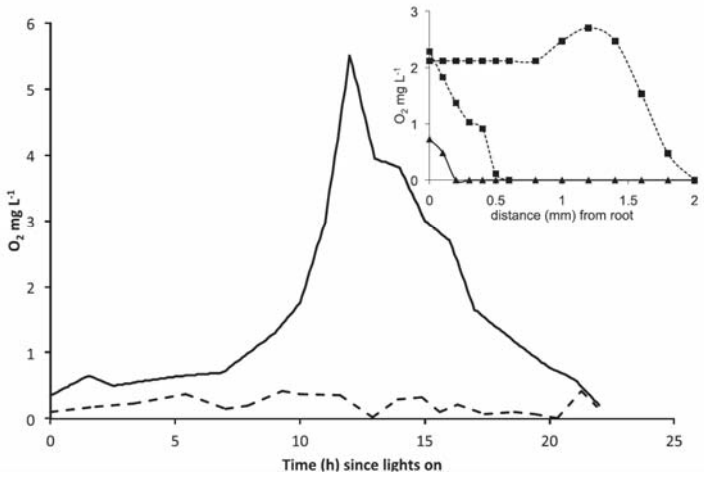

Fig. 2. Oxygen profile at the root tip of a taro plant with no wind (dashed line) and constant wind (solid line) after light initiation. The inset shows O_2 concentration over distance from an old (solid line) and two young (dashed lines) roots under wind treatment 10 h after the onset of light.

from rhizosphere coupling of nitrification and denitrification were highest in the aerenchyma, surface water, and porewater samples collected from the wind treatment immediately following $^{15}NH_4^+$ label introduction and lasted approximately 70 h followed by gradual decrease (Fig. 3). The relation between O_2 flux and N_2 production is illustrated by the 7h lag in both the O_2 flux from the roots and the onset of N_2 production after initiation of the light, as well as the concurrent decrease in

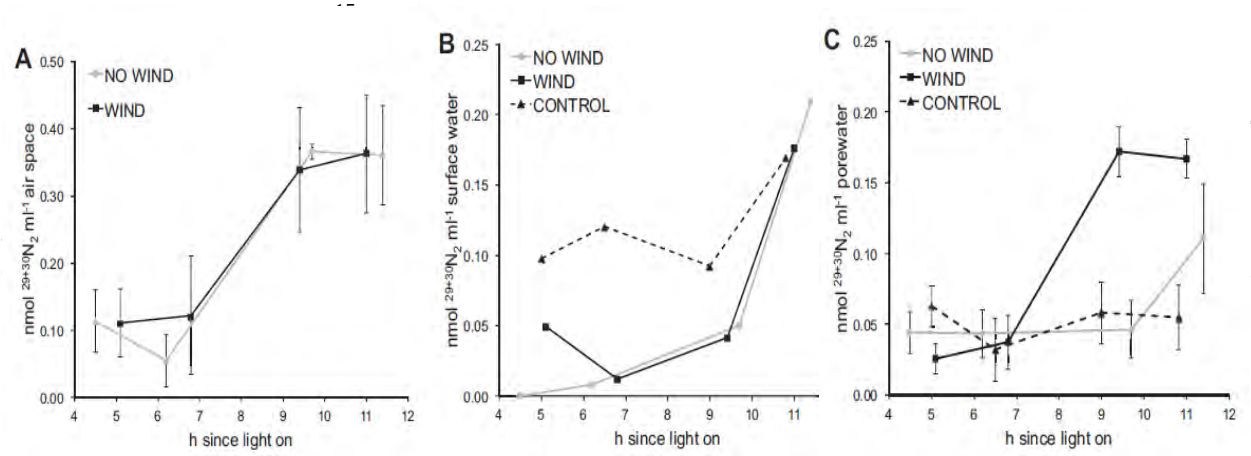

Fig. 3. Light experiment showing measured 29þ30N2 withdrawn from aerenchyma (A), surface water (B), and porewater (C) (average for all depths) for a 4e12 h period followinglights on for the no wind, wind, and control cores.

both O_2 and N_2 flux during the night cycle. The additional lag of the surface water is likely due to N_2 diffusion from the surface oxic/anoxic interface into the overlying water column. Thus, the extensive root O_2 release resulting from a combination of high root density and high O_2 flux distance from root tips and lateral sides during photosynthesis enhanced the couple between nitrification and denitrification in both space and time[15,16].

The logarithmic increase in $^{29+30}N_2$ in the vegetated cores versus the slower, linear accumulation in the control illustrates that the added $^{15}NH_4^+$ is mostly lost immediately following fertilization in vegetated sediments. This is likely due to the influence of subsurface O_2

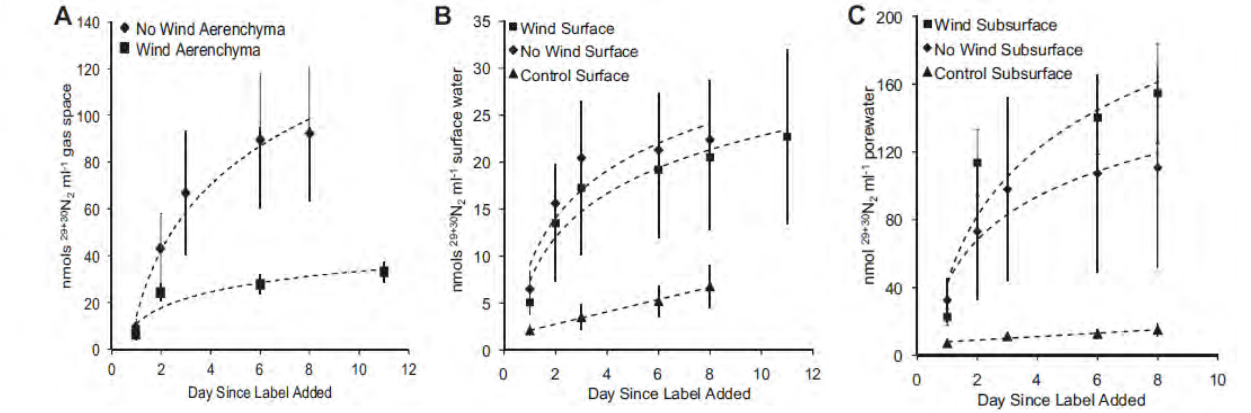

Fig. 4. Accumulated $^{29+30}N_2$ over the incubation period for the three treatments in the aerenchyma (A), surface (B), and porewater (C) samples. Values are means with standard errors.

flux from the rhizosphere versus constant anaerobic conditions in the control cores providing ample sites for subsurface ammonia oxidation as well as a perfusion effect from the even distribution of N label in the porewater. The surface accumulation of $^{29+30}N_2$ reflects both the coupled nitrification-denitrification activity in the surface water and diffusion of N_2 from subsurface denitrification. Although we did not directly measure N_2 flux out of the sediment, the much lower N_2 accumulation in the surface water indicates that the majority of N_2 may remain trapped within the soil matrix due to slow N_2 diffusion rates caused by highly tortuous diffusion pathways. This "trapping" of N_2 in the porewater has also been found in a vegetated wetland system where approximately ~76% of the denitrified N_2 remained trapped within the soil while ~approximately 24% left through the aerenchymous tissue in three aquatic macrophytes[17] and in rice paddies where 2.8% of N_2 lost left through the surface[18].

Nitrogen balance calculations from the whole core experiment found that approximately 68% of the added $^{15}NH_4^+$ was recovered as N_2 gas in the porewater and arenchyma indicating a dominant role of rhizosphere coupling of nitrification and denitrification in the vegetated cores (Fig. 5). In contrast, the non-vegetated cores showed approximately 25% of added $^{15}NH_4^+$ was recovered as N_2. Taro plant uptake efficiency varied from 21-41%, comparable to 33.5% to 50.5% in rice[19,20]. Our work suggests a high potential for NH_4 loss in the subsoil due to coupled nitrification-denitrification in the oxidized rhizosphere, and thus demonstrates that deep placement of urea does not necessarily protect urea-N from loss due to denitrification.

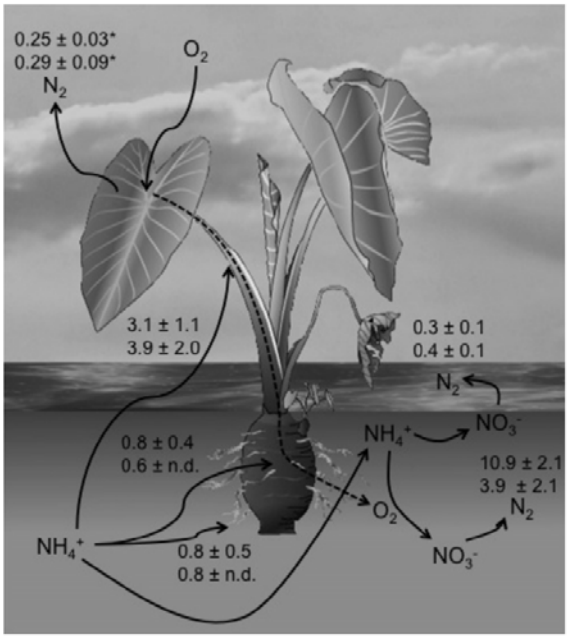

Fig. 5. Illustration of the nitrogen loss and assimilation pathways in the taro wetland system. Top numbers are wind treatment while the bottom numbers represent the no wind treatment. Values are given in mmols assimilated or produced over the course of the incubation period for comparison to total porewater NH_4 loss.

CONCLUSION

Although anammox bacteria were found to be present in these flooded taro agroecosystems, the anammox process was of little importance. This illustrates that presence is not always linked to activity, even at significant abundance and that these linkages should be supported by activity measurements. The intact whole-core experiment using $^{15}NH_4^+$ identified the overwhelming importance of coupled nitrification-denitrification of the sub-surface sediment in vegetated cores compared to bare sediment. Results suggest that fertilizer N loss as N_2 through sub-surface pathways is substantial with greatest losses associated with large pulses of fertilizer $^{15}NH_4^+$. Applying polymer coated ammonium fertilizers or organic fertilizers, which supply ammonium-N in a controlled, slow release pattern may mitigate N loss to coupled nitrification-denitrification in the rhizosphere.

SELECTED REFERENCES

1. Reddy, K.R. 1982. Nitrogen cycling in a flooded-soil ecosystem planted to rice (*Oryza sativa* L.). Plant Soil 67:209-220.

2. Reddy, K.R., and W.H. Patrick. 1984. Nitrogen transformations and loss in flooded soils and sediments. Crit. Rev. Environ. Contr. 13:272-309.
3. Reddy, K.R., W.H. Patrick, and C.W. Lindau. 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. Limnol. Oceanogr. 34:1004-1013.
4. Penton, C.R., A.H. Devol, and J.M. Tiedje. 2006. Molecular evidence for the broad distribution of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. Appl. Environ. Microbiol. 72:1-4.
5. Schubert, C.J., E. Durisch-Kaiser, W. Wehrli, B. Thamdrup, P. Lam, and M.M.M. Kuypers. 2006. Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). Environ. Microbiol. 8:1857-1863.
6. Strous, M., and M.S.M. Jetten. 2004. Anaerobic oxidation of methane and ammonium. Annu. Rev. Microbiol. 58:99-117.
7. Thamdrup, B., and T. Dalsgaard. 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. 68:1312–1318.
8. Zhang, Y., X-.H. Ruan, H.J.M. Op den Camp, T.J.M. Smits, M.S.M. Jetten, and M.C. Schmid. 2007. Diversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in freshwater sediments of the Xinyi River (China). Environ. Microbiol. 9:2375-2382.
9. Zhu, G., S. Wang, Y. Wang, N. Risgaard-Petersen, M.S.M. Jetten, and C. Yin. 2011. Anaerobic ammonia oxidation in a fertilized paddy soil. ISME J. 5:1905-1912.
10. Kuypers, M.M.M., A.O. Sliekers, G. Lavik, M. Schmid, B.B. Jorgensen, J.G. Kuenen, J.S.S. Damste, M. Strous, and M.S.M. Jetten. 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature 422:608-611.
11. Meyer, R.L., N. Risgaard-Petersen, and D.E. Allen. 2005. Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. Appl. Environ. Microbiol. 71:6142-6149.
12. Trimmer, M., J.C. Nicholls, N. Morley, C.A. Davies, and J. Aldridge. 2005. Biphasic behavior of anammox regulated by nitrite and nitrate in an estuarine sediment. Appl. Environ. Microbiol. 71:1923-1930.
13. Penton C.R., Deenik, J.L., Popp, B.N., Bruland, G.L., Engstrom, P., St. Louis, D., Brown, G.A., and Tiedje, J. 2013a. Importance of sub-surface rhizosphere-mediated coupled nitrification-denitrification in a flooded agroecosystem in Hawaii. Soil Biol. Biochem. 57:362-373.
14. Dang, H., R. Chen, L. Wang, L. Guo, P. Chen, Z. Tang, F. Tian, S. Li, and M.G. Klotz. 2010. Environmental factors shape sediment anammox bacterial communities in hypernutrified Jiaozhou Bay, China. Appl. Environ. Microbiol. 76:7036-7047.
15. Risgaard-Petersen, N., Nielsen, L.P, Revsbech, N.P., 1994. Diurnal variation of denitrification and nitrification in sediments colonized with benthic microphytes. Limnology and Oceanography 39, 573-579.
16. Risgaard-Petersen, N., Jensen, K., 1997. Nitrification and denitrification in the rhizosphere of the aquatic macrophyte *Lobelia dortmanna*. Limnology and Oceanography 42, 529-537.
17. Reddy, K.R.R., Patrick Jr., W.H., Lindau, C.W., 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. Limnology and Oceanography 34, 1004-1013.
18. Buresh, R.J., Austin, E.R., 1988. Direct measurement of dinitrogen and nitrous oxide flux in flooded rice fields. Soil Science Society of America Journal 52, 681-688.

19. Cassman, K.G., Kropff, M.J., Gaunt, J., Peng, S., 1993. Nitrogen use efficiency of rice reconsidered: What are the key constraints? Plant and Soil 155, 359-362.
20. Fageria, N.K., de Morais, O.P., dos Santos, A.B., 2010. Nitrogen use efficiency in upland rice genotypes. Journal of Plant Nutrition 33, 1696-1711.