Susceptibility of Ammonia-Oxidizing Bacteria to Nitrification Inhibitors

Douchi Matsuba[a*], Hirotoshi Takazaki[a], Yukiharu Sato[a], Reiji Takahashi[b], Tatsuaki Tokuyama[b], and Ko Wakabayashi[a*]

[a] Graduate School of Agricultural Science, Tamagawa University, Tamagawa-Gakuen, Machida-shi, Tokyo 194-8610, Japan. Fax: +81-42-739-8854.
E-mail: kwaka@agr.tamagawa.ac.jp

[b] Department of Agricultural and Biological Chemistry, College of Bioresource Sciences, Nihon University, Fujisawa-shi, Kanagawa 252-8510, Japan

* Authors for correspondence and reprint requests

Z. Naturforsch. **58 c**, 282–287 (2003); received October 22, 2002

Activity of nitrification inhibitors to several typical ammonia-oxidizing bacteria isolated recently, *i. e. Nitrosococcus*, *Nitrosolobus*, *Nitrosomonas*, *Nitrosospira* and *Nitrosovibrio* species was assayed using 2-amino-4-methyl-6-trichloromethyl-1,3,5-triazine (MAST), 2-amino-4-tribromomethyl-6-trichloromethyl-1,3,5-triazine (Br-MAST), 2-chloro-6-trichloromethyl-pyridine (nitrapyrin) and others, and compared to confirm the adequate control of ammonia-oxidizing bacteria by the inhibitors. The order of activity of the inhibitors to 13 species of ammonia-oxidizing bacteria examined was approximately summarized as Br-MAST $\geq$ nitrapyrin $\geq$ MAST > other inhibitors. Two *Nitrosomonas* strains, *N.* europaea ATCC25978 and *N.* sp. B2, were extremely susceptible to Br-MAST, exhibiting a $pI_{50} \geq 6.40$. These values are the position logarithms of the molar half-inhibition concentration. The 16S rRNA gene sequence similarity for the highly susceptible 4 strains of genus *Nitrosomonas* was 94% to 100% of *Nitrosomonas europaea*, although those of the less susceptible 3 strains of ammonia-oxidizing bacteria, *Nitrosococcus oceanus* C-107 ATCC19707, *Nitrosolobus* sp. PJA1 and *Nitrosolobus multiformis* ATCC25196, were 77.85, 91.53 and 90.29, respectively. However, no clear correlation has been found yet between pI_{50}-values and percent similarity of 16S rRNA gene sequence among ammonia-oxidizing bacteria.

Key words: Ammonia-Oxidizing Bacteria, Susceptibility to Nitrification Inhibitors, Nitrapyrin

Introduction

It is important to slow down processes of ammonia nitrification to nitrate, due to maintenance of soil fertility, prevention of NO_X^- (x = 2 or 3) pollution in ground- and surface-water and suppression of the stratospheric ozone depletion gas (N_2O) in the atmosphere. Effects of nitrification inhibitors, such like 2-chloro-6-(trichloromethyl)pyridine (nitrapyrin), 2-amino-4-methyl-6-trichloromethyl-1,3,5-triazine (MAST) and others, on nitrification activity by the limited species of nitrifying bacteria, *Nitrosomonas europaea* ATCC25978, *Nitrosomonas* sp. TK794 and *Nitrobacter agilis* ATCC14123, have been investigated with intact cells (Murakami *et al.*, 1993; Takagi *et al.*, 1994; Murakami *et al.*, 1995; Takahashi *et al.*, 1997; Ohki *et al.*, 1997) and cell-free extracts (Kasahara *et al.*, 2002), to conclude that the inhibitors affect the ammonia-oxidizing process to nitrate, especially the ammonia monooxygenase (AMO) involved in ammonia oxidation to hydroxylamine, but not the hydroxylamine oxidoreductase (HAO) in hydroxylamine oxidation. This finding supports the assumption that nitrification inhibitors are adequate to act on the ammonia oxidation to hydroxylamine, so that harmful intermediates, such as NH_2OH and NO_2^-, against environmental and human health, do not accumulate.

In these 10 years, more than 20 species of ammonia-oxidizing bacteria have been isolated from upland soil (Takahashi *et al.*, 1992; Tokuyama *et al.*, 1997; Takahashi *et al.*, 2001), paddy soil (Tomiyama *et al.*, 2001), wastewater (Matsuba *et al.*, 2002), sea water (Mizoguchi *et al.*, 1998) and others, by analyzing their 16S ribosomal RNA genes. In this paper, several typical ammonia- oxidizing bacteria isolated, *i. e. Nitrosococcus*, *Nitrosolobus*, *Nitrosomonas*, *Nitrosospira* and *Nitrosovibrio* species were assayed for their susceptibility to nitrification inhibitors and compared to justify an adequate control of ammonia-oxidizing bacteria using the inhibitors.

0939–5075/2003/0300–0282 $ 06.00

Materials and Methods

Chemicals

2-Amino-4-methyl-6-trichloromethyl-1,3,5-triazine (MAST) was prepared by reaction of 2-methyl-4,6-bis(trichloromethyl)-1,3,5-triazine with ammonia water, according to Murakami *et al.* (1993). 2-Amino-4-tribromomethyl-6-trichloromethyl-1,3,5-triazine (Br-MAST) was obtained via bromination of MAST in glacial acetic acid (Ohki *et al.*, 1997). 2-Chloro-6-(trichloromethyl)pyridine (nitrapyrin) was kindly provided by Dow Elanco Japan Ltd. Dicyanodiamide and thiourea were purchased from Wako Pure Chemical Industries Ltd., Tokyo. Analytical grade chemicals for ammonia-oxidizing bacteria cultures and other chemicals were purchased from Kanto Chemical Co. Inc., Tokyo, and Dojindo Laboratories, Kumamoto, Japan.

Ammonia-oxidizing bacteria used in this study

Nitrosococcus oceanus ATCC19707, three strains of *Nitrosolobus* (*N. multiformis* ATCC25196, *N.* sp. PJA1 and *N.* sp. TCH716), four strains of *Nitrosomonas* (*N. europaea* ATCC25978, *N.* sp. IWT514, *N.* sp. TK793 and *N.* sp. B2), two strains of *Nitrosospira* (*N.* sp. GS833 and *N.* sp. NRS527) and three strains of *Nitrosovibrio* (*N.* sp. RY3C, *N.* sp. RY6A and *N.* sp. TYM9) were used as representatives of ammonia-oxidizing bacteria isolated from different habitations for susceptibility assay of nitrification inhibitors. Although the strains with ATCC numbers were purchased from American Type Culture Collection, other ammonia oxidizing bacteria have been isolated by our group (Depository of strains: T. Tokuyama, Department of Agricultural and Biological Chemistry, Nihon University, Fujisawa-shi, Japan. 16S rRNA gene sequences: see NCBI, 2002). All strains were basically incubated in the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) medium before the inhibitory assay, according to Tomiyama *et al.* (2001). After cells were collected by centrifugation and cell suspensions were washed twice, nitrite formation by the cell suspension was adjusted to the rate ($1\ mg \cdot ml^{-1} \cdot 30\ min^{-1}$) with phosphate buffer containing ammonium sulfate (100 mg nitrogen/ml).

Nitrification inhibition assay in cell suspension

Susceptibility of 13 strains of ammonia-oxidizing bacteria to nitrification inhibitors was examined using five nitrification inhibitors, namely MAST, Br-MAST, nitrapyrin, dicyanodiamide and thiourea. Inhibitors were dissolved in acetone and the final concentration of the solvent in the medium was kept below 0.1% (v/v). Ammonium sulfate and hydroxylamine hydrochloride in phosphate buffer (pH 8.0) were employed as substrate for the ammonia-oxidation step and the hydroxylamine-oxidation step of ammonia-oxidizing bacteria, respectively, after Takagi *et al.* (1994) and Murakami *et al.* (1995). Thirty min after incubation at 37 °C with inhibitors, nitrite formation in the culture medium was determined by optical density at 532 nm according to Griess-Ilosvay method (Hewitt and Nicholas, 1964). The molar concentration of the inhibitor, which shows 50% inhibition (molar I_{50}) against the nitrite formation by ammonia-oxidizing bacteria relative to the control, was estimated by probit analysis. The nitrification inhibition indices are expressed as pI_{50}, the negative logarithm of the molar I_{50}. Susceptibility of ammonia-oxidizing bacteria to the inhibitors is discussed with the pI_{50}-values in this paper. For reference, the effect of the nitrification inhibitors on nitrite oxidation by *Nitrobacter agilis* ATCC14123 was also determined according to Takagi *et al.* (1994).

Results and Discussion

Site of action nitrification inhibitors in ammonia-oxidizing bacteria

Nitrite-oxidizing bacteria, *Nitrobacter agilis* ATCC14123, was too insensitive to the three inhibitors, *i.e.* MAST (**1**), Br-MAST (**2**) and nitrapyrin (**3**), to prevent oxidation of nitrite contained in the culture medium as shown by the experiment using MAST in Fig. 1, as reported already for the insensitiveness of inhibitors MAST and nitrapyrin in our previous paper (Takagi *et al.*, 1994), indicating that apparently they affect on ammonia-oxidizing bacteria.

In our previous papers (Murakami *et al.*, 1995; Ohki *et al.*, 1997), the target site of MAST, Br-MAST and nitrapyrin has been elucidated to be on the ammonia-oxidation step from ammonia to hydroxylamine in the experiment using *Nitroso-*

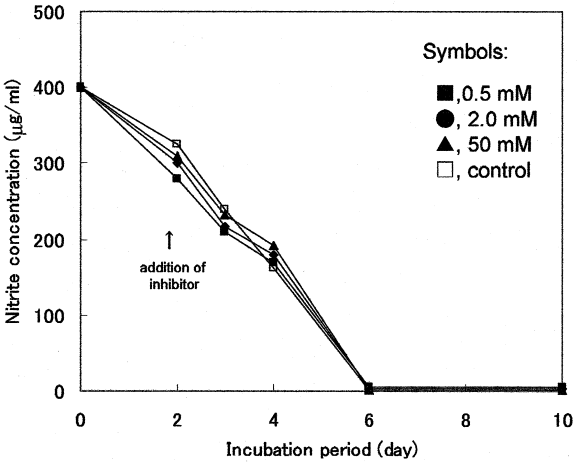

Fig. 1. Effect of nitrification inhibitor (MAST) on nitrite oxidizing activity of *Nitrobacter agilis* ATCC14123. MAST was dissolved in DMSO and the final concentration of DMSO in culture media was kept blow 0.1% (v/v); Effect of DMSO, talc and ethanol without inhibitor were also tested as control.

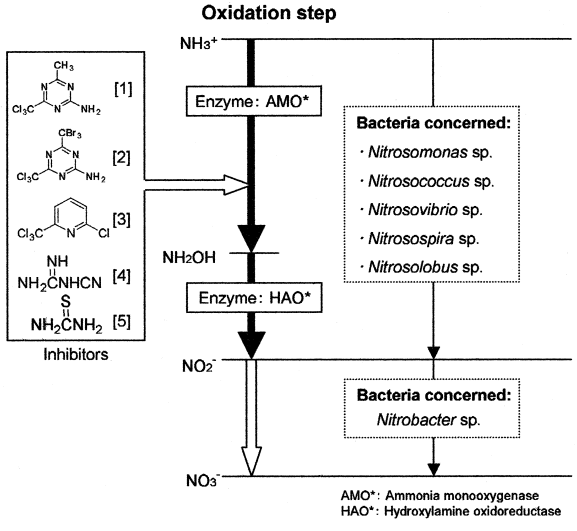

Fig. 2. Effect of inhibitors on nitrification of ammonia.

monas europaea ATCC25978 cells. The three inhibitors were checked in this paper using other 12 strains of ammonia-oxidizing bacteria, whether they were also inhibitors on the ammonia-oxidation step, but no inhibitors for the hydroxylamine-oxidation step. The three inhibitors strongly inhibited nitrite production from ammonia by the bacteria examined at 0.5 to 2.0 μM concentration, indicating 50 to 65% inhibition rates, while they exhibited no inhibition for nitrite production from hydroxylamine even at 50 μM (detailed data: not documented, very analogous to Murakami *et al.*, 1995, see also Fig. 2). Furthermore, our recent experiment using cell-free extract obtained from *Nitrosomonas europaea* ATCC25978 has revealed that MAST, Br-MAST and nitrapyrin are inhibitors of the ammonia-oxidation step from ammonia to hydroxylamine, inhibiting ammonia monooxygenase (AMO), but not affecting hydroxylamine oxidoreductase (HAO) (Kasahara *et al.*, 2002). Thus, inhibitory activity (pI_{50}) of five nitrification inhibitors, *i. e.* MAST (**1**), Br-MAST (**2**), nitrapyrin (**3**), dicyanodiamide (**4**) and thiourea (**5**), in 14 cell-cultures of nitrifying bacteria was assayed for nitrite production from ammonia (results, see Table I).

Susceptibility of ammonia-oxidizing bacteria to inhibitors

Since dicyanodiamide (**4**) and thiourea (**5**) were found to be weak inhibitors with pI_{50} (< 4.35) (see Table I), these two inhibitors are omitted from discussion here. The order of activity of the three inhibitors to 13 ammonia-oxidizing bacteria was approximately summarized Br-MAST (**2**) ≥ nitrapyrin (**3**) ≥ MAST (**1**), with the exception for *Nitrosospira* sp. NRS527. Inhibitor **2** was extremely sensitive to two *Nitrosomonas* strains, *N. europaea* ATCC25978 and *N.* sp. B2, exhibiting $pI_{50} \geq 6.40$. However, MAST (**1**) was ca. 4.7 fold more sensitive to *Nitrosospira* sp. NRS527, isolated from paddy soil, than Br-MAST (**2**) and nitrapyrin (**3**).

The ammonia-oxidizing bacteria isolated from soil (genus *Nitrosomonas* 1, 2 and 3, genus *Nitrosovibrio* 6, 7 and 8, genus *Nitrosospira* 9 and 10, and genus *Nitrosolobus* 11) were highly susceptible ($pI_{50} \geq 5.10$) to the three inhibitors (**1**, **2** and **3**), with an exception of *Nitrosolobus* sp. PJA1 (12, $pI_{50} < 4.80$) isolated from rhizosphere of barley. *Nitrosococcus oceanus* C-107 (5) and *Nitrosolobus multiformis* (13), ammonia-oxidizing bacteria from sea water and wastewater respectively, were about 5 to 10 times less sensitive to the three inhibitors than the *Nitrosomonas europaea* (1). Since the nitrification inhibitors have originally been introduced to control nitrification of ammonia in soils, it is quite desirable that the inhibitors are more sensitive to ammonia-oxidizing bacteria isolated from soils than the bacteria from other sources.

Table I. Susceptibility of inhibitors to nitrifying bacteria.

Nitrifying bacteria	Habitat	pI_{50} values for inhibitors (1)	(2)	(3)	(4)	(5)	Similarity of 16SrRNA (%)
1 *Nitrosomonas europaea* ATCC25978 (AF353160)	upland soil	5.75	7.12	5.66	4.35	< 4	100
2 *Nitrosomonas* sp. TK794 (AF080185)*	upland soil	6.70	–	6.58	–	–	96.64
3 *Nitrosomonas* sp. IWT514 (AF363293)	filter of deodorization equipment	5.80	5.65	5.46	4.22	< 4	94.95
4 *Nitrosomonas* sp. B2 (AB093545)	wastewater	5.35	6.43	6.06	< 4	< 4	97.95
5 *Nitrosococcus oceanus* C-107 ATCC19707 (M96395)	sea water	5.02	–	4.96	–	–	77.85
6 *Nitrosovibrio* sp. TYM9 (AF080256)	woodlands soil	5.36	6.21	5.53	4.11	< 4	90.83
7 *Nitrosovibrio* sp. RY3C (AF363290)	rhizosphere of avocado	5.42	5.13	5.17	< 4	< 4	88.81
8 *Nitrosovibrio* sp. RY6A (AF363291)	rhizosphere of avocado	5.43	5.62	5.53	< 4	< 4	90.03
9 *Nitrosospira* sp. GS833 (AF 353162)	upland soil	5.75	6.39	5.81	4.10	< 4	91.40
10 *Nitrosospira* sp. NRS527 (AF353158)	rhizoplane of paddy rice	6.07	5.39	5.44	4.32	< 4	90.29
11 *Nitrosolobus* sp. TCH716 (AF353156)	alkaline soil	5.13	5.77	5.37	< 4	< 4	91.14
12 *Nitrosolobus* sp. PJA1 (AF353163)	rhizoplane of barley	4.52	4.80	4.48	< 4	< 4	91.53
13 *Nitrosolobus* multiformis ATCC25196 (L35509)	wastewater	4.73	5.48	5.07	< 4	< 4	90.29
14 *Nitrobacter agilis* ATCC14123 (AY055796)	upland soil	< 3	–	< 3	–	–	76.69

* Nitrite formation is not inhibited in the presence of inhibitors in experiments using hydroxylamine as substrate.

Phylogenetic tree of ammonia-oxidizing bacteria and activity of inhibitors

A neighbor-joining phylogenetic tree indicating the relationship among the ammonia-oxidizing bacteria assayed in this study was constructed using their 16S rRNA gene sequences (NCBI, 2002; Saitou and Nei, 1987). Four strains of ammonia-oxidizing bacteria belonging to genus *Nitrosomonas* were in a close relation in the phylogenetic tree (Fig. 3) and were found highly susceptible to the three nitrification inhibitors (MAST, Br-MAST and nitrapyrin), although three bacteria of genus *Nitrosolobus* showing a distant relation from the genus *Nitrosomonas* were about 10 times less sensitive against the inhibitors. The activity of three inhibitors to genera *Nitrosococcus*, *Nitrosovibrio* and *Nitrosospira* were intermediate between the genus *Nitrosomonas* and *Nitrosolobus*.

The 16S rRNA gene sequence similarity for the highly susceptible 4 strains of genus *Nitrosomonas* is 94% to 100% of *Nitrosomonas europaea* as shown in Table I, although those of the less susceptible 3 strains of ammonia-oxidizing bacteria, *Nitrosococcus oceanus* C-107 ATCC19707, *Nitrosolobus* sp. PJA1 and *Nitrosolobus multiformis* ATCC25196, are 77.85, 91.53 and 90.29, respectively. However, no clear correlation has been found yet between pI_{50}-values and

$$pI_{50}(\text{MAST}) = 0.045 \times [\text{similarity}] + 1.273 \ (\pm\ 0.064) \quad [n = 13, r = 0.430, s = 0.542] \quad (1)$$

$$pI_{50}(\text{Br-MAST}) = 0.134 \times [\text{similarity}] - 6.572 \ (\pm\ 0.097) \quad [n = 11, r = 0.721, s = 0.486] \quad (2)$$

$$pI_{50}(\text{nitrapyrin}) = 0.055 \times [\text{similarity}] + 0.451 \ (\pm\ 0.052) \quad [n = 13, r = 0.570, s = 0.446] \quad (3)$$

percent similarity of 16S rRNA gene sequence, as shown in the following equations (1) to (3).

Accordingly, the different susceptibility of the 13 strains of ammonia-oxidizing bacteria assayed to the inhibitors may be due to (1) different permeation of inhibitors through the intracytoplasmic membrane system (Takahashi *et al.*, 1992), (2) different detoxifying metabolism of inhibitors, and/or (3) different ammonia monooxygenase activities against inhibitors. To clear up this discussion, further experiments, including isolation of AMO and its inhibition by inhibitors, are under way in our laboratories.

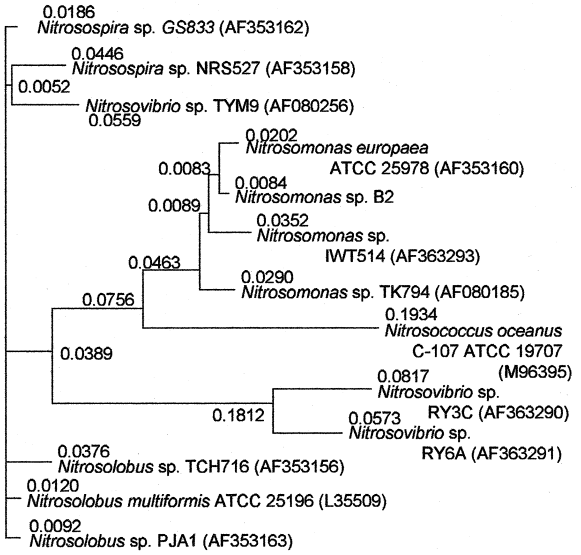

Fig. 3. Calculated phylogenetic tree using 16S rRNA gene sequences by neighbor-joining method.

Acknowledgements

The authors wish to thank Dr. Shinpei Ohki and Prof. Dr. Peter Böger, Universität Konstanz, for their suitable advice and suggestion for the present study. This work was supported by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture of Japan.

Hewitt E. J., and Nicholas D. J. D. (1964), Enzymes of inorganic nitrogen metabolism. In: Modern Method of Plant Analysis (Linskens H. F., Sanwal B. D., and Tracy M. V., eds.). Vol. **7**, pp. 167–172.

Kasahara Y., Ohki S., Sato Y., Takahashi R., Tokuyama T., Takeshima S., Vonk J. W., and Wakabayashi K. (2002), Influence of 1,3,5-triazine compounds on ammonia oxidizing activity of cell-free extracts from *Nitrosomonas europaea*. J. Pestic. Sci. **27**, 133–135

Matsuba D., Ogawa H., Takazaki H., Sato Y., Tokuyama T., Vonk J. W., and Wakabayashi K. (2002), *Nitrosomonas* sp. B2, an ammonia oxidizing bacterium, with a high rate nitrite formation. Arch. Microbiol. submitted.

Mizoguchi M., Omotani J., Mizuno Y., Takahashi R., Kanehira T., Shinohara M., and Tokuyama T. (1998), Newly isolated marine ammonia-oxidizing bacterium, *Nitrosomonas* sp. TNO632. J. Ferment. Bioeng. **86**, 406–409.

Murakami M., Takagi S., Takahashi I., Tokuyama T., Sato Y., Vonk J. W., and Wakabayashi K. (1995), A possible target site of 1,3,5-triazine nitrification inhibitors. J. Pestic. Sci. **20**, 471–477.

Murakami M., Tsuji A., Miyamoto Y., Yamazaki C., Ogawa H., Takeshima S., and Wakabayashi K. (1993), Synthesis of trichloromethyl-1,3,5-triazines and their nitrification-inhibitory activity. J. Pestic. Sci. **18**, 147–154.

NCBI (2002), 16S rRNA gene. http://www.ncbi.nlm.nih.gov/

Ohki S., Kasahara Y., Murakami M., Miyamoto Y., Tokuyama T., Vonk J. W., Sato Y., and Wakabayashi K. (1997), Effects of halomethyl-1,3,5-triazines on nitrification. J. Pestic. Sci. **22**, 95–101.

Saitou N., and Nei M. (1987), A neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**, 406–425.

Takagi S., Murakami M., Sato Y., Takahashi R., Tokuyama T., and Wakabayashi K. (1994), Effect of nitrification inhibitors on *Nitrosomonas* and *Nitrobacter*. J. Pestic. Sci. **19**, 19–23.

Takahashi I., Ohki S., Murakami M., Takagi S., Sato Y., Vonk J. W., and Wakabayashi K. (1997), Mode of action and QSAR studies of nitrification inhibitors: Effect of trichloromethyl-1,3,5-triazines on ammonia-oxidizing bacteria. J. Pestic. Sci. **22**, 27–32.

Takahashi R., Kondo N., Usui K., Kanehira T., Shinohara M., and Tokuyama T. (1992), Pure isolation of a new chemoautotrophic ammonia-oxidizing bacterium on gellan gum plate. J. Ferment. Bioeng. **74**, 52–54.

Takahashi R., Ohishi M., Ohshima M., Saitoh M., Omata K., and Tokuyama T. (2001), Characteristics of an ammonia-oxidizing bacterium from alkaline soils and its phylogenetic relationship. J. Biosci. Bioeng. **92**, 232–236.

Tokuyama T., Yoshida N., Matsuishi T., Takahashi N., Takahashi R., Kanehira T., and Shinohara M. (1997), A new psychrotrophic ammonia-oxidizing bacterium, *Nitrosovibrio* sp. TYM9. J. Ferment. Bioeng. **83**, 377–380.

Tomiyama H., Ohshima M., Ishii S., Satoh K., Takahashi R., Isobe K., Iwano H., and Tokuyama T. (2001), Characteristics of newly isolated nitrifying bacteria from rhizoplane of paddy rice. Microbes and Environments, Japan, **16**, 101–108.