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Original Research Article

Morphology and Morphogenetic Description of an Indian Population of a Soil Ciliate *Notohymena gangwonensis* from Rithala Wastewater Treatment Plant (WWTP), Delhi, India

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ABSTRACT

Ciliates are single celled eukaryotes which have been discovered from a wide variety of habitats. Their free-living forms are present in aquatic as well as terrestrial habitats. A large number of ciliates have also been recorded from wastewater treatment plants throughout the world. In WWTP these species are found from the final effluent as well as from the sewage sludge. In the present study, an Indian population of *Notohymena gangwonensis* was isolated from the sewage sludge samples collected from Rithala wastewater treatment plant (WWTP), Delhi, India, and is described on the basis of its morphology and morphogenesis. Morphological features of *N. gangwonensis* are as follows: flexible body; anterior lanceolate and posterior rounded end; body size about 51 x 15 µm in protargol preparations with body length:width ratio of around 3.5:1; large and deep buccal cavity; undulating membranes in *Notohymena* pattern; adoral zone with 19 (average) adoral membranelles; 18 frontal-ventral-transverse (FVT) cirri; one right and one left marginal row with about 14 and 13 cirri respectively; 6 dorsal rows consisting of 4 dorsal kineties (DKs) and two dorso-marginals (DMs), the fourth row of dorsal kineties (DK_4) being unique as it is *'curved'*, and occupies 2/3rd of the cell; 3 caudal cirri; two macronuclear nodules; 1–4 micronuclei. The morphogenetic study showed *de novo* origin of oral primordium between left marginal cirral row and post oral ventral cirri, and involvement of five parental cirri (three frontals and two ventrals) in the formation of streaks I–VI for the proter and opisthe. Their morphological and morphogenetic characters were compared with the other members of the genus and it confirmed that the present species is *Notohymena gangwonensis* belonging to the subfamily Oxytrichinae, which is a new record from the Indian subcontinent. The morphogenesis of *N. gangwonensis* has also been described here for the first time.

Keywords: Morphology, morphogenesis, *Notohymena*, Oxytrichidae

1. INTRODUCTION

Ciliated protists are single celled and heterokaryotic eukaryotes. Their presence has been recorded from varied aquatic as well as terrestrial habitats such as soil, desert sands and forest litter (Corliss, 1979; Foissner, 1987; Patterson et al., 1989; Foissner et al., 1994; Foissner, 1995; Foissner, 1998). Though many scientific studies point out that the soil contains a wide variety of ciliates, not much is known about the biodiversity of these organisms as a whole (Venter et al. 2018).

In a testament for their wilderness in distribution, a large number of species of ciliates have been discovered from the wastewater-treatment plants (WWTP) world-wide (Roberts et al., 2004; Madoni, 2011; Moreira et al., 2022). Their presence has been recorded from the treated final effluent as well as even in the sewage sludge, which is a semi-solid waste by-product containing a rich mix of organic and inorganic nutrients from human waste, food waste and inorganic solids, providing a conducive idyllically nutrient-rich environment sustaining the

supplies required for the growth of these unicellular organisms. While in the effluent, they feed on bacteria and purify the final effluent (Moreira et al., 2022).

In the present study, an Indian population of *Notohymena gangwonensis* was recorded from the sludge of WWTP, Rithala, Delhi, India. Previously, we reported another species, the *Notohymena limus* from the same sample (Naqvi et al., 2016). Blatterer and Foissner (1988) were credited with providing the first description of the Genus *'Notohymena'*. Prior to their contribution, it was nearly impossible to differentiate this genus from another look-alike doppelgänger genus, the *'Oxytricha' in vivo* due to their striking similarities. The distinctive and conspicuously prominent features of the genus *Notohymena* include flexible and slightly contractile bodies, with 18 or less frontal-ventral-transverse (FVT) cirri, a question mark (**?**) shaped Adoral Zone of Membranelles (AZMs), presence of 3 caudal cirri, undulating membranes (UMs) in a peculiar pattern typical to *Notohymena* (Figure 1A), and one right and one left marginal row of cirri (Blatterer & Foissner, 1988; Foissner, 1997; Berger, 1999; Foissner, 1999).

In the *Notohymena* pattern of UMs, the paroral membrane is distinctly curved at the anterior end and thus appears hook-shaped. The buccal cavity is moderately wide and rather deep. Eight species of this genus are reported so far (Hemberger, 1985; Blatterer & Foissner, 1988; Foissner, 1996; Berger, 1999; Küppers et al., 2007; Kamra & Kumar 2010; Naqvi et al., 2016; Kim et al., 2019; Moon et al., 2020).

Surprisingly, the majority of the species have been reported from soil samples, with the exception of a few amphibious species that were isolated from both soil and water samples. One was also reported from sewage sludge (Naqvi et al., 2016).

The current study's goal is to report an Indian population of *Notohymena gangwonensis* that was isolated from sewage sludge obtained from Rithala WWTP in Delhi, India. This species is isolated for the first time from the Indian subcontinent. The morphology and morphogenesis of this species were described based on the 'protargol' impregnation studies.

2. MATERIALS AND METHODS

2.1. Soil sampling

Sewage sludge was collected from the Delhi Jal Board's WWTP located in Rithala (Latitude: 28°43'12" N, Longitude: 77°6'3" E) region of Delhi, India. This sewage sludge comprises debris, sand particles as well as litter which arises after the recycling of water by WWTP. This treated recycled water is redistributed to various areas of Delhi for industrial and agricultural purposes. The sludge samples thus collected were transported to the laboratory, air dried for 15 days and analysed with the "non-flooded Petri dish method" as described by Foissner (Foissner 1987, 1992).

2.2. Laboratory culturing and maintenance of ciliates

Live ciliates were identified and isolated under the microscope to raise clonal cultures for the species under investigation. They were cultured in Pringsheim's medium (Chapman-Anderson, 1958; Pringsheim, 1964). The composition of the Pringsheim's medium was $Ca(NO_3)_2.4H_2O$ (0.85mM), KCl (0.35 mM), $MgSO_4.7H_2O$ (0.08mM) & $Na_2HPO_4.2H_2O$ (0.11mM). Cells were fed daily on green alga *Chlorogonium elongatum*. Cells were kept at 23 ± 1 ºC temperature in B.O.D. with sufficient food.

2.3. Protargol staining of the cells

The Protargol (chemically it is 'Silver Proteinate' or 'Silver Albumose') staining technique which is a far more effective alternative to the prevailing but niggly silver nitrate solutions, was first developed in 1897 by chemist Arthur Eichengrün. Ever since, it underwent several changes by several workers, starting from 1940 by Cole and Day, through 1975 by Wilbert, and so on (Pan et al., 2013). The Protargol impregnation protocol used in the present study was that of Wilbert's (Wilbert, 1975), with minor modifications by Kamra and Sapra (1990) for revealing the infraciliature and the ciliary bases present on the surface of the cells. Briefly, the ciliates were fixed in regular Bouin's fixative. Following fixation, these cells were coated with albumin-glycerine mixture and bleached in 0.6% sodium hypochlorite (NaOCl) solution. The cells were then impregnated with 2% freshly prepared Protargol (Silver Proteinate) solution. Following impregnation, the slides were dipped in the 'slow developer' solution, a departure from the original protocol, to allow the stain to develop the anticipated colour. Silver stain was then fixed by dipping the slides in 5% sodium thiosulphate ($Na_2S_2O_3.5H_2O$). The slides were finally alcohol dehydrated, xylene cleared and mounted in D.P.X.

2.4. Morphometry and developmental morphogenesis

Though the species *Notohymena gangwonensis* was discovered and reported earlier (Kim et al., 2019) along with its morphology and morphometry, its developmental morphogenetic characterisation, description and reporting of the Indian population is being done for the first time in the present study. The infraciliature of the organism was studied using Protargol impregnation following the method of Kamra and Sapra (1990) and Wilbert (1975) as mentioned above. Following silver impregnation, a total of 20 impregnated cells were counted and measured under the oil immersion objective lens (1000X). The terminology used for describing the species in the present study was in conformity with other standard reference studies including those of Berger (2008), Foissner and Stoeck (2011) and Küppers et al. (2011); while, the cirri numbering system used was the one developed by Wallengren (1900), Borror (1972), Martin (1982) and Hemberger (1985).

3. RESULTS

The ciliate species discovered was an Indian population of recently discovered *Notohymena gangwonensis*, following standard practices of binomial nomenclature.

3.1. Occurrence

Indian population of *Notohymena gangwonensis* was isolated from the sewage sludge collected from Delhi Jal Board's WWTP from Rithala, Delhi, India.

3.2. Characteristic features

The average size of the protargol-stained cells was observed to be about 51x15µm. Other features unique to this species include flexible body; lanceolate anterior end and rounded posterior end; UMs arranged in *Notohymena* pattern; a large, deep buccal cavity; presence of two macronuclear nodules along with 1- 4 micronuclei; a total of 18 frontal-ventral-transverse ($F_{1-8}V_{1-5}T_{1-5}$) cirri; 14 right marginal cirri; 13 left marginal cirri; an average of 19 adoral membranelles; 6 dorsal kineties (DKs) of bristles including two dorso-marginals (DMs), *i.e.*, ($DK_{1-4}DM_{1-2}$), and 3 caudal cirri.

3.3. Description of the species *N. gangwonensis* Indian population

The size of protargol-stained non-dividing cells was around 51x15 µm. The anterior end of the cell was lanceolate while the posterior one was rounded. The cells have parallel margins. The body of these ciliates is generally flexible, dorsoventrally flattened with the length to width ratio of 3.5:1.

Macronuclear nodules measure 10x6 µm each. One to four spherical compact micronuclei measuring around 4 µm each were also observed attached to the macronuclear nodules at variable positions. The morphometric characteristic features are summarised in table 1.

The AZM consisted of about 19 membranelles, and occupies almost 34% of the body length. The buccal cavity was noticed to be comparatively a larger and deeper one with undulating membranes (UMs) arranged in the typical *Notohymena* pattern. The ventral ciliature consists of 18 FVT cirri. The anterior frontal cirri were slightly larger than those of ventral and transverse cirri. The three postoral ventral cirri (V_1–V_3) can be found closer to the cytostome, with V_1 and V_2 appearing together, while V_3 situated far away from the other two. The pre-transverse ventrals were found near the transverse cirri and were smaller than the transverse as well as the other ventral cirri. T_1-T_4 were found arranged in an oblique-linear row adjoined by T_5 forming a hook shape arrangement. Both the Right and Left Marginal Cirri (RMC & LMC) rows converge slightly posteriorly (Figures 1A & 2A).

The dorsal ciliature was arranged into 6 rows namely the DK_{1-4} and DM_{1-2}. The first three (DK_{1-3}) ciliary rows were complete, with a curvature in the posterior half of the cell. Of these three, the DK_1 was anteriorly shortened as compared to the rest of the dorsal rows. Though the DK_4 was also found curved, it is very short and consists of only 4-5 bristles and occupies the posterior 2/3rd region of the cell. The curvature of the DK_4 was opposite to that of the three longer kineties (DK_{1-3}). The rest of the two rows, the $DM_{1\ \&\ 2}$, are short and cover the cytostomal region of the cell. Another important observation was that the $DK_{1,2\ \&\ 4}$ were found to be ending in one each of the three prominent caudal cirri (Figures 1D & 2C).

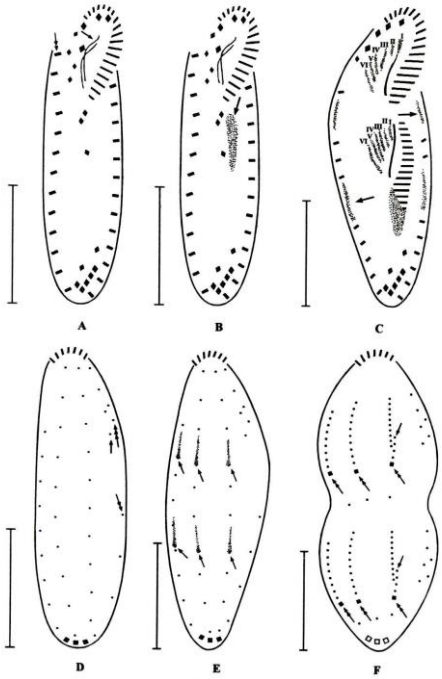

Figure 1: Line diagrams of protargol impregnated cells of *N. gangwonensis* Indian population. showing vegetative surface (A, D) and division morphogenesis on ventral (B, C) and dorsal (E, F) surface. **A.** Vegetative ventral surface: UMs in *Notohymena* pattern (arrow), beginning of RMC row (double arrow). **B.** Origin of OP (arrow). **C.** Two sets of six streaks (I-VI), within the row formation of LMCP and RMCP (arrow). **D.** Vegetative dorsal surface: Shortened DK_4 (double arrow), DM_1 (arrow), very short DM_2 (triple arrow). **E.** Within row proliferation of 2 sets of 3DP and localised proliferation of kinetosomes at their posterior ends (arrows). **F.** Unequal split of DK_3 (arrows), newly formed cc at the ends of $DK_{1,2\&4}$ (double arrows)

3.4. Divisional morphogenesis

During the cell division and morphogenesis of this species, the stomatogenesis was observed to begin with the *de novo* appearance of kinetosomes between the left marginal (LMC) and post-oral ventral (POVC) rows of cirri. Further proliferation of kinetosomes consequently resulted in the formation of a long anarchic field of Oral Primordia (OP).

In case of proter, the parental UMs served as the 'streak I', whereas the disintegrating F_1 and OP formed the 'streak II', thus showing its composite origin. The disaggregating F_7 and F_8 resulted in the formation of 'streak III' and 'streak IV', respectively. The origins of the 'streaks V & VI', however, could not be confirmed during the divisional morphogenesis. In the opisthe, on the other hand, the 'streaks I & II' originated from OP, 'streak III' from V_1, whereas, the 'streaks IV, V, & VI' were then derived from the disaggregating parental V_2 and V_3. In all, two sets of six streaks each were formed in the two daughter cells. In this manner, the cirri differentiated into these streaks in the typical oxytrichid 1,3,3,3,4,4 pattern, forming a total of 18 FVT cirri for each daughter cell as described for sub-family Oxytrichinae (Berger & Foissner, 1997; Berger, 1999; Shao et al., 2015).

Further, the marginal ciliature of the daughter cells developed from two sets of linear streaks, which develop within the marginal rows (RMC & LMC) at their anterior and equatorial ends as described for the family Oxytrichidae (Arora et al., 1999; Berger, 1999; Gupta et al., 2001, 2002, 2003, 2006; Naqvi et al., 2006). These primordia then elongated and differentiated into new marginal rows for the two daughter cells (Figures 1B, 1C & 2B).

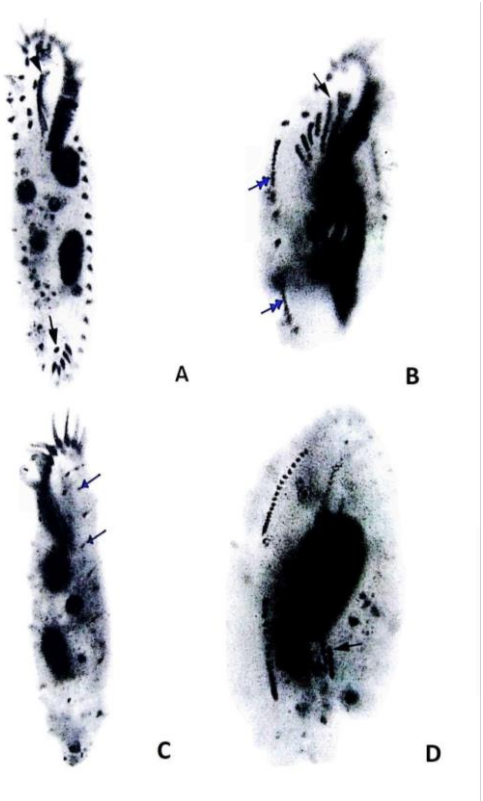

Figure 2: Photomicrographs of protargol impregnated cells of *N. gangwonensis* Indian population. **A.** Vegetative ventral surface: presence of V_5 (arrow). **B.** Streak II of proter formed by the joining of the kinetosomes of OP with disaggregating F1 (arrow), within row formation of the LMCP and RMCP (double arrow). **C.** Vegetative dorsal surface: Distantly placed dorsal bristles (arrows). **D.** Dorsal surface showing the formation of new dorsal kineties rows (arrow).

Development of the dorsal ciliature began within the row by the proliferation of kinetosomes at two levels in DK_{1-3} to form two sets of three dorsal primordia (DP_{1-3}), one each for the daughter cells. These primordia elongate and the DP_3 splits into two unequal parts. The smaller part formed the DK_4. The posterior most kinetosomes of $DK_{1,2\&4}$ have undergone enlargement in size and formed a caudal cirrus in each row. The two DM rows, on the other hand, were formed from the primordia that arise near the right marginal primordia and later moved to the dorsal surface (Figures 1E, 1F & 2D).

Table 1: Morphometric analysis of Indian population of *Notohymena gangwonensis*

Character		**Mean**	**Min**	**Max**	**SD**	**CV**	**N**
Body length		51.1	43.1	58.8	5.26	10.30	20
Body width		14.9	10.8	18.2	1.99	13.32	20
Body length/Body width		3.5	2.9	4.1	0.34	9.86	20
Macronuclear, number		2.0	2.0	2	0.00	0.00	20
Macronuclear, length		9.6	7.8	11.4	1.07	11.12	20
Macronuclear, width		5.5	4.5	7.1	0.76	13.77	20
Micronuclear, number		2.1	1	4	0.61	29.76	20
Micronuclear, diameter		3.4	3.3	3.7	0.11	3.24	20
AM, number		19.3	16	22	1.78	9.22	20
Adoral length		17.5	14.7	19.6	1.29	7.39	20
Adoral length/Body length		0.3	0.3	0.4	0.03	8.82	20
Frontal cirri, number		8.0	8	8	0.00	0.00	20
Ventral cirri, number		5.0	5	5	0.00	0.00	20
Transverse cirri, number		5.0	5	5	0.00	0.00	20
LMC number		13.3	10	17	1.81	13.61	20
RMC number		13.5	10	16	1.64	12.15	20
DKs, number		4.0	4	4	0.00	0.00	20
DMs, number		2.0	2	2	0.00	0.00	20
Dorsal bristles number in	DK_1	9.0	7	10	1.16	12.89	10
	DK_2	9.5	8	11	1.08	11.37	10
	DK_3	9.8	8	12	1.23	12.55	10
	DK_4	4.2	4	05	0.42	10.00	10
	DM_1	4.2	3	05	0.63	15.00	10
	DM_2	2.2	2	3	0.42	19.0	10
Total number of bristles		39.2	33	40	3.52	8.97	10
Caudal cirri, number		3.0	3	3	0.00	0.00	10

4. DISCUSSION

4.1. Occurrence

Indian population of *N. gangwonensis* was discovered from the sewage sludge collected from Delhi Jal Board's WWTP from Rithala, Delhi, India.

4.2. Comparison with other species

Comparative features of the Indian population of *N. gangwonensis* with other reported species of this genus are summarised in the Table 2.

Table 2: Comparison of *Notohymena gangwonensis (*Indian population) with other known species of the genus in terms of morphometry and morphogenesis

Characters	***N. gangwonensis*** Indian population **(Present investigation)**	***N. antarctica*** (Berger, 1999; Foissner, 1996)	***N. selvetica*** (Blatterer & Foissner, 1988; Berger, 1999; Hemberger, 1985)	***N. rubescens*** ((Blatterer & Foissner, 1988; Berger, 1999)	***N. pampasica*** (Küppers et al., 2007)	***N. sapraii*** (Kamra& Kumar, 2010)	***N. limus*** (Naqvi et al., 2016)	***N. gangwonensis*** (Kim et al., 2019)	***N. gangwonensis*** (Moon et al., 2020)
Body shape	Anterior lanceolate & posterior rounded end	Prolate & ellipsoidal	Anterior end rounded& posterior tapering	Left margin convex & right straight	Elliptical	Oblong	Ellipsoidal with anterior end rounded & posterior end lanceolate	*	Elongated and elliptical
Body length (mm)	51	85.5	180	84	96.4	149.20	61.50	74.3	64.3
Body width	15	30.4	65	33.4	38.8	48.80	21.90	25.3	20.0
Body length to width ratio	3.45:1	2.8:1	*	2.5:1	2.48:1	3.05:1	2.9:1	*	*
Ma number	2	2	2	2	2	4	4	2	2
Ma length	10	11-18	*	9-15	13.3	13.80	7.7	10.8	10.5
Ma width	6	7-8	*	6-9	9.7	9.10	6.50	5.7	5.5
Mi number	1-4	2-3	2-3	1-4	2-3	4-6	2	1-3	1-4
AM, number	19	30.2	*	27	22-29	52.70	25.80	25.1	26.3
LMC, number	13	17.9	*	17	17.1	43.90	14.50	14.8	15.1
RMC, number	14	16.8	*	18.3	17.4	43.10	15.60	14.8	14.7
Dorsal kineties	6	6.0	6	6	6	6	6	6	6
OP origin	De-novo	*	*	From transverse	*	De-novo	De-novo	*	*
Caudal cirri	3	3	3	3	3	3	3	3	3
Granules	Absent	Present; arranged in groups; yellow to yellow green in colour	Absent	Present; ruby coloured	Present; colourless	Present; dark green in colour	Colourless sub-pellicular granule; present in groups; arranged around the bases of dorsal bristles	Colourless, 2 types, irregular distribution on cortex	Colourless, 2 types, distributed along the kineties,

Note: * not reported in the literature

4.2.1. Morphology and morphometry

The Indian population of *N. gangwonensis* is easily distinguishable from the other reported species of this genus. The most distinctive feature is its shape- a lanceolate anterior end and a rounded posterior end- which differs considerably from other reported species. The Indian population of *N. gangwonensis* also differs from other *Notohymena* species in several other morphometric features such as body size (length & width), body length-to-width ratio, number of micronuclei and macronuclear nodules, number of AZM, LMC, RMC, cortical granules, and origin of oral primordia as well as the number of dorsal bristles in dorsal kineties. This species also bears resemblances to the other Indian species such as *N. Limus* in terms of its body size (Naqvi et al., 2006), while differs from it in that the latter contains four macronuclear nodules and 1-4 micro nuclei, whereas the former contains only 2 macro nuclear nodules and mostly 2 and rarely 4 micronuclei. Moreover, the Indian population of *N. gangwonensis* matches well with *N. gangwonensis* (Kim et al., 2019; Moon et al., 2020) in its size, body length to width ratio, LMC and RMC numbers, the number and size of micronuclei. However, it differs from *N. gangwonensis* (Kim et al., 2019; Moon et al., 2020) in the absence of cytoplasmic granules which are colourless. The colourlessness of these granules might possibly be because of their inability to get impregnated by the protargol. So, these cells may have cytoplasmic granules which could not be observed in the absence of live cell observations.

4.2.2. Morphogenesis

The morphogenetic development of this species, which is being reported for the first time, differs from that of the other local species, *N. limus*. In the present study, we have found that in the Indian population of *N. gangwonensis*, the morphogenesis starts with the *de novo* appearance of kinetosomes between POVC and LMC. On the contrary, in *N. limus* the morphogenesis process starts with the *de novo* emergence of kinetosomes in the area between V_4 and LMC and above the transverse cirri (Naqvi et al., 2016).

Interestingly, another Indian species, the *N. sapraii* also bears resemblances with the present species under study, the Indian population of *N. gangwonensis*, in morphogenetic developmental aspects, where the OP arises between LMC and three POVC (Kamra & Kumar, 2010), though it is much bigger in size in comparison to the newly discovered Indian population of *N. gangwonensis*. The other known species *N. rubescens* also differs from the current species in that the OP forms in close proximity to the topmost transverse cirrus T_1 (Berger, 1999). In conclusion, because of these striking differences between known species of *Notohymena* discussed above, and because of the stark similarities between the current species and *N. gangwonensis*, the species discovered in the present study would be regarded as the 'Indian population of the *N. gangwonensis*' (Kim et al., 2019; Moon et al., 2020).

5. CONCLUSION

On the basis of above experimental morphological, morphometric, and morphogenetic observations which showed a close resemblance to *N. gangwonensis*, the described *Notohymena* sp. isolated from the sludge samples of Rithala WWTP, Delhi, India is concluded as the Indian population of the *Notohymena gangwonensis*, reported from South Korea.

STATEMENTS AND DECLARATIONS

All authors of this article have made substantial contributions towards the conception and design of the work, acquisition, analysis, and interpretation of data; as well as for the preparation of draft and editing of the manuscript and approved the final version for submission.

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CONFLICT OF INTEREST

The authors further declare that there are no financial or non-financial interests that are directly or indirectly related to the work submitted for publication; the work was not supported by any governmental or non-governmental financial institutions; and the authors declare no conflicting interests.

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