Method Article

Alzheimer and depressive cognitive-like behaviors in male and female rats: A new method for exposure to ambient air pollution

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A B S T R A C T

In the previous studies regarding the effects of exposure to ambient air pollution on biological markers and/or behavior of animals, the gaseous pollutants are not separated from the particulate matter (PM). Hence the synergetic effect of gaseous pollutants and PM was not considered. In this regard, current study was aimed to develop a new method for separation of PM from gaseous pollutants. Also, the effect of exposure to fine particulate matter (PM\textsubscript{2.5}) on the Alzheimer and depressive cognitive-like behaviors in rats after 3 and 6 months were investigated. Three chambers were designed including exposure group 1 (PM\textsubscript{2.5} plus gaseous pollutants alone), exposure group 2 (gaseous pollutants alone) and control group (clean air). Exposure time was 5 h per day (9.00 a.m.–2.00 p.m.) for 4 days per week. The concentration of PM\textsubscript{2.5} and gaseous pollutants (O\textsubscript{3}, NO\textsubscript{2}, and SO\textsubscript{2}) were monitored in the exposure hours, continuously. Concentration of PM\textsubscript{2.5} by beta attenuation method and concentration of O\textsubscript{3}, NO\textsubscript{2}, and SO\textsubscript{2} by UV fluorescence was monitored. Also, the concentration of metals including Al, Cr, Mn, Pb, Cd, Ni, Fe, and Cu and 16-polycyclic aromatic hydrocarbons (PAHs) bound PM\textsubscript{2.5} by inductively coupled plasma mass spectrometry (ICP-MS) and gas chromatography-mass spectrometry (GC—MS) were analyzed, respectively. Cognitive-like behavior related to Alzheimer and depressive behaviors were determined by Y maze and Force swimming. The concentration of PM\textsubscript{2.5} in the 3 and 6 months exposure was higher than WHO guideline, significantly (p-value <0.05). The concentration of O\textsubscript{3}, NO\textsubscript{2} and SO\textsubscript{2} in the 3 and 6 months exposure was lower than WHO guideline, significantly (p-value <0.05). The order of metals in the PM\textsubscript{2.5} according to mean concentration Al > Ca > Cu > Cd > Na > Fe > Cr > Ni > Mn > Pb. Also, the sum concentration of 16-PAHs in the PM\textsubscript{2.5} in the 3 and 6 months exposure was 45.7 ± 37.15 ng/m\textsuperscript{3} and 30.04 ± 25.27 ng/m\textsuperscript{3}, respectively. Exposure to PM\textsubscript{2.5} cannot significantly increase Alzheimer and depressive cognitive-like behaviors in the rats. Also, a significant difference between male and female in Alzheimer and depressive cognitive-like behaviors not observed.

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A new method for separation of PM$_{2.5}$ from other PM in the ambient air by ECO-PM sampler was presented.

- A new method for separation of PM$_{2.5}$ from gaseous pollutants in the ambient air by HEPA filter and active carbon was presented.

- Tow exposure groups including exposure 1: PM$_{2.5}$ plus gaseous pollutants and exposure 2: gaseous pollutants only were designed for increased accuracy of the in-vivo study.

- Exposure to PM$_{2.5}$ cannot cause significant increased Alzheimer and depressive cognitive-like behaviors in the rats.

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**Specifications Table**

| Subject area: | Environmental Science |
|---------------|-----------------------|
| More specific subject area: | A new method of exposure to Air pollution in the in-vivo model study. |
| Method name: | In-vivo model; Alzheimer; Depressive and Cognitive-like behaviors |
| Reagents/tools: | Y-maze and forced swimming |
| Experimental design: | Alzheimer and Depressive like behaviors by Y-maze and force swimming was investigated, and concentration of PM$_{2.5}$, O$_3$, SO$_2$ and NO$_2$, PAHs and metals bound to PM$_{2.5}$ based on approved methods was detected during the exposure time. |
| Trial registration: | Not applicable |
| Ethics: | It was approved |
| **Value of the Protocol:** | 
  - The data presented in this article can be useful for managers of air pollution in the urban areas and health management organization. 
  - Due to adverse health effect of air pollution and its consequence on human health effect and increases prevalence Alzheimer and depressive symptoms in the urban areas, investigation on the effect of exposure to air pollution on the Alzheimer and depressive cognitive-like behaviors is necessary. |

**Method details**

Air pollution is a complex mixture of gases, particulate matter (PM), metals and organic compounds [1]. Recently global concerns increased due to the adverse effects of air pollution on human health [2].

In this regard, the International Agency for Research on Cancer (IARC) declared that air pollution is definitive carcinogenic (Group 1) for humans [3]. The relationship between the exposure to air pollution and autoimmune diseases [4], cardiovascular diseases [5], asthma [6], chronic obstructive pulmonary disease (COPD) [7], and cognitive disorders [8] were well approved.

PM classified as the ultrafine (≤0.1 μm), fine (≤2.5 μm) and coarse (2.5–10 μm), and particles [9]. Due to disperse uniformly, remaining as suspended in the air for a long period time, capability of penetration into the deepest respiratory system exposure to PM$_{2.5}$ is a matter of concern [10]. Also, due to the high surface area of PM$_{2.5}$, it can bind with metals, heavy metals, polycyclic aromatic hydrocarbons (PAHs), metalloids, elemental carbon, organic carbon, ammonium, sulfate, nitrate, viruses, bacteria, which could increase adverse health effects [11]. Approximately 7.6% (42 million) of the total number of deaths worldwide is related to Exposure to PM$_{2.5}$ [12].
PAHs in the air and bound PM$_{2.5}$ have mutagenic and/or carcinogenic properties [13] and the metals include chromium (Cr), lead (Pb), arsenic (As), cadmium (Cd), nickel (Ni), manganese (Mn) and copper (Cu) bound with PM$_{2.5}$ induced to oxidative stress [14], following with an increase in DNA lesions [15].

The association between air pollution and neuroinflammatory and neurodegenerative as one of the routes for increasing neurodegenerative disorders (ND) was approved [16,17]. However, pathophysiological mechanisms on the central nervous system (CNS) due to PM exposure is elucidated [18]. Alzheimer’s disease (AD) is progressive and irreversible ND that is most observable in older adults peoples [19]. Almost 30 million persons in the worldwide and more than 4 million in the USA are suffered by the AD [20]. Advanced age is of the main pathogenic factors for AD. However, other common pathogenic factors are included such as family history [21], sex [22], down syndrome [23], cardiovascular disease [24], poor educational level and environmental pollutants [25]. Also, in some societies, Alzheimer’s like behaviors has been associated with air pollution exposure [26]. Exposure to air pollution can be related to depressive diseases [27]. Potential biochemical mechanisms relating to depressive diseases is including oxidative stress; dysfunction of neurotransmitter systems; alterations of neurohumoral, and autonomic regulation [28]. Previous empirical studies have shown that exposure to air pollution can be increases the prevalence of depressive diseases in the Netherlands, Korea and Japan [29]. Also, an increasing number of emergency visits due to depression in Korea and Canada have been observed [30].

In previous investigations regarding the effects of exposure to ambient air pollution on biological markers and/or behavior of animals, the gaseous pollutants are not separated from the particulate matter (PM). Hence, the synergetic effect of gaseous pollutants and PM was not considered. Previous research demonstrated that exposure to ambient PM following gaseous pollutants can have a synergetic effect on the health animals [31,32]. Therefore perform of study on effect of exposure to ambient PM and gaseous pollutants separately is necessary. Hence, in the current study, a new method for separation of ambient PM from gaseous pollutants was presented. Also, changes of Alzheimer and depressive-like behaviors in the male and female rats due to inhalation PM$_{2.5}$ in ambient air by Y-maze and Force swimming cognitive-like behavior tests were investigated.

Material and methods

Rats

Ninety-six six-week-old male (n = 48) and female (n = 48) Wistar rats (with an average weight of 85 ± 10 g) were purchased from Pasteur Institute, Tehran, Iran. Rats were maintained in the laboratory standard conditions including supplying the water, food, libitum, and light (12 h)/dark (12 h) cycles. Also, before the starting study, rats were maintained for one week in similar environmental conditions including temperature (20–25 °C) and relative humidity (40–60%). The protocols of the current study were confirmed by the laboratory animal research principles by the Animal Welfare Act [33]. Ethics approval by Shahid Beheshti University of Medical Sciences Ethics Committee and the number of rats were minimized as far as possible.

Study location and method of exposure

The pilot animal room is located in the roof school of public health in Shahid Beheshti University (35.7991°N, 51.3947°E) at an altitude of 20 m above the ground.

In the pilot animal room three chambers were designed including exposure group 1 (PM$_{2.5}$ plus gaseous pollutants alone), exposure group 2 (gaseous pollutants alone) and control group (clean air). Exposure group 1: Urban ambient air with 12 l/min flow rate was introduced into the chamber by Echo PM (TCR Tecora Italy) low volume sampler (LVS) without sampling filter. It is noteworthy that LVS was designed for sampling of PM in the ambient air (PM$_{10}$ and PM$_{2.5}$), but when the sampling filter removed, outlet air will contain PM (Fig. 1) [34]. In the current study, PM$_{2.5}$ warhead was used; hence, output air contains ambient PM$_{2.5}$ plus gaseous pollutants. Exposure group 2: urban ambient air with 12 l/min flow rate was passed into the chamber by a vacuum pump (Model LFS-113; Gillian Instrument
For removing of PM, HEPA filter model H13 is located in the inlet valve of the vacuum pump. HEPA filter class H13 (Camfill-FARR, Switzerland) removes PM with 99.97%. Therefore, outlet air contains gaseous pollutants alone. Control group: In order removing PM and gaseous pollutants, ambient air with 12 l/min flow rate was subjected into the chamber by a vacuum pump with HEPA filter model H13 (Sungjin Co., Ltd., Korea) and active carbon air filter in the inlet and outlet valves, respectively (Fig. 1).

**Exposure periods**

A number of rats in each group was equal to 32 rats (males = 16 and females = 16). A two-period study including; 3 months (31 December, 2017–30 March, 2018) and 6 months (31 December, 2017–1 July, 2018) was selected (Fig. 2). Similar to the in-vivo model study on the neurological system effect due to air pollution [35,36], exposure time was 5 h per day (9.00 a.m.–2.00 p.m.) for 4 days per week.

**Monitoring PM$_{2.5}$ and gaseous pollutants**

The concentration of PM$_{2.5}$ and gaseous pollutants (O$_3$, NO$_2$, and SO$_2$) were monitored in the exposure days continuously in the ambient air of adjacent of pilot animal room. Concentration of PM$_{2.5}$ by beta attenuation monitoring technique [37] and concentration of gaseous pollutants (O$_3$, NO$_2$, and SO$_2$) by UV fluorescence (Horiba AP-370 series) was monitored which are certified instruments based on EU directives [38].
**Sampling of PM$_{2.5}$**

The sampling of PM$_{2.5}$ ambient air of the pilot animal room was conducted using LVS (Echo PM) from 31 December 2017 to 1 July 2018. The sampling was performed at 20 L/min flow rate with 24 h (9:00 A.M.–9:00 A.M.) Quartz microfiber filters (47 mm diameter, Whatman International Ltd) was used for a collect of PM$_{2.5}$. Before sampling, Quartz microfiber filters (47 mm diameter, Whatman International Ltd) was washed with distilled water and was located in an oven (105 °C, 2 h) [39]. Afterward, QM filters were subjected to relative humidity equal to 40–50% in the room temperature equal to 18–20 °C for 24 h [40]. After sampling, until analysis, to prevent evaporation and photodegradation, QM filters were stored in the –10 °C in the aluminum foil [41].

**Digestion and analysis of metals procedure**

One-fourth of each QM filter was shredded into 15 mL Teflon container. Then, 2.5 mL HNO$_3$ (69%) and 2.5 mL of concentrated of HClO$_4$ (70%) were added in Teflon container and then at 170 °C for 4 h heated until dryness. Hence, the solution was dried at 95–100 °C on a hot plate. Afterward, 2.5 mL of HNO$_3$ and 2.5 mL of distilled water were added to the samples. Samples were shaken at 180 rpm for 30 min [42]. The solutions were filtered via Whatman filter (paper No 42; 125 mm) and diluted up to 10 mL and then were stored in a plastic vial at 4 °C until analysis day. For assess of validity digestion method, blank sample (clean Whatman filter) following with other samples were digested. Based on a previously conducted method [43], metals analysis was conducted via inductively coupled plasma mass spectrometry (ICP-MS Model 7900 Agilent Technologies, Santa Clara, CA). Operating parameters of ICP-MS was presented in Table 1s.

**Digestion and analysis of PAHs procedure**

One-fourth of each QM filter was shredded into 15 mL Teflon container. Then, 2.5 mL CH$_2$Cl$_2$ and 2.5 mL of CHCl$_3$ were added in Teflon container and was sonicated in an ultrasonic bath with 20 kHz for 30 min (Elmasonic S 80H). Finally, solutions were filtered via 0.22 μm Millipore PTFE filters (Hesperia, CA, USA). The concentration of 16-PAHs was performed using gas chromatography–mass spectrometry (GC–MS) (Agilent model 5890A). Operating parameters of GC–MS presented was demonstrated in Table 2s.
Quality assurance

Heavy metals
The concentration of metals in the sample was detected at 3 replications and the mean was calculated. The recovery rate of metals ranged from 83 to 98% (RSD: 2–6%). The limit of detection (LOD) and limit of quantitation (LOQ) for Pb was 5 µg/L and 1 µg/L. However, these values for other metals were 25 µg/L and 5 µg/L, respectively (Table 3s).

16-PAHs
The concentration of 16-PAHs in the sample was detected at 3 replications, and the mean was calculated. The recovery rate of 16 PAHs ranged from 79 to 98% (RSD: 4–8%). PAHs were not observed in any of the analytical blanks. LOD and LOQ for 16 PAHs was <2 ng/l and <10 ng/l, respectively (Table 4s).

Cognitive-like behavior assessment via Y maze
To investigate cognitive-like behavior, the Y maze test was conducted with at least 8 rats in groups. Y maze test was performed in the light phase of the cycle for least to stressful [44]. The custom-made Y maze has designed as a ‘Y’ shape with 3 arms (A, B and C) (10 cm wide, 60 cm long and 25 cm high) which are at 120° angle. Custom-made Y maze was located at the center of a behavior room at the low light conditions (Figs. 1 and 2).

Y maze test was performed in 2 steps [45]. In step 1, rats were placed in the long arm (A-arm) with lateral closed for 20 s and in step 2, lateral A-arm is open for 8 min. Rats were excluded from the test when had lower than 12 arm entries and/or have not new entries for 2 min during 8 min period. Healthy rat with hippocampal memory and learning have a preference for the search of new locations [46]. Hence rat with higher alternation indicates more healthy hippocampal memory and learning [46]. When rat completed entries into a new arm before returning to the visited arm successful alternation was happened [47]. The results of the test were presented as alternation (%), that was calculated by the following equation [46,47]:

\[
\text{alternation} = \frac{\text{Number of successful arm entries}}{\text{Total arm entries} - 2} \times 100
\]

Cognitive-like behavior assessment via force swimming
In the forced swimming test, the duration of immobilization is equivalent to depression and its reduction as an antidepressant effect. After exposure, the rats were placed separately in a transparent cylindrical container with 30 cm diameter and 80 cm depth. The depth of water was 20 cm, and its temperature was 25 °C. Stopping the movement’s hands and feet of the rat was considered as a time of immobilization. The whole test was 6 min, which was 2 min for adaptation of the rat and in the 4 min was recorded immobilization, swimming and climbing time of rats via chronometer [48].

Statistical analysis
Distribution of concentration of PM2.5, gaseous pollutants (O3, NO2, and SO2), alternation and immobilization time was determined by Kolmogorov–Smirnov (KS) test. Compare concentration of PM2.5, gaseous pollutants (O3, NO2, and SO2) with standard limits was performed by one sample t-test. Difference alternation (%) and immobilization time among three groups of rats were determined by two-way analysis of variance (ANOVA) analysis with Tukey’s test. All statistical analyses and graph design were conducted using GraphPad PRISM version 5.01 software (Inc., San Diego, CA. USA). All results mentioned as the mean ± standard error of the mean (M ± SEM). If p-value <0.05 selected as statistically significant.
Table 1
Concentration of PM$_{2.5}$ and gaseous pollutants in ambient air of pilot animal room.

| Contaminants | Duration of exposure | Unit | Mean | Range | WHO guideline |
|--------------|----------------------|------|------|-------|---------------|
| PM$_{2.5}$   | 3 Months             | µg/m$^3$ | 37.76 ± 11.96 | 13.17–58.57 | 25 |
|              | 6 Months             | µg/m$^3$ | 31.61 ± 11.20 | 10.01–58.57 | |
| O$_3$        | 3 Months             | ppb  | 16.55 ± 2.30  | 12.00–23.00 | 100 |
|              | 6 Months             | ppb  | 23.37 ± 7.72  | 12.00–39.00 | |
| SO$_2$       | 3 Months             | ppb  | 5.90 ± 1.30   | 4.00–8.00   | 20 |
|              | 6 Months             | ppb  | 5.37 ± 1.23   | 3.00–8.00   | |
| NO$_2$       | 3 Months             | ppb  | 56.88 ± 7.82  | 43.00–77.00 | 100 |
|              | 6 Months             | ppb  | 53.28 ± 8.73  | 37.00–77.00 | |

Results

The concentration of PM$_{2.5}$, O$_3$, NO$_2$, and SO$_2$

The concentration of PM$_{2.5}$ in the 3 and 6 months of exposure was presented in Table 1. The mean concentration of PM$_{2.5}$ in the 3 and 6 months exposure was 37.76 ± 11.96 and 31.61 ± 11.20 µg/m$^3$, respectively. Concentration of PM$_{2.5}$ in the 3 and 6 months exposure was higher than WHO guideline (25 µg/m$^3$), significantly (p-value <0.05) [49]. The mean concentration of O$_3$ in the 3 and 6 months exposure was 16.55 ± 2.30 and 23.37 ± 7.72 ppb, respectively. The concentration of O$_3$ in the 3 and 6 months exposure was lower than WHO guideline (100 ppb), significantly (p-value <0.05) [49]. The mean concentration of SO$_2$ in the 3 and 6 months exposure was 5.90 ± 1.30 and 5.37 ± 1.23 ppb, respectively. The concentration of SO$_2$ in the 3 and 6 months exposure was lower than WHO guideline (20 ppb), significantly (p-value <0.05) [49]. The mean concentration of NO$_2$ in the 3 and 6 months exposure 56.88 ± 7.82 and 53.28 ± 8.73 ppb, respectively. The concentration of NO$_2$ in the 3 and 6 months exposure was lower than WHO guideline (100 ppb), significantly (p-value <0.05) [49].

In the current study, unlike gaseous pollutants, concentration of PM$_{2.5}$ was higher than WHO guideline (Table 1) mainly due to their higher emission from sources. Emission sources of PM$_{2.5}$ in the urban areas are including natural (dust), anthropogenic (combustion of fuel in vehicles) and marginal sources (PM of factories around the urban area) but gaseous pollutants emitted from anthropogenic sources mainly [2]. On the other hand, gaseous pollutants are rapidly converted to other compounds due to their reactivity [9,50].

The concentration of heavy metals bound with PM$_{2.5}$

The concentration of heavy metals bound with PM$_{2.5}$ was presented in Table 2. The order of heavy metals bound with PM$_{2.5}$ based on mean concentration in first 3 months exposure was ranked as
Al > Ca > Cu > Cd > Na > Fe > Cr > Ni > Mn > Pb (Table 2). Also, the order of metals bound with PM$_{2.5}$ based on mean concentration in 6 months exposure was Ca > Al > Cu > Cd > Na > Fe > Cr > Ni > Mn > Pb. The results showed that the concentration of Al was highest while compared with other metals bound PM$_{2.5}$ after 3 months exposure while the concentration of Ca was the highest among other metals bound PM$_{2.5}$ after 6 months exposure.

The concentration of PAHs bound with PM$_{2.5}$

The concentration of PAHs bound with PM$_{2.5}$ was presented in Table 3. The sum concentration of 16 PAHs bound PM$_{2.5}$ in the 3, and 6 months exposure was 45.7 ± 37.15 ng/m$^3$ and 30.04 ± 25.27 ng/m$^3$, respectively. The order of 16-PAHs was Phenanthrene > Naphtalene > Chrysene > Anthracene > Acenaphthylene > B(a)P > Acenaphten > Florene > Pyrene > Fluorantene > Benzo(a)ant > B(k)F > Indeno (1,2,3-cd)Pyrene > B(b)F > Dibenzo(a,h)Anthracene > Benzo g,h,i Perylene. According to findings, the concentration of Phenanthrene was higher than other PAHs bound PM$_{2.5}$ that were inhalated by rats after both 3 and 6 months exposure.

| 16-PAHs                  | 3 Months  | 6 Months  |
|--------------------------|-----------|-----------|
| Naphtalene               | 7.17 ± 16.17 | 4.39 ± 11.4 |
| Acenaphthen              | 1.99 ± 0.8  | 2.57 ± 0.43 |
| Florene                  | 2.96 ± 1.92 | 1.67 ± 1.34 |
| Phenanthrene             | 0.96 ± 0.57 | 1.33 ± 0.34 |
| Anthracene               | 18.98 ± 10.35 | 9.86 ± 7.26 |
| Fluorantene              | 5.3 ± 3.27  | 2.89 ± 2.3  |
| Pyrene                   | 1 ± 0.57    | 0.86 ± 0.38 |
| Benzo(a)ant              | 1.74 ± 0.78 | 0.87 ± 0.55 |
| Chrysene                 | 3.05 ± 1.27 | 3.05 ± 1.27 |
| B(b)F                    | 0.35 ± 0    | 0.35 ± 0   |
| B(k)F                    | 2.2 ± 1.45  | 2.2 ± 1.45 |
| Dibenzo(a,h) Anthracene  | ND         | ND         |
| Benzo(g,h,i) Perylene    | ND         | ND         |
| Indeno(1,2,3-cd)Pyrene    | ND         | ND         |
| **Sum**                  | **45.7 ± 37.15** | **30.04 ± 25.27** |

Fig. 3. Compare changes alternation (%) in the male and female rats after 3 months exposure.
Fig. 4. Compare changes alternation (%) in the male and female rats after 6 months exposure.
Fig. 5. Compare changes immobilization time (s) in the male and female rats after 3 months exposure.
Fig. 6. Compare changes immobilization time (s) in the male and female rats after 6 months exposure.
Cognitive-like behavior assessment

Y maze and force swimming

Exposure to ambient PM$_{2.5}$ plus gaseous pollutants did not affect alternation in the male and female rats after in both 3 and 6 months periods study (p-value >0.05) (Figs. 3 and 4). Exposure to gaseous pollutants alone had no significant effects on alternation in both male and female rats (p-value >0.05) (Figs. 3 and 4). Also, a significant difference between alternation in male and female rats in both periods study was not observed (p-value >0.05) (Figs. 3 and 4).

Exposure to ambient PM$_{2.5}$ plus gaseous pollutants did not affect immobilization time in the male and female rats after in both 3 and 6 months periods study (p-value >0.05) (Figs. 5 and 6). Exposure to gaseous pollutants alone had no significant effects on immobilization time in both male and female rats (p-value >0.05) (Figs. 5 and 6). Also, a significant difference between immobilization time in male and female rats in both periods study was not observed (p-value >0.05) (Figs. 5 and 6). The alternation and immobilization time in the exposed groups was not significantly higher than the control group (p-value >0.05) (Figs. 3–6).

Conclusion

In the current study, effect of exposure to PM$_{2.5}$ and gaseous pollutants on the Alzheimer and depressive cognitive-like behaviors in the rats after 3 and 6 months of exposure by approaching a new method exposure was investigated. The concentration of PM$_{2.5}$ in the both 3 and 6 months exposure was higher than WHO guideline, significantly (p-value <0.05) however concentration of O$_3$, NO$_2$, and SO$_2$ was lower than the WHO guideline, significantly (p-value <0.05). Among metals bound PM$_{2.5}$, the concentration of Al and Ca was higher than other metals and also among 16-PAHs bound PM$_{2.5}$, the concentration of Phenanthrene was higher than other PAHs. Exposure to PM$_{2.5}$ and gaseous pollutants separately cannot significantly increase Alzheimer and depressive cognitive-like behaviors in the rats after both 3 and 6 months exposure. Also, a significant difference between male and female in Alzheimer and depressive cognitive-like behaviors not observed.

Conflict of interest

The authors of this article declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mex.2019.03.018.

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