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Comparison of spectrophotometric methods using cuvette tests and national standard methods for analysis of wastewater samples

Sonya Dimitrova, Nadejda Taneva*, Kapka Bojilova, Vesela Zaharieva, Svetlana Lazarova, Mariana Koleva, Rumen Arsov and Tony Venelinov

University of Architecture, Civil Engineering and Geodesy, 1 Christo Smirnenski blvd., 1046 Sofia, Bulgaria.

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The suitability of spectrophotometric methods using cuvette tests (CT) for determination of ammonia (NH_4^+-N), nitrates (NO_3^--N), total phosphates (PO_4^{3-}-P) and chemical oxygen demand (COD) in real wastewater were evaluated by comparison with corresponding Bulgarian national standard methods (BS). The CT methods are based on measuring of ready-to-use cuvettes (bar-coded reagent vials) in wavelength range of 340 to 900 nm. Nine wastewater samples from the inlet of the wastewater treatment plant of Sofia city were collected in the period of three months and the above mentioned quality indicators were measured in parallel both with CT and BS methods. Mean values of ten replicate determinations for each of the samples were compared statistically using Dixon's t-test and linear regression model. Excellent linear correlation ($R^2 > 0.99$) was found. As another mean of comparison, all the methods (CT and BS) for the determination of all the quality indicators were validated and uncertainties were estimated. Based on this, all data were compared and proved statistically equivalent.

Key words: Method comparison, method validation, uncertainty estimation, cuvette tests, linear regression, spectrophotometer, wastewater.

INTRODUCTION

The management of the urban water systems is relied generally on chemical analysis of a set of wastewater quality indicators. Chemical oxygen demand is an integrated parameter, which gives information for the level of organic contamination of wastewater. COD tests are traditionally used for assessing the effectiveness of the biological wastewater treatment and the organic load of the treated wastewater. Nitrogen and phosphorus are also among the important indicators which are controlled at the wastewater treatment plants.

There are many analytical methods for measuring the above mentioned indicators. COD, NH_4^+-N, NO_3^--N and PO_4^{3-}-P at laboratory scale set-up of samples collected manually and/or by automatic samplers, are usually carried out according to the national and international standard methods. These methods are well studied and documented, but they are too sophisticated and time consuming for operational control and require considerable practical experience and skill to get reproducible results. Furthermore, most of them require the use of toxic substances, which can be harmful to the analyst and their subsequent utilization can be dangerous to environment. Therefore, it is an emerging need for new rapid, low cost and nono-toxic substance consuming, reliable and precise analytical techniques and instruments applicable for real-time control.

Over last decades many new automated analytical methods and instruments have been developed and evaluated in respect of their applicability for wastewater control - UV/Visible spectroscopy (Ferree and Shannon,

*Corresponding author. E-mail: nadejda.taneva@abv.bg.

2001; Langergrabe et al., 2003), ion chromatography (Karmarkar, 1999) and others.

Spectrophotometric methods for water analysis, based on ready-to-use cuvette tests, can be an alternative to the time-consuming reference methods. Practically all compounds and indicators of water environment could be measured directly or after suitable preliminary treatment using contemporary spectrophotometers and portable photometers. The cuvette tests quality is demonstrated by the fact that for the first time a COD cuvette test has been accepted as a reference method (ISO/IEC 15705, 2002).

The aim of this study was to evaluate the comparability of the NH_4^+-N, NO_3^--N, PO_4^{3-}-P and COD data obtained by cuvette tests and the corresponding Bulgarian standard methods (BDS 17.1.4.02-77, 1977; BDS 17.1.4.10-79, 1979; BDS ISO 7890-3:1997, 1998; BDS EN ISO 6878:2004, 2005) for analysis of real wastewater samples.

MATERIALS AND METHODS

The study was carried out on nine wastewater samples collected at the inlet of the wastewater treatment plant of Sofia city, which serves about 1 200 000 habitants. Samples were collected weekly during a three-month period. Each sample was analyzed on the day upon receipt. Ten replicate determinations were performed on each of the 9 samples, both by CT and BS. The cuvette tests were performed according to the user's manual issued by instrument manufacturer. The spectrophotometric measurements of the PO_4^{3-}-P and NO_3^--N by the Bulgarian standard methods were realized in quartz cuvettes on the same spectrophotometer after appropriate calibration at 680 and 410 nm, respectively.

Equipment

DR 2800 - portable spectrophotometer (Hach Lange GmbH**)** with 340 to 900 nm wave length range (tungsten halogen lamp) and referent ray to compensate lamp wear and power fluctuation was used. The devise has integrated system for barcodes reading of the prepared tests, with ten measurements for rotation and elimination of wrong reading caused by prepared cuvettes wasting.

LT 200 - thermo-reactor (Hach Lange GmbH). TenSette plus - Electronic pipette 0.2 to 5 mL (Hach-Lange GmbH).

Determination methods

Chemical oxygen demand (COD)

The method for the determination of COD in wastewater samples according to the Bulgarian National Standard (BSS 17.1.4.02-77) is based on the titrimetry, whereby to 20 mL of the samples Ag_2SO_4, $K_2Cr_2O_7$ and H_2SO_4 are added. After boiling under reflux for 2 h, the samples are cooled down and indicator is added, before titration with $FeNH_4(SO_4)_2$.

The method for the determination of COD in wastewater samples using cuvette tests is based on addition of 2 mL of sample to the cuvette, which is heated in thermo-reactor for 2 h at 148 ± 50°C. After cooling, the cuvette is inserted into the spectrophotometer and measured. Depending on the concentration, cuvette test for COD LCK 314 (15 to 150 mgO_2/L) and LCK 114 (150 to 1000 mgO_2/L) are used.

Stock solution of 1000 ± 1 mg/L COD (Hach-Lange GmbH) and CRM (RTC COD 500-500) with certified value for COD of 500.00 ± 7.65 mgO_2/L -(LOT No. 016203) were used for the validation studies.

Nitrates

The method for the determination of nitrates in wastewater samples according to the Bulgarian National Standard Bulgarian National Standard (BDS ISO 7890-3:1998) is based on spectrophotometric determination at 410 nm of the color intensity of the formed substance between the nitrates and the sulfosalicylic acid in presence of alkali base and Na_2EDTA and NaN_3.

The method for the determination of Nitrates in wastewater samples using cuvette tests is based on the reaction between nitrate ions with 2,6-dimethylphenol in presence of sulfuric acid and phosphorus acid. Cuvette tests for nitrates used were LCK 339 (0.23 to 13.50 mg/L). Stock solution of 10 ± 0.1 mg/L NO_3-N (Hach-Lange GmbH) and CRM (CertiPrep) with certified value for NO_3-N of 1005 ± 3 mg/L (LOT No. 2-78NO_3N-2) were used for the validation studies.

Total phosphates

The method for the determination of Phosphates in wastewater samples according to the Bulgarian National Standard (BDS EN ISO 6878:2004) is based on spectrophotometric determination at 880 nm of the color intensity of the formed substance between orthophosphates, ammonium molibdate and antimony in presence of ascorbic acid and sulfuric acid.

The method for the determination of total phosphates in wastewater samples using cuvette tests is based on the reaction between phosphate ions and molybdate ions and subsequent reduction by ascorbic acid. Cuvette tests for total phosphates used were LCK 348 (0.5 to 5.0 mg/L). Stock solution of 1000 ± 1 mg/L P tot (Hach-Lange GmbH) and CRM (RTC TPO 1000 to 500 ML) with certified value for P total of 1000.0 ± 15.5 mg/L (LOT No. 017605) were used for the validation studies.

Ammonia

The method for the determination of ammonia in wastewater samples according to Bulgarian National Standard (BDS 17.1.4.10-79) is based on distillation in presence of phosphate buffer and boric acid and titration using sulfuric acid and methylrod and methileneblou as mixed indicator.

The method for the determination of ammonia in wastewater samples using cuvette tests is based on the reaction between the ammonium ions and hypochlorite ions and salicylic ions in presence of sodium nitroprucide. Cuvette tests for ammonia used were LCK 303 (2 to 47 mg/L]). Stock solution of 1000 ± 1 mg/L NH_4-N (Hach-Lange GmbH) and CRM (SPEX Ammonium Standard) with certified value for NH_4-N of 1002 ± 3 mg/L (LOT No. 2-95NH_4N-2) were used for the validation studies. Distilled water was used throughout.

Statistical data treatment

The least squares method and Dixon's t-test were used to establish the relationships between measurements and data obtained by the two methods, as well as for evaluation of correlation and significance of any founded discrepancies.

Table 1. Mean concentrations for NH_4^+-N, NO_3^- -N, PO_4^{3-}-P and COD with respective RSDs (n = 10).

Sample	NH_4^+-N (mg/L)		NO_3^- -N (mg/L)		PO_4^{3-}-P (mg/L)		COD (mgO_2/L)	
	BS	***CT***	***BS***	***CT***	***BS***	***CT***	***BS***	***CT***
1	6.75±0.33	7.17±0.07	0.49±0.05	0.54±0.03	2.04±0.18	1.97±0.06	368±15	372±8
2	6.78±0.25	7.19±0.08	0.81±0.02	0.90±0.02	2.13±0.01	1.95±0.08	104±18	101±8
3	3.83±0.14	3.98±0.12	2.49±0.06	2.55±0.03	1.07±0.07	0.93±0.03	73±7	69±14
4	5.24±0.27	6.07±0.28	0.79±0.02	0.88±0.02	2.00±0.14	1.95±0.05	40±5	46±3
5	13.74±0.34	15.39±0.24	0.18±0.04	0.25±0.04	5.04±0.21	4.92±0.10	62±10	68±2
6	13.89±0.32	14.40±0.76	0.16±0.01	0.11±0.07	6.97±0.19	6.81±0.18	277±8	273±7
7	13.62±0.16	14.21±0.21	0.22±0.01	0.22±0.04	4.95±0.27	4.39±0.07	266±7	284±7
8	14.78±0.34	15.48±0.27	0.25±0.02	0.30±0.03	5.18±0.33	4.86±0.16	303±9	323±10
9	14.07±0.30	15.03±0.55	0.28±0.04	0.31±0.02	5.10±0.22	4.91±0.14	276±13	274±4

RESULTS AND DISCUSSION

Table 1 shows mean values and their standard deviations (n=10) for ammonia (NH_4^+- N), nitrates (NO_3^- -N), total phosphates(PO_4^{3-}-P) and chemical oxygen demand (COD) for cuvette test (CT) and standard methods (BS) for replicate measurements of the 9 sampling weeks without any outliers removal.

As can be seen, generally the RSDs of BS and CT methods overlap, but 60% of COD, 80% of NO_3^- -N and all NH_4^+-N mean values measured by CT methods are higher than those measured by standard methods. This is not the case with phosphorus measurements.

The method performance was inspected for any potential systematic errors as data obtained for NH_4^+-N, NO_3^--N, PO_4^{3-}-P and COD by cuvette tests were plotted against the corresponding mean values of the standard methods (Figures 1 to 4). The equations of the best-fit lines trough the data were determined using the method of least squares. It was found that the relationships between the measurements by both methods are approximated very well by linear regression equation:

$$Y\ (CT) = a + b\ x\ (BS)$$

The coefficients of determination (R^2) are close to 1 (>0.99): for NH_4^+-N - 0.9936; for NO_3^--N - 0.9970; for PO_4^{3-}-P - 0.9948 and for COD – 0.9923. The obtained regression lines for each one of measured indicators were statistically evaluated by their comparison with a hypothetic ideal line the slope of which is unit and the intercept is zero. The zero-hypothesis H_0: $a = 0$; $b = 1$ was revised according to the t- criteria:

$t_{statistic} < t_{critcal}$ (P = 95 %);
$t_{statistic}(b) = (b-1)/S_b$;
$t_{statistic}(a) = (a-0)/S_a$,

where P is significance level; S_a and S_b –standard deviations of the estimators a and b, respectively.
Regression lines parameters (slope, *y* intercept and the standard error of estimate in *y* direction) provide specific estimatation of errors, but only in case of approved linear relationships (Simeonov, 1997; Westgard and Hunt, 1973). A study of the regression straight line gives a possibility to evaluate at least three kinds of errors: random, proportional and constant. If the zero-hypothesis is not realized for the line slope it is an indication for a proportional systematic error occurrence. If the zero-hypothesis is not realized for the line intercept, this suggests a constant systematic error.

The results from statistical assessment of the regression lines are given in Table 2. Two methods are admitted as statistically equivalent if zero-hypothesis is realized, therefore it can be concluded that there are no systematic errors for the data obtained by the both methods.

As a second means of comparison, Dixon's t-test was performed on both the CT and BS data (as dependent data-sets), and compared with the tabular value. Here, the zero-hypothesis H_0: $\bar{x}_d = 0$ is checked against H_{alt}: $\bar{x}_d \neq 0$ using Equation 1:

$$t_{\exp} = \frac{\bar{d}}{\frac{s_d}{\sqrt{n}}} \quad (1)$$

where $d_i = x_{1i} - x_{2i}$, s_d – standard deviation of the mean, *n* - number of samples.

The zero-hypothesis is realized if the experimentally obtained value is lower than the tabular value for the same degrees of freedom. Calculations show experimental values of -4.79 for NH_4^+–N; -3.04 for NO_3^--N; 3.82 for PO_4^{3-}-P and -1.50 for COD with tabular value of 2.31 (df=8). The calculations show that only for COD the H_0 hypothesis proved true.

According to the EU Application note 1 (European

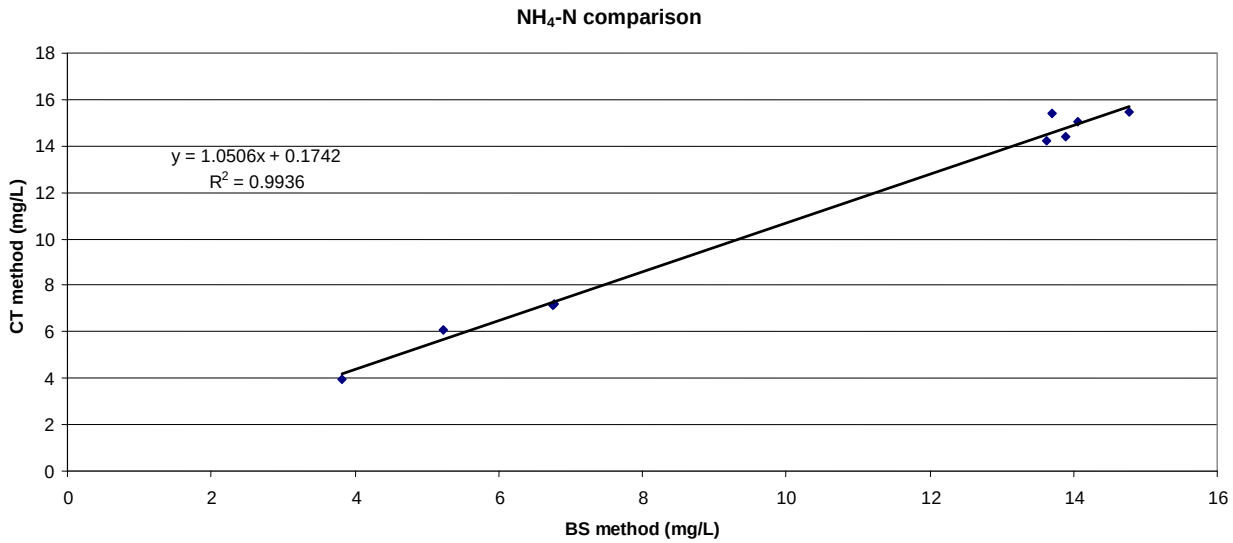

Figure 1. Comparison of mean values for NH_4^+-N using a cuvette test method and the Bulgarian standard method.

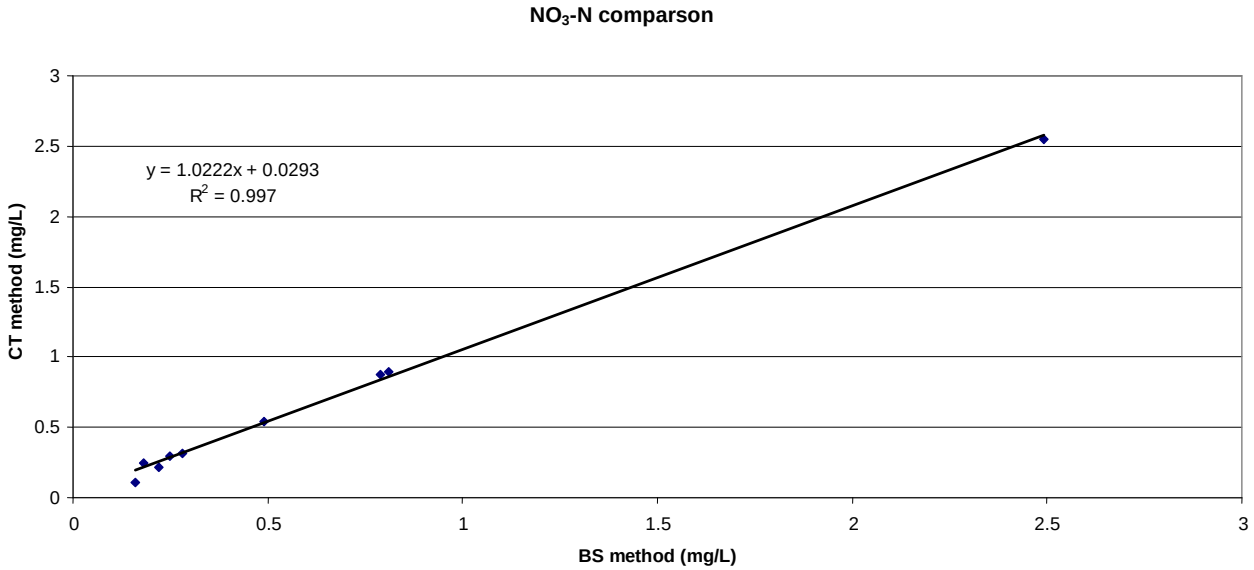

Figure 2. Comparison of mean values for NO_3^--N using a cuvette test method and the Bulgarian standard method.

Commission, 2005), two mean values are best compared using their uncertainties. Uncertainty is best estimated during method validation. The experimental design was set up in a way so that the repeatability, reproducibility and trueness estimates are used for measurement uncertainty estimation (ISO/IEC 21748:2010, 2010).

Trueness was proven by measurement of three independent samples of a certified reference material on two different days. From these data the uncertainty of trueness and method bias were calculated. Repeatability and intermediate precision were determined by replicate analysis and assessment of between-day effects. This was achieved by preparation of three independent samples of a certified reference material on three extra days. Combination from these data and the data obtained for trueness were used for calculation of the uncertainties of repeatability and due to intermediate precision.

Measurement uncertainty components of repeatability and due to intermediate precision can easily be calculated using the ANOVA function in the Microsoft Excel (Equations 2 and 3):

$$u_{rw} = \frac{s_{rw}}{\sqrt{n}} = \frac{\sqrt{MS_{within}}}{\bar{x}} \quad (2)$$

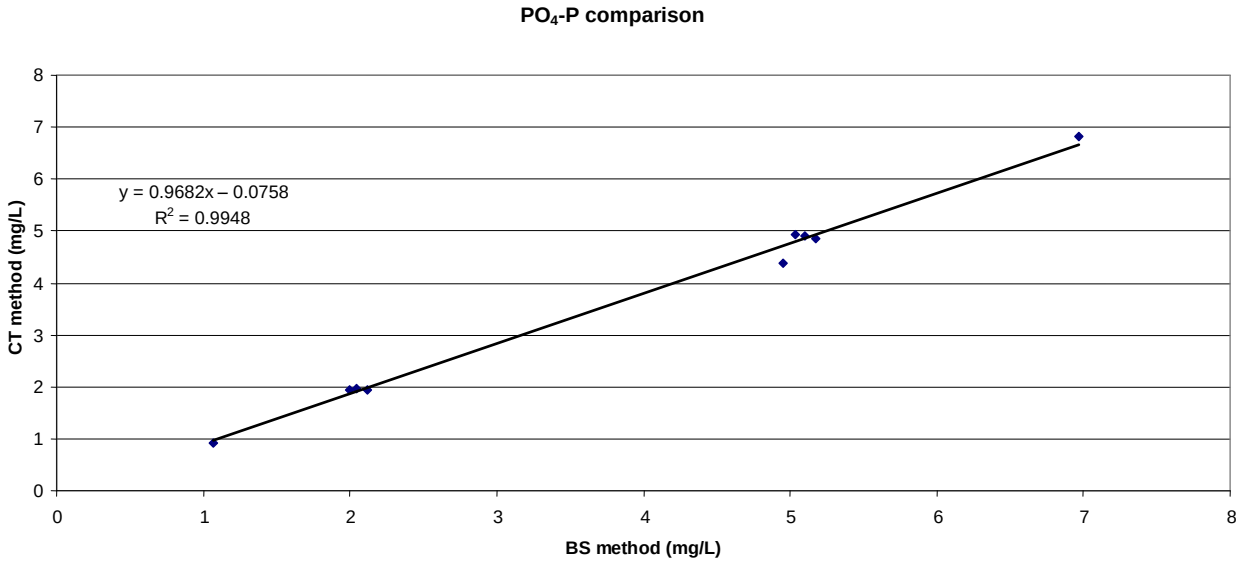

Figure 3. Comparison of mean values for PO_4^{3-}-P using a cuvette test method and the Bulgarian standard method.

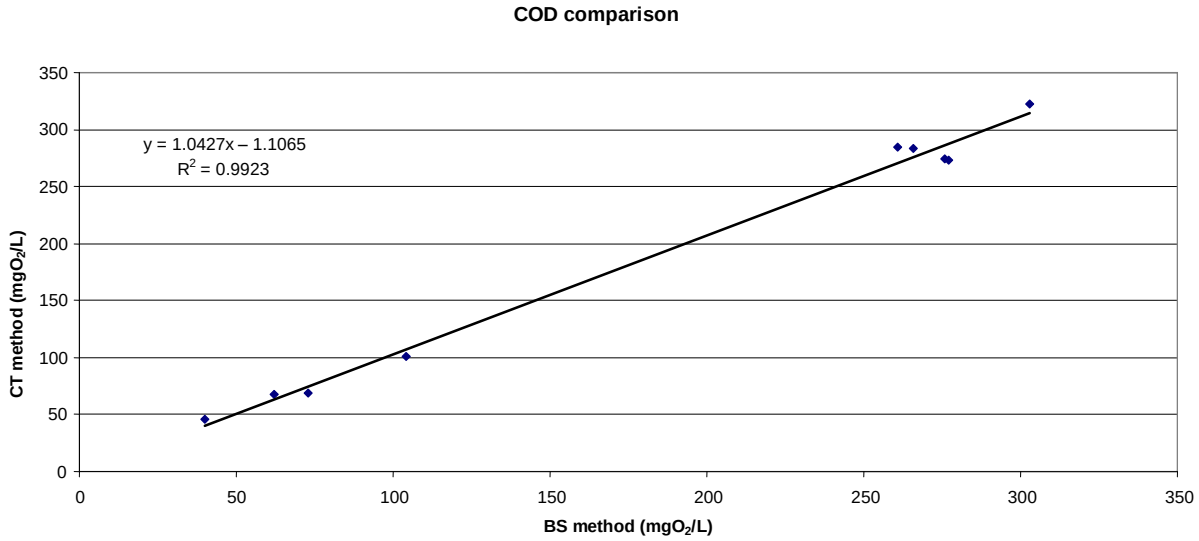

Figure 4. Comparison of mean values for COD using a cuvette test method and the Bulgarian standard method.

where u_r is the uncertainty of repeatability, s_r is the SD of all the repeatability measurements, and n is the number of replicates and $\bar{x}$ is the mean of all measurements performed.

$$u_{ip} = \frac{s_d}{\sqrt{d}} = \frac{\sqrt{MS_{Between} - MS_{within}}}{\bar{x}} \quad (3)$$

where u_{ip} is the uncertainty due to intermediate precision, s_d is the day-to-day variation, d is the number of measurement days, and n is the number of replicates and $\bar{x}$ is the mean of all measurements performed.

The uncertainty of trueness (u_t) is calculated using the Equation 4:

$$u_t = \frac{s_t}{\sqrt{n}} = \sqrt{\frac{s_t^2}{n} + \frac{\sum_m^2}{n_{mc}^2}} \quad (4)$$

Table 2. Estimation of the regression coefficients $T_c = t\ (95\%;\ f_1,\ f_2)$

Parameter	NH_4^+-N	NO_3^--N	PO_4^{3-} P	COD
n	9	9	9	9
Intercept (a)	0.177	0.032	-0.079	0.592
Slope (b)	1.050	1.021	0.969	1.024
Sa	0.172	0.016	0.091	5.511
Sb	0.173	0.017	0.021	0.024
$t_{\text{statistic (a)}}$	0.511	1.595	-0.693	0.086
$t_{\text{statistic (b)}}$	1.611	0.985	-1.182	0.781
t_{critcal}	2.36	2.36	2.36	2.36
Proportional systematic error	No	No	No	No
Constant systematic error	No	No	No	No

Table 3. Mean concentrations for NH_4^+-N, NO_3^- -N, PO_4^{3-}-P and COD with respective uncertainties (k=2).

Samples	NH_4^+-N (mg/L)		NO_3^--N (mg/L)		PO_4^{3-}-P (mg/L)		COD (mgO_2/L)	
	BS	***CT***	***BS***	***CT***	***BS***	***CT***	***BS***	***CT***
1	6.75±0.44	7.17±0.49	0.49±0.05	0.54±0.03	2.04±0.29	1.97±0.32	368±15	372±11
2	6.78±0.44	7.19±0.49	0.81±0.90	0.90±0.05	2.12±0.30	1.95±0.31	104±3	101±14
3	3.83±0.25	3.98±0.27	2.49±0.26	2.55±0.15	1.07±0.15	0.93±0.15	73±2	69±9
4	5.24±0.34	6.07±0.41	0.79±0.08	0.88±0.05	2.00±0.28	1.95±0.31	40±1	46±6
5	13.74±0.89	15.39±1.05	0.19±0.02	0.25±0.02	5.04±0.71	4.92±0.79	62±2	68±9
6	13.89±0.90	14.40±0.98	0.16±0.02	0.11±0.01	6.97±0.98	6.81±1.09	277±9	273±8
7	13.62±0.89	14.21±0.97	0.22±0.02	0.22±0.01	4.95±0.69	4.39±0.70	261±9	285±9
8	14.78±0.96	15.48±1.05	0.25±0.03	0.30±0.02	5.18±0.73	4.86±0.78	303±10	323±10
9	14.07±0.91	15.03±1.02	0.28±0.03	0.31±0.02	5.10±0.71	4.91±0.79	276±9	274±8

where s_t is the SD, n_t is the number of replicates, u_{mat} is the uncertainty of the certified value of the CRM used and n_{mat} is the number of the CRMs used.

The combined uncertainty (u_c) is then calculated using Equation 5:

$$u_c = \sqrt{u_t^2 + u_p^2 + u_l^2} \quad (5)$$

After calculation of the measurement uncertainties for all the methods, the mean values for all the parameters were compared using Equation 6:

$$\bar{x} = \mu \pm 2\sqrt{u_{meas}^2 + u_{CRM}^2} \quad (6)$$

where $\bar{x}$ - average content, μ - certified value, u_{meas} - combined expanded uncertainty of the measurement and u_{CRM} - combined expanded uncertainty of CRM used.

The results from the 9 weeks of samplings with their uncertainties are presented in Table 3. In all chases the results obtained by the two methods yield statistically equivalent data. Only for NO_3^--N two of the nine samples do not fulfill Equation 6, since they are marginally out.

In order to find which of the methods (CT and BS) is more precise and reliable, method accuracy and trueness were estimated by performing students' t-test comparing the methods' performance on a certified reference material (CRM). Data from repeatability assessment was used. Here, the zero-hypothesis H_0: $\mu_{BS} = \mu_{CRM}$ and $\mu_{CT} = \mu_{CRM}$ is checked against H_{alt}: $\mu_{BS} \neq \mu_{CRM}$ and $\mu_{CT} \neq \mu_{CRM}$ using Equation 7:

$$t_{\exp} = \frac{\left|\bar{x} - \mu\right|}{\frac{s}{\sqrt{n}}} \quad (7)$$

where $\bar{x}$ is the mean of all measurements performed, μ is the certified value of the used CRM, s is the standard deviation of the mean and n=15.

The zero hypothesis is realized if the calculated $t_{exp} < t_{tabl}$ and the data is presented in Table 4. Calculations show that both CT and BS methods yield statistically equivalent data and can be used as an alternatives for the measurement of PO_4^{3-}-P and COD. Based on the statistical data treatment, it is advised to use the CT method for the determination of NH_4^+-N and NO_3^--N.

Table 4. Calculated t_{emp} using repeatability data and Equation 7.

Parameter	NH_4^+-N		NO_3^--N		PO_4^{3-}-P		COD	
	BS method	CT method	BS method	CT method	BS method	CT method	BS method	CT method
Mean Value ± standard deviation	986.8±27.0	1000.4±31.0	1077.3±47.3	1011±29	0.54±0.03	999.7±68.7	498.3±6.7	503.2±5.9
Certified value of a CRM	1002	1002	1005	1005	0.52	1005	500	500
t_{exp}	2.18	0.21	6.40	0.80	1.50	0.30	0.96	2.13
t_{tabl}	2.14	2.14	2.14	2.14	2.14	2.14	2.14	2.14

Conclusion

Different wastewater quality indicators - ammonia (NH_4^+-N), nitrates (NO_3^--N), total phosphates(PO_4^{3-}P) and chemical oxygen demand (COD) were measured using spectrophotometric method using cuvette tests (CT) and Bulgarian standard methods (BS), and compared. The methods showed generally similar results for real wastewater samples obtained at the inlet of this wastewater treatment plant. Statistical evaluation indicated that the CT and BS methods are comparable. The spectrophotometric methods with cuvette tests are convenient and easy to use with some advantages (that is, lower sample volumes, lower chemical reagents needs and respectively less waste production.

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