

Inhibition of the activated sludge-associated enzyme phosphatase by transition metal oxyanions

Beatriz Böger, Marion Wacht, Michaela Leuck, Raquel de O. Vilhena, Maria Riese and Klaus Fischer

ABSTRACT

Organic esters of phosphoric acid and other organophosphorous compounds are enzymatically hydrolyzed during wastewater treatment by microbial phosphoesterases, especially by phosphomonoesterase (phosphatase). For physiological reasons, the enzyme is inhibited by its main inorganic reaction product, ortho-phosphate. It is known that oxyanions of transition metals, resembling the molecular topology of ortho-phosphate, e.g. vanadate and tungstate, are more potent inhibitors for microbial alkaline phosphatase than phosphate. To proof this effect for activated sludge, a multitude of samples from a communal wastewater treatment plant was exposed at pH values from 7.00 to 8.50 to tungstate, vanadate, and molybdate. Inhibition effects were determined by a sensitive fluorimetric microplate assay and characteristic parameters (IC_{50} and IC_{20} concentrations) were deduced from modelled dose-response functions. Mean inhibitor concentrations (in brackets: ranges) causing 50% inactivation (IC_{50}) at pH 7.50 were 2.5 (1.3–4.1) μM tungstate, 2.9 (1.6–5.5) μM vanadate, and 41.4 (33.6–56.7) μM molybdate. Vanadate and tungstate concentrations between 0.6 and 0.7 μM provoked a 20% (IC_{20}) inhibition. The inhibition efficiency of tungstate and molybdate decreased with increasing pH, whereas vanadate reacted pH independently. These results underline the necessity to consider enzyme inhibition assessing the limitations and potentials of biological wastewater treatment processes.

Key words | activated sludge, alkaline phosphatase, enzyme inhibition, tungstate, vanadate, wastewater treatment

HIGHLIGHTS

- Sensitive fluorimetric microplate assay applied to analyze phosphatase inhibition.
- Vanadate and tungstate are strong inhibitors of sludge-associated alkaline phosphatase.
- Molybdate has lower inhibition potency than the other oxyanions.
- Inhibition efficiency of tungstate and molybdate decreased with increasing pH.
- Enzyme inhibition needs more attention in the performance assessment of wastewater treatment.

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INTRODUCTION

During wastewater treatment, most of the organic wastewater components are microbially degraded in the biological treatment stage by enzymatic hydrolysis (Nybroe *et al.* 1992; Burgess & Pletschke 2008). Many of the involved enzymes belong to the class of hydrolases, constituting one of the seven main enzyme classes according to the Enzyme Commission (EC) classification system. Phosphohydrolases form a subgroup of this enzyme class. They preferentially cleave organic esters of phosphoric acid. The phosphohydrolases are subdivided further with regard to the molecular properties of the transformed substrates. Alkaline phosphatase (APA, EC 3.1.3.1), a phosphomonoesterase, has highest importance for the biotransformation of organic phosphorus compounds during wastewater treatment. It mainly hydrolyses phosphoric acid monoesters like nucleotides, terminal nucleic acid phosphate bonds, phosphoproteins and phosphorylated carbohydrates, releasing phosphorus in the easily bioavailable form as (ortho-)phosphate (O'Brien & Herschlag 2001). Synthetic organic phosphoric acid monoesters such as alkyl phosphates and phosphotensides are cleaved by APA also. Thus, APA serves to secure the microbial P supply and it contributes to the elimination of phosphorus-containing trace pollutants.

Due to its pivotal role in microbial phosphorus acquisition and metabolization, APA was included in several studies dealing with enzyme activities in wastewater treatment plants (WWTP). Together with other hydrolases, Richards *et al.* (1984), Boczar *et al.* (1992), and Hassard *et al.* (2018) determined the APA activity as a general measure for microbial vitality. Fischer *et al.* (2013) analyzed enzyme activity profiles of 6 communal wastewater treatment plants. They found that APA activity was highest within the group of phosphohydrolases. Goel *et al.* (1998) and Anupama *et al.* (2008) compared enzyme activities, including APA, under aerobic and anaerobic treatment conditions. Further research topics were potential correlations between APA activities and the distribution of P species in activated sludge particles (Xie *et al.* 2011; Huang *et al.* 2015), and the involvement of APA in the biological P removal (Lotter & van der Merwe 1987; Seviour *et al.* 2003; Zafiriadis *et al.* 2013). All of these studies substantiated the essential role of APA in the reduction of the organic P load of wastewaters. Thus, APA activity is crucial for the compliance with P threshold values specified for effluent quality.

To maintain high P removal rate effects, causing a decrease of the catalytic efficiency of APA should be avoided or, at least, alleviated. One of several detrimental

factors is the occurrence of enzyme inhibitors in wastewaters. Three main APA inhibition mechanisms exist, which define related chemical groups of inhibitors (Marcos & Townshend 1994):

- (i) Metal cations, such as Be^{2+} and Cd^{2+} , acting as competitors with the Zn^{2+} ions in the catalytic cavity of the enzyme or inactivating the enzyme by a distortion of its conformation
- (ii) Chelating agents like EDTA, competing with the ligating enzyme functions for coordinative bonding of Zn^{2+}
- (iii) Oxyanions such as AsO_4^{3-} and CrO_4^{2-} , acting as analogous of the transition state formed during the enzymatic transformation of organic phosphoric acid monoesters. Orthophosphate induces a product inhibition of the enzyme. The high APA inhibition efficiency of such metal oxyanions was tested and confirmed with many isolated enzymes stemming from various (micro-)organisms (Lopez *et al.* 1976; Stankiewicz & Gresser 1988; Crans 2015; Danikowski & Cheng 2018).

Whereas some knowledge is available regarding effects of group (i) and (ii) inhibitors on activated sludge APA, only one study considered oxyanions as potential inhibitors (Flint & Hopton 1977). Clearly those trace compounds seem to be of lesser importance for communal wastewaters than metal cations and chelating agents, but they are relevant for various industrial wastewaters. Li *et al.* (2019) reported vanadium concentrations, mainly occurring as vanadate, of about 10 mg L^{-1} in wastewaters of the vanadium processing industry. Effluents from a chemical plant, generating and purifying synthesis gas, contained roughly 50 mg L^{-1} V (Leiviskä *et al.* 2015). According to Stephanie *et al.* (2020) and with reference to data from the International Molybdenum Association (IMoA) 10%, corresponding to around 25,300 tons, of the global Mo production is transferred into wastewater leading to a maximal Mo burden of up to $1,000 \text{ mg L}^{-1}$. In a survey of the USEPA (2009), Mo was found in all 84 sewage sludge samples with concentrations ranging from 2.5 to 132 mg L^{-1} . Tungstate concentrations in wastewaters from galvanization facilities can reach several hundred milligrams per litre (Plattes *et al.* 2007). The authors raised concern about possible deterioration of the performance of biological wastewater treatment systems due to adverse effects of solved tungsten on activated sludge microorganisms.

In the study of Flint & Hopton (1977), cell-free extracts of activated sludge samples from one wastewater treatment

plant were exposed to high (range: 10–1,000 mM) concentrations of molybdate, tungstate, and arsenate, respectively. At 100 mM concentrations and pH 7.2, APA inhibition ranged from 71% (tungstate) to 18% (molybdate). Neither were integer sludge samples exposed nor dose-response functions recorded.

To fill this knowledge gap, the present study aimed at conducting an in-depth investigation of the inhibition effects of vanadate, tungstate, and molybdate, respectively, on activated-sludge associated extracellular APA in the pH range from 7.00 to 8.50. A multitude of samples from aerated tanks of a communal wastewater treatment plant were collected within two sampling campaigns, the first stretching over a period of eight months. To cover a wide inhibitor concentration range, necessary for the determination of dose-response functions, and to achieve a high analytical sensitivity for particle-containing samples, a microplate-established fluorimetric assay was applied for that purpose for the first time.

MATERIALS AND METHODS

Site description

The study was conducted during two periods, the first from June 2018 to January 2019 and the second in spring 2020, taking activated sludge samples from aerated tanks of the WWTP of the city of Trier, Germany. The plant has a capacity of approximately 170,000 population equivalent. The wastewater mainly stems from municipal sources and contains discharges from three sparkling wine producers, one tobacco factory, and one rolling mill. The latter discharges pretreated wastewater. The biological treatment combines nitrifying conditions provided by aerated tanks with denitrifying conditions established in non-aerated tanks.

Preparation of solutions

Stock solutions of the analytical APA substrate 4-methylumbelliferyl (4-MUF) phosphate were prepared by solving the required amounts in 0.05 M aqueous solutions of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, adjusted to the desired pH by addition of NaHCO_3 or NaOH. The fluorogenic product of the enzymatic reaction, 4-MUF, was applied as calibration standard for the fluorimetric quantification of the enzyme activity. Stock solutions were prepared in methanol and working solutions were generated by dilution with the required volumes of 0.05 M HEPES solutions.

The transition metal oxyanions vanadate, molybdate, and tungstate were applied in the form of their analytical grade sodium salts. Stock solutions contained 10 mM of the respective salts. Dilutions were made by addition of HEPES solutions. All stock solutions were protected against light and stored at 4 °C.

Sludge sampling and sample pretreatment

For every test series, six samples of activated sludge with an approximate volume of 1 L were taken from different positions within the first tank of one of the six biological treatment channels at around 8 a.m. by submersion of a cleaned HDPE canister. After that, an aggregate sample was generated by volume-proportional mixing of the individual samples. This sample was stored prior to further treatment at 4 °C. Generally, inhibition tests were performed at sampling day. Immediately before start of the analytical procedure, 100 mL of the resuspended sludge was treated in an ultrasonic bath for 10 min at 35 kHz to disperse the sludge flocs and to release occluded extracellular phosphatase molecules. Finally, the suspension was diluted (1:5) and adjusted to the desired pH by addition of pH aligned 0.05 M HEPES buffer.

APA inhibition assay

The inhibition assay is based on the determination of APA reaction rates (enzymatically generated 4-MUF per minute and sample volume) in samples with and without addition of metal oxyanions. To analyze a sample in five parallels simultaneously, the wells of five horizontal rows of a 96 well microplate were filled with 100 μL of the diluted sludge. Then 50 μL of the inhibitor solutions were added in the sequence of increasing inhibitor concentrations to the well numbers 2–12 of each of the five rows, adjusting 11 different inhibitor concentrations within a range of 3 orders of magnitude; for example, from 0.1 to 100.0 μM effective concentrations in the wells. The first well (control) of every row received 50 μL inhibitor-free HEPES solution. One further row was filled with blanks (corresponding inhibitor concentrations plus 4-MUF-phosphate, constituted in HEPES buffer). The remaining two rows served for matrix-specific calibration. These calibration wells were filled with 100 μL of the diluted sludge samples and with 100 μL of the differentially concentrated 4-MUF solutions. Next, the plate is equilibrated during 15 min at 30 °C in the reader. Then, 50 μL of 40 μM substrate solutions, tempered at 30 °C, were quickly added to the wells of the

parallel test series. Afterwards, the fluorescence emission at 460 nm (excitation wavelength 360 nm) was measured every 2 minutes within 20 minutes to record the reaction progress.

The calculation of the reaction rates is based on corrected fluorescence emission intensities (raw signal minus blank minus background fluorescence of the samples at time 'zero' of the reaction). After averaging of the corrected fluorescence signals of the five parallel samples, the spectroscopic data were converted into 4-MUF amounts using the simultaneously determined linear calibration functions. The linear regression of the increasing 4-MUF amounts over time yielded the reaction rates in the dimension 'mU' (transformation of 1 nM of substrate per minute and mL). These data were multiplied with the overall sludge dilution factor (10) to yield the enzyme activities of the undiluted samples.

The percentage inhibition of the APA activity is calculated applying the following equation:

$$\text{inhibition}[\%] = 100 \times [1 - (r_i/r_0)] \quad (1)$$

where r_i is the reaction rate at given inhibitor concentration and r_0 is the rate in the absence of an inhibitor (controls).

Inhibitor concentrations causing a 50% (IC_{50}) or a 20% (IC_{20}) decrease of the APA activity were calculated by modelling of these data with the standard dose-response function ('four parameter logistic equation'), implemented in GraphPad Prism, version 8.0. In the case of molybdate, the dose-response function 'normalized response - variable slope' resulted in a higher modelling quality; that is, higher determination coefficients.

Statistical analysis

For each data group, resulting from analysis of five sub-samples processed in parallel, the mean, the standard deviation, and the relative standard deviation (% RSD) were calculated. The quality of the modelled regression functions was assessed by the coefficient of determination.

To account for the natural inhomogeneity of the sludge samples, all data were analysed by Friedman test. A significance level (p) of <0.05 was accepted to indicate significant value differences. Spearman's rank-order correlation was employed to evaluate the strength of the relationship between the enzyme activities of the controls and the parameters IC_{50} , IC_{20} , and pH, respectively, at the 95% confidence level. Comparison between Spearman's ρ and Spearman's critical value for the two datasets was used as a measure of how closely these two datasets are correlated with each other. Spearman's ρ values from 0.4 to 0.59 express a moderate, from 0.6 to 0.79 a strong, and ≥ 0.8 a very strong correlation. Additionally, the t -test was applied to evaluate the IC_{50} 's of vanadate and tungstate ($p < 0.05$). All statistical tests were performed with Graphpad Prism 8.0.

RESULTS AND DISCUSSION

Vanadate

Eight sludge samples collected between July 2018 and January 2019 were used to investigate the inhibition of activated sludge-associated APA by vanadate at pH 7.5 (Table 1). To receive additional information about the variability of the

Table 1 | Inhibition of activated sludge-associated APA by vanadate at pH 7.5

Sampling day	4-MUF control [μM] ($\pm\text{SD}$)	APA activity control [mU mL^{-1}]	IC_{50} [μM] ($\pm\text{SD}$)	r^2 (IC_{50})	IC_{20} [μM] ($\pm\text{SD}$)
18.07.18	1.45 \pm 0.09	0.73	2.26 \pm 1.05	0.991	0.56 \pm 0.30
25.07.18	1.52 \pm 0.03	0.76	5.53 \pm 0.55	0.980	1.38 \pm 0.14
26.07.18	1.67 \pm 0.04	0.84	2.41 \pm 1.23	0.972	0.60 \pm 0.19
09.08.18	1.54 \pm 0.11	0.77	2.88 \pm 1.38	0.973	0.72 \pm 0.66
14.08.18	1.09 \pm 0.07	0.55	1.59 \pm 1.12	0.987	0.39 \pm 0.28
16.08.18	1.65 \pm 0.04	0.83	2.01 \pm 0.54	0.978	0.50 \pm 0.39
16.12.19	3.81 \pm 0.16	1.91	3.37 \pm 0.92	0.990	0.84 \pm 0.23
16.01.19/TS-1	3.80 \pm 0.11	1.90	3.60 \pm 1.60	0.990	0.90 \pm 0.40
16.01.19/TS-2	2.35 \pm 0.22	1.18	3.34 \pm 0.97	0.911	0.84 \pm 0.16
16.01.19/TS-3	2.85 \pm 0.51	1.43	1.81 \pm 0.34	0.978	0.45 \pm 0.43

Inhibitor concentration range: 0.025–50 μM , SD, standard deviation; TS, test series.

enzyme activity in various sludge subsamples three test series were sequentially processed at the last sampling day. The IC_{50} and IC_{20} concentrations resulting from these tests were averaged before calculation of the statistical parameters for the whole data set (Table 1).

The mean IC_{50} concentration of vanadate at given reaction conditions was $2.87 \pm 1.21 \mu\text{M}$ (range: 1.59 to 5.53 μM , RSD: 42.0%). The mean vanadate concentration effecting a 20% inactivation (IC_{20}) was $0.72 \pm 0.30 \mu\text{M}$ (0.39–1.38 μM), corresponding to a mass concentration of 82.9 $\mu\text{g/L}$. Thus, it is justified to assess vanadate as a strong inhibitor of activated sludge APA.

Without compromising the relevance of the inhibition concentrations determined in the present study, one has to consider that these parameters should be seen as conditional ‘apparent’ values rather than constants unequivocally attributable to a defined reaction of a specific protein. The applied enzymatic assay – and this holds for most of the photometric or fluorimetric assays – does not provide the specificity necessary to target one particular type of enzyme exclusively.

The four-parameter dose response function applied for the modelling of the effect concentrations offered mostly a very good fit to the experimental data, expressed by the high values of the coefficients of determination (r^2). Often, the simpler model (‘normalized response – variable slope’) returned almost identical IC values with a slightly lesser modelling quality. A semi-logarithmic plot of the vanadate inhibition functions, based on the means of data from eight test series (TS-1 to TS-3/16.01.2019 treated as above noticed), is depicted in Figure 1.

The relative standard deviation (42%) of the mean IC_{50} concentration seems to be low with respect to the time span covered and the related potential modifications of the

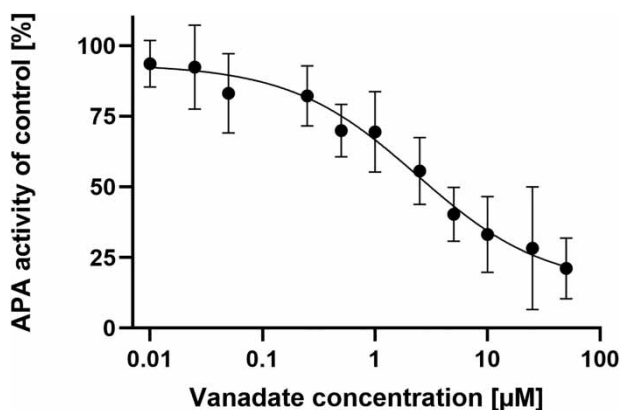


Figure 1 | Inhibition of activated sludge associated alkaline phosphatase by vanadate at pH 7.5. Means and standard deviations of eight test series (TS 1–3/16.01.2019 averaged and treated as one test series).

composition and of the metabolic activity of the microbial sludge consortium. The variability of the inhibition efficiency of vanadate corresponds to the variability of the APA activity in the controls (mean: 0.99 mU mL^{-1} , RSD: 47.3%). The relatively constant inhibition efficiency of vanadate is corroborated by the results of preceding tests conducted under deviating reaction conditions; that is, sludge dilution, substrate concentration, inhibitor concentration range, reaction time, and sequence of reagent addition (details not shown). The mean IC_{50} concentration stemming from those investigations was 3.54 μM , corresponding to a slightly elevated APA activity of the controls (1.15 mU mL^{-1}). Nevertheless, the statistical analysis gave low correlation between the effect concentrations and the APA activity of the controls (IC_{50} : $\rho = 0.38$, $p = 0.27$; IC_{20} : $\rho = 0.36$, $p = 0.29$).

Albeit not directly comparable, Stankiewicz & Gresser (1988) assessed vanadate as a potent competitive inhibitor of isolated *Escherichia coli* APA in the pH range from 7.0 to 8.0. Dose-response functions were not determined by these authors. Since further data on inhibition of sludge-bound APA by the relevant oxyanions are very scarce, one has to take other reaction media and even other, but closely related enzymes into account to classify the achieved results. Such a procedure might provide some indications but the direct comparability is very limited due to essential differences in reaction conditions, reaction mechanisms, and thermodynamics of catalytic transition states. For instance, Xu et al. (2018) and Ni et al. (2019) reported IC_{50} concentrations of Na_3VO_4 for the inhibition of calf intestine APA in the range of 70–80 μM . The enzymatic substrate was pyrophosphate. Vanadate was also proven to efficiently inhibit different variants of acid phosphatase (EC 3.1.3.2) and of protein tyrosine phosphatase (PTP, EC 3.1.3.48). A 0.5 m solution of vanadate inhibited the acid phosphatase of the bacteria *Burkholderia gladioli* almost completely at pH 6.0 and surpassed the inhibition efficiency of arsenate (Rombola et al. 2014). At pH 7.5, IC_{50} concentrations for PTP inhibition in rat hepatocytes stretched between 4 and 50 μM , depending on the selected PTP fraction and on the added substrate (Pugazhenti et al. 1996).

Tungstate

Inhibition tests with tungstate were conducted under conditions nearly identical with those applied for vanadate. The tungstate concentration range was shifted to slightly higher values. Except two test series, the investigations were performed with the same bulk samples as for the vanadate experiments.

The experimental results are summarized in Table 2. Figure 2 illustrates the dose-response function calculated with the means of the eight test series (TS 1–3 treated as above).

With a mean IC_{50} concentration of $2.45 \pm 0.90 \mu\text{M}$ (range: 1.51–3.88 μM), a mean IC_{20} of $0.60 \pm 0.21 \mu\text{M}$ (range: 0.32–0.97 μM), and a mean APA activity of $0.81 \pm 0.22 \text{ mU mL}^{-1}$ of the controls, the findings are very close to the vanadate figures. All of these figures are smaller than for vanadate. The differences were moderately statistically significant ($\rho = 0.64$; $p < 0.05$). The correlations between the effect concentrations and the APA activity of the controls were on a low level of significance (IC_{50} : $\rho = 0.43$, $p = 0.21$; IC_{20} : $\rho = 0.36$, $p = 0.29$).

The results achieved here suggest that the APA inhibition potency of tungstate is considerably higher than reported in the study of Flint & Hopton (1977). Exposing particle-free sludge extracts to a 1,000 μM tungstate solution at pH 8.5, they ascertained a reduction of the APA activity by 41%, whereas an inhibition of roughly 80% caused by a tungstate concentration of 75 μM was ascertained for the same pH in the present study. Stankiewicz & Gresser (1988) described a high inhibition efficiency of tungstate against *Escherichia coli* APA at pH 7.4, which surpassed that of vanadate slightly. With an IC_{50} concentration of 31.7 μM , APA from rabbit intestine was strongly inhibited by tungstate at pH 10.0 (Hazarika et al. 2008).

Molybdate

The reaction of molybdate with APA exhibited some distinctive features. Several times, the maximal inhibition degree did not exceed 50% making a reliable modelling of the

IC_{50} concentration impossible. A maximum inhibition effect of 63% was achieved in the majority of the test series but not necessarily evoked by the highest molybdate concentration. The inhibition effects, especially for molybdate concentrations $\leq 5 \mu\text{M}$, were strongly time-dependent and variable in several test series.

The results achieved with such low molybdate concentrations were inconsistent. Both an (apparent) activation of the enzyme and a significant inhibition with IC_{20} concentrations between 1.0 and 2.5 μM were observed. Also, the goodness of fit by the four parameter dose-response function was considerably lower than for tungstate and vanadate in most cases. A better representation of the analytical data was achieved for several test series by the three-parameter dose-response function.

The important figures of the test series with molybdate are compiled in Table 3. Only those experiments were

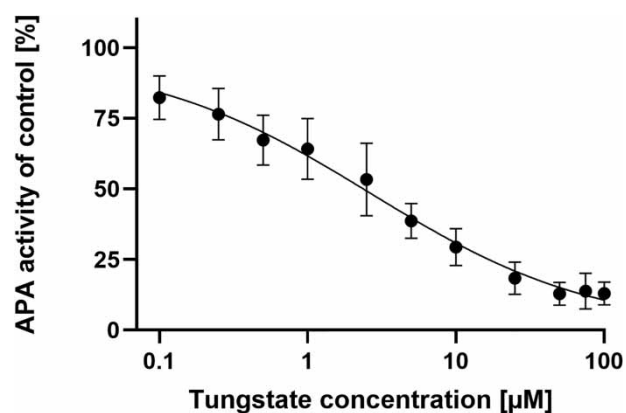


Figure 2 | Inhibition of activated sludge associated alkaline phosphatase by tungstate at pH 7.5. Means and standard deviations of eight test series (TS 1–3/16–17.01.2019 averaged and treated as one test series).

Table 2 | Inhibition of activated sludge-associated APA by tungstate at pH 7.5

Sampling day	4-MUF control [μM] ($\pm\text{SD}$)	APA activity control [mU mL^{-1}]	IC_{50} [μM] ($\pm\text{SD}$)	r^2 (IC_{50})	IC_{20} [μM] ($\pm\text{SD}$)
07.06.18	2.05 ± 0.11	1.03	2.20 ± 0.22	0.982	0.55 ± 0.05
18.07.18	1.25 ± 0.04	0.63	3.88 ± 1.49	0.993	0.97 ± 0.48
19.07.18	1.50 ± 0.06	0.75	2.16 ± 0.60	0.985	0.54 ± 0.15
25.07.18	1.72 ± 0.04	0.86	2.72 ± 1.34	0.992	0.68 ± 0.34
09.08.18	1.52 ± 0.04	0.76	1.27 ± 0.61	0.970	0.32 ± 0.18
14.08.18	1.11 ± 0.05	0.56	1.51 ± 0.35	0.989	0.38 ± 0.09
16.08.18	1.37 ± 0.04	0.69	2.37 ± 0.61	0.997	0.59 ± 0.63
16.01.19/TS-1	2.48 ± 0.18	1.24	3.20 ± 1.38	0.997	0.8 ± 0.35
16.01.19/TS-2	2.54 ± 0.34	1.27	3.13 ± 1.22	0.986	0.70 ± 0.31
17.01.19/TS-3	2.29 ± 0.15	1.15	4.11 ± 1.43	0.949	0.78 ± 0.36

Inhibitor concentration range: 0.1–100 μM , SD, standard deviation; TS, test series.

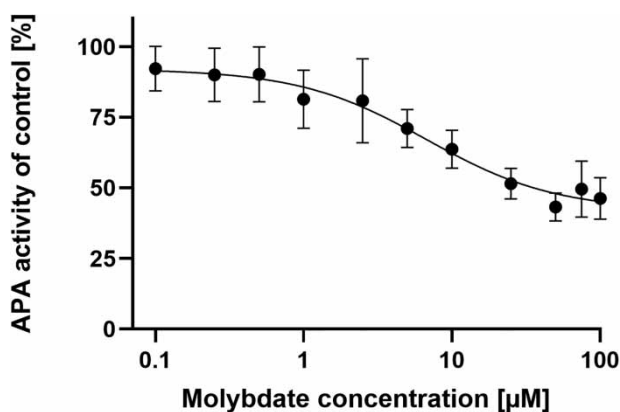
Table 3 | Inhibition of activated sludge associated APA by molybdate at pH 7.5

Sampling day	4-MUF control [μM] ($\pm\text{SD}$)	APA activity control [mU mL^{-1}]	IC_{50} [μM] ($\pm\text{SD}$)	r^2 (IC_{50})	IC_{20} [μM] ($\pm\text{SD}$)	Maximal inhibition [%]
10.09.18	1.38 ± 0.21	0.69	33.6 ± 0.73	0.909	8.4 ± 0.80	63
20.11.18	1.72 ± 0.04	0.86	41.7 ± 5.58	0.924	10.4 ± 1.40	63
28.11.18	1.94 ± 0.06	0.97	o. r.	–	–	51
4.12.18	1.95 ± 0.06	0.98	56.7 ± 1.66	0.927	14.17 ± 0.58	55
17.01.19	3.19 ± 0.55	1.60	33.6 ± 0.41	0.987	8.4 ± 0.55	63

Inhibitor concentration range: 0.1–100 μM , SD: standard deviation, o. r.: outside MoO_4^{2-} test concentration range.

included in the calculation of the mean IC_{50} value, which gave a maximal inhibition degree >50% and enabled the modelling of a dose-response ('normalized response – variable slope') function with $r^2 \geq 0.9$. With those constraints a mean IC_{50} of 41.4 ± 10.9 (range: 33.6–56.7 μM) resulted. The related mean APA activity in the controls was 1.03 mU mL^{-1} . Figure 3 illustrates the highly variable inhibition effects provided by low molybdate concentrations. No significant correlation between the effect concentrations and the APA activity of the controls was found (IC_{50} : $\rho = 0.12$, $p = 0.9$; IC_{20} : $\rho = 0.10$, $p = 0.9$).

Acting on a recombinant protein tyrosine phosphatase at pH 7.5, molybdate (IC_{50} : 4.2 μM) exerted an essentially lower inhibition effect than vanadate (IC_{50} : 0.2 μM , Heo et al. 2002). A similar activity difference was reported by Pugazenthi et al. (1996) for rat liver PTP. Several data are available regarding the action of molybdate on the acid phosphatase of various plants. Reacting on the acid phosphatase of *Hypericum perforatum L.* at pH 5.0, an IC_{50} concentration of 30 μM resulted (Demir et al. 2004). According to Kuang et al. (2009), a molybdate concentration of 250 μM completely abolished the purple acid phosphatase of *Arabidopsis thaliana* at pH 4.5. Here, the inhibition efficiency of molybdate

**Figure 3** | Inhibition of activated sludge-associated alkaline phosphatase by molybdate at pH 7.5. Means and standard deviations of five test series.

surpassed that of phosphate and fluoride considerably. These findings demonstrate that molybdate executes moderate to elevated inhibition strength against various members of the phosphatase superfamily but often lacks the high inhibition efficiency of vanadate. This corroborates the reactivity difference between molybdate and the other oxyanions established in the present study, which is in line with the findings of Flint & Hopton (1977).

pH dependency of inhibition efficiency

The pH dependency of the inhibition efficiency of the metal oxyanions was examined for the pH range from 7.00 to 8.50 with 0.25 increments, conducting four (tungstate) or five (vanadate, molybdate) test series. These assays were carried out more than one year later than the former investigations, providing a completely independent second data set. Furthermore, the sludges were sampled during the spring season, whereas the samples of the earlier examinations stemmed from the three other seasons. The essential findings are compiled in Table 4.

The inhibition efficiency of tungstate, expressed by the IC_{50} concentrations, decreased regularly with increasing pH ($\rho = 1.0$, $p = 0.004$). The IC_{50} concentration at pH 8.50 (5.19 μM) was roughly the 5-fold of pH 7.00 (0.97 μM). The relative standard deviations (n: 4) of the effect concentrations spanned between 10.5 and 24.6%, indicating a relatively constant inhibition activity.

A similar pH dependence was observed for molybdate, at least in the form of a trend. Repeatedly, no stringent correlation between molybdate concentration and inhibition strength was found, making a proper modelling with conventional dose-response functions impossible. Frequently, such an uncoupling between molybdate concentration and inhibition effects was encountered at $\text{pH} \geq 7.75$.

A distinct pH influence on the inhibition reaction of vanadate was not discernible. The statistical analysis indicated a very weak negative correlation between the IC_{50} concentration and

Table 4 | pH dependent IC₅₀ concentrations [μM] of the inhibition of activated sludge associated alkaline phosphatase by metal oxyanions

	pH						
	7.00	7.25	7.50	7.75	8.00	8.25	8.50
WO ₄ ²⁻	0.97 ± 0.24	1.03 ± 0.11	1.83 ± 0.41	2.11 ± 0.27	3.37 ± 0.48	3.75 ± 0.86	5.19 ± 0.59
VO ₄ ³⁻	2.57 ± 1.62	1.02 ± 0.47	1.37 ± 0.84	2.00 ± 0.65	1.17 ± 0.38	1.73 ± 0.61	1.36 ± 0.31
MoO ₄ ²⁻	20.9 ± 4.6 ^a	30.0 ± 11.9 ^a	36.8 ± 16.4 ^a	(43.8) ^b	(83.3) ^b	105.8 ± 36.1 ^c	152.8 ± 46.0 ^c

Test series (n): tungstate: 4, vanadate and molybdate: 5.

^aone test series excluded ($r^2/IC_{50} < 0.90$).

^bone test series with $r^2 \geq 0.90$.

^ctwo test series with $r^2 \geq 0.90$.

the pH value ($\rho = 0.21$, $p = 0.66$). The IC₅₀ concentrations stretched from 1.0 to 2.6 μM. The relative standard deviations were considerably higher than for tungstate and spanned between 22.8 and 63.0%. Except pH 7.00, the IC₅₀ concentrations of vanadate were equal or lower than for tungstate. The APA activity in the controls increased slightly with increasing pH. Typically, APA activity at pH 8.50 was 20–30% higher than at pH 7.00. The pH-dependent activity variations, determined for the pH range 7.00–8.50 with pH-adjusted subsamples from one bulk sample, were lower than those ascertained for the entire test series at fixed pH 7.50.

Stankiewicz & Gresser (1988) determined inhibition constants for the oxyanions reacting with *Escherichia coli* APA at pH 7.0, 7.4 and 8.0, applying para-nitrophenyl phosphate as substrate. The affinities of tungstate and molybdate to the enzyme were highest at pH 7.4, but the inhibitor-specific constants were all in the same order of magnitude. In contrast to that, a significant increase of the inhibition efficiency with pH was ascertained for vanadate.

Taking the interval between the two sludge sampling periods into account, the differences in the mean IC₅₀ concentrations at pH 7.50 seem to be remarkably low, as the following juxtaposition clarifies (the second figure stems from the first test period): tungstate 1.83 ± 0.41 μM/2.45 ± 0.90 μM, vanadate 1.37 ± 0.84 μM/2.87 ± 1.21 μM, and molybdate 36.8 ± 16.4 μM/41.4 ± 10.9 μM.

This underlines the constancy and relevance of the inhibition capability of the oxyanions.

Comparative interpretation of the inhibition properties of the oxyanions

An important outcome of this study is the substantiation of the reproducibly high inhibition efficiency of vanadate and tungstate against activated sludge bound APA. IC₂₀ concentrations below 1 μM indicate that traces of the oxyanions are

able to inhibit APA significantly. Differences in the inhibition strengths of both anions were not significant. This corresponds to the findings of Stankiewicz & Gresser (1988), who reported almost identical inhibition constants of these anions for *Escherichia coli* APA at pH 7.4. The essentially lower inhibition potency of molybdate was ascertained by these authors, by Flint & Hopton (1977), and Bedu et al. (1982). To interpret this phenomenon, it is necessary to refer to the probable inhibition mechanism. Vanadate and tungstate form pentavalent trigonal bipyramidal structures in the catalytic cavity of the enzyme, despite their tetrahedral geometries in their energetic ground states. The pentavalent complex, which is also formed by phosphate and phosphate esters, behaves as a transition state analogue in the enzymatic reaction (Peck et al. 2016). During the lifetime of the complex, the reactive cavity remains occupied preventing the bonding and subsequent transformation of natural substrate molecules. According to Stankiewicz & Gresser (1988) and Heo et al. (2002), molybdate has a higher tendency to adopt a distorted hexavalent octahedral coordination geometry. Potentially more energy is needed to form such a structure, leading to a less stable transition state.

There are a few approaches to interpret differences in the pH dependency of the inhibition strengths of the oxyanions. Stankiewicz & Gresser (1988) argued that the dianionic form of the ions is the species relevant for the inhibition process. The formation of this species depends on the second dissociation constant (pK_2) of the related acids. Consequently, the acids H₂WO₄ and H₂MoO₄ should have donated their protons almost completely at pH ≥ 7.0, whereas the dissociation of the less acidic H₃VO₄ is incomplete at pH 7.00 and increasing with increasing pH. Based on these considerations, one would expect a pH dependency for vanadate, but not for tungstate and molybdate. Actually, the reverse was established here. In contrast to pure enzymes, which were applied by the mentioned authors,

activated sludge suspensions offer much more reaction possibilities including pH-dependent adsorption, heteropolyanion formation, and precipitation of insoluble compounds. Thus, the real ion speciation and fractionation in this matrix might deviate from those applicable to simpler reaction conditions.

The relevance of the APA inhibition capability of the oxyanions becomes clear by comparison with the inhibition effects of metal cations. According to Flint & Hopton (1977), tungstate was a stronger inhibitor than all of the investigated divalent cations, including Cd^{2+} and Hg^{2+} . Xie *et al.* (2010) examined the inhibition effects of several metal ions on APA at pH 7.6, treating purified activated sludge samples. A 500 μM solution of Cu^{2+} ions provoked a reduction of the APA activity by 11.3%. Similar effects are achievable with three orders of magnitude lesser concentrated vanadate or tungstate solutions.

All prior investigations of APA inhibition were conducted selecting p-nitrophenyl phosphate as analytical substrate for photometric reaction recording. The fluorimetric method applied here proved to be superior in various respects. Most importantly, the separation of particles; that is, sludge flocs, is not required, allowing continuous monitoring of inhibition kinetics. Secondly, the analytical sensitivity is higher, enabling the quantitation of very low ($<0.1 \mu\text{M}$) product concentrations in the initial reaction stage at high inhibitor concentrations. Finally, matrix effects, such as background absorbance of sludge components and of added test substances, are of minor importance.

To evaluate the practical relevance of the reported findings, some aspects need to be critically reflected. The tenfold sludge dilution led to a corresponding increase of the molar inhibitor to enzyme ratio. Since the inhibition mode exerted by the oxyanions is 'competitive' predominantly (Stankiewicz & Gresser 1988), this ratio might be of lesser importance than the adjusted range of inhibitor to substrate ratios. Another experimental artefact is the replacement of the wastewater phase by the buffer solution. This altered the ion strength and ion composition of the aqueous phase. It might have also disturbed the distribution of sludge components including enzymes between the liquid and solid phase. Potential impacts on the APA activity are not known. Finally, competitive inhibition assumed, the inhibition efficiency depends on the ratio of the enzyme affinities (Michaelis Menten constants) for the substrate and the inhibitor. In cases where the enzyme has a higher affinity (lower Michaelis Menten constant) for a natural substrate than for the analytical substrate, applied in the inhibition assay, resulting inhibition effects are probably lower and vice versa.

CONCLUSIONS

The enzyme alkaline phosphatase is essential for the microbial breakdown of organic phosphorus compounds and as such decisive for the efficiency of the biological P removal from wastewaters. It could be demonstrated that this activated sludge associated enzyme is highly vulnerable against inhibition by some transition metal oxyanions, i.e. tungstate and vanadate. The application of a sensitive fluorimetric assay allowed for the determination of inhibition effects caused by oxyanion concentrations $<1 \mu\text{M}$. These effects were not restricted to a fixed pH value but occurred over the whole pH range relevant for wastewater treatment. With respect to the relatively long sampling period and the high number of performed assays, it seems to be justified to conclude that the ascertained inhibition effects are comparably constant and more or less independent from the APA activity of the uninhibited sludge.

Despite the assumption that vanadate and tungstate concentrations in communal wastewaters are usually too low to exert measurable effects on APA, the findings point to a hitherto underestimated reaction type potentially influencing the efficiency of the microbial P elimination. The practical relevance might be greater for treatment plants receiving industrial wastewaters. Finally, the potential inhibition of microbial enzymes by trace pollutants during wastewater treatment merits more attention and investigation.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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