Activated Sludge Respiration Activity Inhibition Caused by Mobile Toilet Chemicals

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Abstract: Ensuring high quality drinking water sources is important task nowadays. To reach this task, knowledge about the impact of different chemicals on aerobic wastewater treatment is mandatory. A mixture of different chemicals reaches wastewater treatment plant every day. With the growing discharge volume of mobile toilet chemicals, active substances in these products in the past years have been recorded. The respiratory activity of activated sludge was determined to show how mobile toilet chemicals and their active substances may affect the biological wastewater treatment process. The results show negative effect of formaldehyde and bronopol on respiratory activity of activated sludge. The wastewater treatment plants influent composition and size also play important roles. Results shows that activated sludge microorganisms at a wastewater treatment plant in industrial urban area may be adapted to the higher pollutants concentration. When mobile toilet tanks are directly discharged at smaller wastewater treatment plant, an activated sludge process can be affected. For treating mobile toilet wastewater, bacterial degraders can be used. During our respiratory activity experiments, potential degraders were searched. Ralstonia sp. prevails in all samples and it is therefore a potential mobile toilet chemicals degrader.

Keywords: activated sludge; bronopol; formaldehyde; chemical toilets; biological oxygen demand

1. Introduction

Safe and readily available water is important for public health, whether it is used for drinking, domestic use, food production, or recreational purposes. Inadequate management of urban, industrial, and agricultural wastewater means the drinking-water of hundreds of millions of people is dangerously contaminated or chemically polluted [1]. Wastewater treatment can be affected by many different substances, which can be discharged to the sewer system or directly at the wastewater treatment plant. This is particularly important in small wastewater treatment operations for which side-streams might constitute a significant portion of influent. One group of chemical substances with a possible impact on the activated sludge process during sewage treatment can be found in cesspools and septic tanks, and during the servicing of chemical toilets. Even 20 years ago, the use of mobile toilets was very limited. In last decades massive usage of chemical toilets in different areas of human activities is obvious. Nowadays, mobile toilets are part of every construction site, the organization of festivals has expanded for thousands of citizens, tourism has increased. Mobile toilets are used in recreation vehicles, boats, recreational facilities, etc. For this reason, wastewater is burdened more and more. The average production of wastewater in the EU is 150 L per person per day. Production from other sources (cesspools, septic tanks and chemical toilets) can represent, in connection with
the size of the treatment plant, up to 20% of the inflow. The high strength wastewater produced from the sources above is rich in biological oxygen demand (BOD), chemical oxygen demand (COD), total solids (TS), and nitrogen [2–4]. Synthetic organic chemicals such as those found in chemical toilets are usually hardly biodegradable in activated sludge process and can be toxic to activated sludge microorganisms without proper acclimation [5–7]. Based on our screening, common commercial additives for chemical toilets contain different types of chemical compounds. Most frequently used are formaldehyde, bronopol, limonene, and citric acid. The limonene and citric acid were used as deodorants. Formaldehyde is very widely used in industry. In chemical toilets, it is used for its biocides properties [8]. Relatively high formaldehyde concentrations can be found in industrial wastewater 0.2–10 g/L [9–11]. Formaldehyde-rich wastewater at municipal wastewater treatment plant may cause inhibition of microbial activity during aerobic biological wastewater treatment [6,7]. Bronopol is used as an active biocide, surfactant and preservative in cosmetics [8,12]. In aqueous solutions, bronopol degrades to various products with biocide effect on activated sludge [13–15]. Limonene is one of the most widespread monoterpenes [16], it is found mostly in the essential oils of citrus fruits. Antimicrobial activity of limonene was also reported [17–19], however its hydrophobicity may be a limitation in aqueous environments. Citric acid is the most widely used acid in the food industry. Citric acid has antimicrobial properties due to its acidulation. Studies have indicated that the chelating effect of citric acid also inhibits bacteria [20].

The aim of the present work was to study the possible toxic effect of chemicals commonly used in mobile chemical toilets on microbial activated sludge respiratory activity during wastewater treatment process. The water solution incurred after BOD tests was used for screening bacteria as the potential degrader of toxic chemical substances in mobile toilet products. Such information is useful for optimizing the treatment of high strength wastewater originated from chemical toilets contains.

2. Materials and Methods

2.1. Mobile Toilet Chemicals

For activated sludge respiratory activity assays, the following mobile toilet chemicals commonly available on the market were used (Table 1). Most of the products contain bronopol as an active substance.

| Product          | Manufacturer         | Prevailed Chemical Substance                  |
|------------------|----------------------|-----------------------------------------------|
| Qualicar STACHEMA CZ, CZ | formaldehyde (<16% w/w) |
| Instagreen Spezial Campingaz, FR | citric acid (>5% w/w) |
| Elsan Double Blue Elsan Limited, UK | formaldehyde (20–30% w/w) |
| Aqua Kem Blue Thetford, US | formaldehyde (20–30% w/w) |
| Aqua Rinse Spray Thetford, US | bronopol (2-bromo-2-nitropropan-1,3-diol) (0.1–1% w/w) |
| Aqua Kem Sachets Thetford, US | bronopol (2-bromo-2-nitropropan-1,3-diol) (20–30% w/w) |
| Porta-Pak * Walex, US | bronopol (2-bromo-2-nitropropan-1,3-diol) (3–8% w/w) |
| Power Care Tabs Dometic, SE | bronopol (2-bromo-2-nitropropan-1,3-diol) (1–10% w/w) |
| Safe-T-Fresh 5000 Satellite, US | bronopol (2-bromo-2-nitropropan-1,3-diol) (1–10% w/w) |
| Safe-T-Fresh 4000 * Satellite, US | bronopol (2-bromo-2-nitropropan-1,3-diol) (1–10% w/w) |

* used for activated sludge respiratory activity testing, most frequently used by mobile toilet operators in the monitored area

2.2. Other Chemicals

Based on the mobile toilet chemicals composition, following pure chemicals for activated sludge respiratory activity assays have been used. (R)-(−)-Limonene 97% (Merck, Darmstadt, Germany), 2-Bromo-2-nitro-1,3-propanediol (Bronopol, BNP, purity ≥ 99%) (Merck, Darmstadt, Germany), Formaldehyde p.a. (PENTA, Prague, Czech Republic), Citric Acid p.a. (PENTA, Prague, Czech Republic).
2.3. Sludge Samples

Activated sludge samples were taken at the wastewater treatment plants (WWTPs) Brno-Modřice, 513,000 PE (population equivalent), Czech Republic and Moravany 5000 PE, Czech Republic. The activated sludge was directly collected from the activation sludge tank at the wastewater treatment plant. After the collection in sterile containers, the activated sludge samples were in cooling box immediately transported to the laboratory. Activated sludge samples were used for respiratory activity tests and subsequently for bacteria isolation.

2.4. Respiratory Activity Testing

In general, respiration activity can be followed by the determination of CO$_2$ production and oxygen uptake to allow the identification of the beginning and end of biodegradation. Continuous measurement of activated sludge respiratory activity was determined by an OxiTop Control system (WTW GmbH, Weilheim, Germany) in accordance with Organisation for Economic Co-operation and Development (OECD) Guideline 301 F [21]. The test determines the complete biodegradability of organic substances in an aqueous basic medium by determining the oxygen consumption in a closed respirometer. The test specimen is added to medium as the single carbon source. The CO$_2$ developing due to oxygen consumption is absorbed by concentrated NaOH and manometrically measured as a negative pressure. The system determines biological oxygen demand (BOD) indirectly. The results are expressed in mg O$_2$/L of the sample.

For sterile basic medium preparation, 1 g malt extract (OXOID CZ Ltd., Brno, Czech Republic), 0.5 g peptone (OXOID CZ Ltd., Brno, Czech Republic), and 1000 mL of deionized water were used. Each sample bottle (510 mL) was filled with activated sludge (30 mL), sterile basic medium (50 mL), deionized water (17 mL). After the addition of the tested chemical, the final volume in each sample bottle was adjusted to 97 mL. The sample bottles were placed in a cooling thermostat Liebherr FKU 1800 (Liebherr, Bulle, Switzerland) situated on an inductive stirring plate and the temperature was maintained at 20 °C for 120 h.

In the first sets of experiments two mobile toilet chemicals were used (Table 1, marked by asterisk). The maximal volume, thus maximal load of active substance, of mobile toilet chemicals used for respiratory activity test was based on the maximal possible volume which can be present in wastewater tank, according to the manufacturer instruction. The experiments were conducted during 2018 and in total 10 assays for each WWTP were done. In the second sets of experiments pure chemicals prevailed in the mobile toilet chemicals were used. Calculated dose for individual compounds were, bronopol 0.05, 0.2, 0.3, 0.4, 0.5 mM; formaldehyde 0.3, 0.5, 0.6, 0.8, 1 mM; citric acid 0.2, 0.4, 0.6, 0.8, 1.0 mM; limonene 0.01, 0.1, 0.4, 0.7, 1.0 mM. All tests were done in triplicates. Acclimation period and plots were calculated and built using software package Origin7.0 (OriginLab Corporation, Northampton, UK) and MS Office.

2.5. Isolation of Pure Bacterial Strains

After first sets of activated sludge respiratory activity tests the remaining solution was used for bacteria isolation. Solution containing both activated sludge samples (WWTP Modřice and WWTP Moravany) were used. Briefly, 1 mL of solution was diluted by 10-fold serial dilution and dilution $10^{-5}$–$10^{-8}$ were plated on Nutrient agar (Peptone 5.0 g/L; Meat extract 3.0 g/L; Agar 15.0 g/L). pH was adjusted to 7.0. Plates were incubated at 20 °C for 7 days. Pure bacterial strains were isolated.

2.6. Bacterial Strains Identification

Bacterial DNA was isolated from pure bacterial cultures by freeze-thaw method and amplified using the PSP® Spin Stool DNA Kit (Stratec, Birkenfeld, Germany). The success of PCR amplification was confirmed by gel electrophoresis. DNA was sequenced by the Sanger sequencing method. DNA sequences were analyzed by BLAST in NCBI database.
2.7. Statistical Analysis

Using the experimental data, the basic statistical parameters (M—mean, SE—standard error, M ± SE) were calculated. The accurate approximation was when p ≤ 0.05 [22]. To more understanding the effect of the compounds studied above on respiratory activity of activated sludge, the principal component analysis (PCA) was carried out. PCA is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The algorithm of the PCA of each compound of different concentration is shown in the Figure 1.

![Principal component analysis (PCA)](image)

**Figure 1.** The algorithm of principal component analysis (PCA) of each compound of different concentration.

PCA was done using Statistica 13.5 software (https://it.muni.cz/sluzby/software/statistica) based on concentrations of each compound (formaldehyde, bronopol, limonene, and citric acid) separately in WWTP Modřice and Moravany. PCA was also carried out based on the data on the effect of different concentrations of compounds together, for individual WWTPs. To more understanding the effect of all compounds and their concentrations in complex, the PCA based on the all parameters together (formaldehyde, bronopol, limonene, and citric acid) in WWTPs Modřice and Moravany. The plots were built by software package Origin7.0 (OriginLab Corporation, Northampton, UK).

3. Results

Our results show that the concentration of mobile toilet chemicals in the wastewater affected the respiratory activity of microorganisms in the activated sludge. Moreover, it is interesting to compare the length of acclimation period of the microbial communities in activated sludge at these different chemical agents used (Figure 2a,b). The acclimation period length represents the microbial adaptation to the activated sludge environment and their ability to degrade organic pollutants in sludge. The acclimation periods increased progressively as the concentration of toilet agents were increased. The principal active ingredient in both products was bronopol. It should be noted that the mobile toilet chemical Porta–Pak was dosed in lower concentrations compared to Safe—T-Fresh.
4000 according the manufacturer instructions. Figure 2a shows the results for Porta-Pak. The results clearly show that the acclimation period of microorganisms in activated sludge is longer in the case of a wastewater treatment plant Moravany (5000 PE). Activated sludge microorganisms in the Modřice wastewater treatment plant (513,000 PE) are more resistant to changes in external factors or the presence of pollutants. The results are similar for Safe-T—Fresh for higher concentrations of toilet chemicals (Figure 2b). Contrary, the results for low concentrations of mobile toilet chemicals are different in this graph. No acclimation periods for the activated sludge microorganisms originated from Moravany WWTP were recorded for 3000 and 7000 µL/L dose of Safe-T—Fresh product.

![Graph](image)

**Figure 2.** Activated sludge acclimation period based on different dose of mobile toilet products.

After the initial experiments with mobile toilet chemical products, the role of the individual active substances in the products were investigated. The respiratory activity of activated sludge was determined for four individual active substances commonly present in the mobile toilet chemicals. Formaldehyde, bronopol, limonene and citric acid were prevailing chemicals in most toilet chemical products (Table 1). The results of the experiments are shown in the in Figure 3. Formaldehyde and bronopol are chemicals that significantly affect the respiratory activity of activated sludge. As the concentration of these substances increases, the respiratory activity of microorganisms in the WWTP Modřice (513,000 PE) and WWTP Moravany (5000 PE) decreases. In particular, formaldehyde and bronopol have a strong toxic effect on microorganisms, which results in a prolonged acclimation phase of the community and a decrease in overall respiratory activity. For the highest (1 mM) formaldehyde concentration, a 37% decrease in activated sludge respiration activity for WWTP Modřice and 95% decrease in activated sludge respiration activity for WWTP Moravany was determined. Bronopol at the highest concentration (0.05 mM) showed decrease in activated sludge respiration activity 37% for WWTP Modřice and 48% for WWTP Moravany. Respiratory activity of activated sludge microorganisms was not significantly affected in experiments with different concentrations of limonene or citric acid (Figure 3). No significant differences were observed between the activated sludge respiratory of a small and large wastewater treatment plant, depending on the increasing concentration of these chemical substances.
Figure 3. Development of the respiratory activity biological oxygen demand (BOD) of activated sludge in presence of various concentrations of active substances used in chemical toilets.
To more understanding the effect of the compounds studied above on respiratory activity of activated sludge, the principal component analysis (PCA) was carried out. PCA was done based on concentrations of each compound (formaldehyde, bronopol, limonene, and citric acid) separately in WWTP Modřice and Moravany (Figure 4). Different concentration of formaldehyde form two separated clusters in WWTP Modřice; first cluster shows that control was close with concentration 0.3 mM of formaldehyde and higher concentrations from 0.5 to 1 mM were combined in second cluster. However, it should be noted that formaldehyde of 0.3–0.6 mM concentrations formed separately points of each in WWTP Moravany and does not form cluster. It is the same case with higher concentrations 0.8–1 mM. The concentrations of bronopol (0.05–0.5 mM) were not differing in WWTPs Modřice and Moravany. Each concentration form separated cluster in both WWTPs. The controls also formed one cluster. Other pattern was observed with limonene; clusters were formed in two planes for WWTP Modřice and Moravany separated. Similar observation as limonene can be seen in the case with citric acid, but the concentration of 1 mM WWTP Modřice formed the cluster with control of WWTP Moravany. Perhaps, this concentration could be more toxic for WWTP Moravany.

![Figure 4. Principal component analysis (PCA) based on the effect of formaldehyde, bronopol, limonene, and citric acid (blue indicates the concentrations of the compounds in WWTP Modřice and black indicates the concentrations in WWTP Moravany).](image-url)

PCA was also carried out based on the data on the effect of different concentrations of compounds together, for individual WWTPs. As it can be seen from Figure 5, bronopol in the highest concentration 0.5 mM formed outlying point for WWTP Modřice. In this case, the lower concentrations of bronopol (0.2–0.4 mM) formed two clusters together with the concentrations of formaldehyde (0.5–1 mM). Citric acid did not show distribution into clusters by PCA based on all parameters in WWTP Modřice. Different concentration of limonene formed two close but separated clusters. One cluster formed with concentrations of 0.01, 0.4, and 1 mM and the second was control with 0.1 mM of limonene and 0.3 mM of formaldehyde. Not less interesting pattern was observed in WWTP Moravany, where concentration of citric acid, limonene, and bronopol formed one main cluster. However, formaldehyde
in the concentration of 0.3 mM formed the cluster with bronopol (0.3 mM) and the same cluster was observed in the case of 0.5–0.6 mM of formaldehyde with 0.3 mM of bronopol in WWTP Moravany.

**Figure 5.** PCA based on the effect of different concentrations of compounds separately in WWTP Modřice and WWTP Moravany: the color indicates the concentrations of the formaldehyde (brown), bronopol (green), limonene (red), and citric acid (violet).

PCA based on the effect of all active substances together (formaldehyde, bronopol, limonene, and citric acid) in different concentrations in WWTPs Modřice and Moravany is shown in Figure 6. As can be seen, limonene and citric acid formed one cluster in both WWTPs. The second cluster in both WWTPs was formed by bronopol. Formaldehyde formed outlying points for both WWTPs.

**Figure 6.** PCA based on the effect of all parameters of formaldehyde, bronopol, limonene, and citric acid (blue indicates the compounds in WWTP Modřice and black indicates compounds in WWTP Moravany).

Activated sludge used for the first set of respiratory activity tests was diluted and inoculated on agar media for subsequent pure bacterial cultures isolation. Both WWTPs activated sludge were used for bacteria isolation. Bacteria isolated as pure cultures were stained (Gram staining) and observed in optical microscope (Figure 7).
Figure 7. Pure bacterial activated sludge cultures: *Paracoccus aminophilus* MT003978 (A), *Phyllobacterium brassicacearum* MT005284 (B), *Ralstonia pickettii* MT003984 (C), *Acinetobacter johnsonii* MT004766 (D), *Sphingomonas* sp. MT004768 (E), *Novosphingobium* sp. MT004815 (F), *Psychrobacter maritimus* MT004816 (G), *Ralstonia solanacearum* MT004912 (H), magnification 1000×.
To accurately identify isolated bacterial species, DNA was isolated and Sanger sequencing method was performed using 16S rRNA primer sets. The identity of sequenced DNA samples with the reference species in GenBank were found from 99.27% to 100%. The genomic sequences are available in GenBank, access No.: MT003978, MT005284, MT003984, MT004766, MT004768, MT004815, MT004816, MT004912.

Bacteria repeatedly isolated from WWTP Modrice and WWTP Moravany activated sludge belong to genera Paracoccus, Phylobacterium, Ralstonia, Acinetobacter, Sphingomonas, Novosphingobium, and Psychrobacter. Ralstonia sp. prevailed in all studied samples from both WWTPs.

4. Discussion

We considered the problem of continually increasing wastewater pollution, and we wondered how adaptable the micro-organisms providing wastewater treatment are. The working hypothesis was based on the assumption that chemicals contained in mobile toilet fillings have a toxic effect on activated sludge microorganisms. This hypothesis has been confirmed, especially for the chemicals bronopol and formaldehyde. Other chemicals in mobile toilets tend to act as deodorants, eliminating odors. These include mainly limonene. Also, citric acid is not a major problem for microorganisms. Although it lowers the pH of the environment, it is a source of carbon and energy for microorganisms to grow. An interesting effect was observed depending on the size of the wastewater treatment plant. The activated sludge microorganisms of a smaller wastewater treatment plant are much more sensitive to any changes, such as changes in temperature or salinity [5,23]. This was also confirmed in this study. The sensitivity of activated sludge microorganisms is best reflected in the change of their acclimatization phase. The microorganisms of a small WWTP take much longer to adapt to higher pollutant concentrations, pH fluctuations, temperature changes and other external factors. High concentrations of strong pollutants (bronopol, formaldehyde), however, have the same negative impact on small and large WWTP.

Another aim of this study was to find suitable degraders of chemicals contained in mobile toilet fillings. Although we have isolated several bacterial species, we have repeatedly and in large quantities encountered the isolation of Ralstonia sp. Ralstonia sp. is an aerobic, Gram-negative, oxidase-positive, non-fermentative rod and is a ubiquitous micro-organism found in water and soil [24]. R. pickettii has been shown to have biodegradative abilities. Already known compounds, which can be degraded by R. pickettii were reported previously and are mentioned in publication [25]. R. pickettii can grow for example on aromatic hydrocarbons, trichloroethylene and use them as the sole source of carbon and energy. The pathway responsible for the degradation is mostly toluene-3-monoxygenase pathway (tbu pathway). In the follow-up study, we will focus on this bacterium. It is important to determine its degradation ability, to describe its growth on selected chemical substances. The aim will be to use its capabilities for the targeted decontamination of wastewater or contaminated soil.

5. Conclusions

Mobile toilet chemicals can pose a risk to biological wastewater treatment. The respiratory activity of microorganisms present in activated sludge was chosen as an indicator for biological wastewater treatment efficiency. Beside other factors, which can play significant role, commercial products had a negative effect on the respiratory activity of activated sludge at the concentrations recommended by the manufacturers. This applies to both selected wastewater treatment plants (513,000 PE and 5000 PE). Supporting chemicals (limonene and citric acid) did not affect the respiratory activity of the sludge. In contrast, active substances (bronopol, formaldehyde) negatively influenced the activated sludge respiratory activity. From the results of respiratory activity, it can be concluded that activated sludge from WWTP (5000 PE) was more sensitive to the presence of tested chemical substances. We have isolated potential degraders from activated sludge exposure with mobile toilet chemicals. Bacterium Ralstonia sp. appears to be promising degrader, further tests must be carried out. Operators of WWTPs should avoid direct discharge of mobile toilet chemicals to the technology. For large WWTPs,
dilution with partial degradation can take place due to the long residence time of wastewater in sewer system. With small WWTPs, direct intake of these waters should be banned or strictly regulated.

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