Evaluation of single cell gel electrophoresis data: Combination of variance analysis with sum of ranking differences

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Abstract

Specimens of *Mytilus galloprovincialis* were collected from five sites in the Boka Kotorska Bay (Adriatic Sea, Montenegro) during the period summer 2011 – autumn 2012. Three types of tissues, haemolymph, gills and digestive gland were used for assessing of DNA damage. Images of randomly selected cells were analyzed with a fluorescence microscope and image analysis by Comet Assay IV Image analysis system. Three parameters tail length; tail intensity and Olive tail moment were analyzed on 4200 nuclei per cell type. Sum of ranking differences (SRD) was implemented to compare use of different type of cells and different measure of comet tail per nucleus. Numerical scales were transferred into ranks, range scaling between 0 and 1; standardization and normalization were carried out.

We have observed variations in the level of DNA damage in mussels collected at different sites and seasonal variations in response as well.

SRD selected the best (and worst) combinations: Tail moment is the best for all data treatment and for all organs; second best is tail length, and intensity is the third (except for digestive gland). The differences were significant at the 5% level. Whereas gills and heamolymph cells do not differ significantly, cells of digestive gland are much better for genotoxicity estimation. Variance analysis decomposed the effect of different factors on the SRD values. This unique combination has provided not only the relative importance of factors, but an overall evaluation: the best evaluation method, the best data pretreatment, etc. were chosen even for partially contradictory data.

The rank transformation is far better than any other way of scaling proven by ordering the SRD values by SRD again and by cross validation.

Keywords: Ecogenotoxicity, Comet assay, Analysis of variance, Ranking, Fluorescence, mussels

1. Introduction

The mussels of the *Mytilus* sp. are commonly used as sentinel organisms for the screening of pollution and potential environmental harm [1-3]. As members of cosmopolitan species, they have been employed in numerous environmental studies from all parts of the world [4]. Several characteristics such as filter feeding, sessile life form and ability to accumulate pollutants in addition with a wide distribution, makes them favored organisms for estimating environmental pollution levels [5,6]. Showing a range of physiological, histological and molecular responses, including abnormal morphology, alterations of antioxidative status, induction of DNA strand breaks, etc. gave them applicability for *in situ* and *ex situ* assessment of the effects of the pollutants present in environment [7-9]. Most importantly, they are widely employed for assessing genotoxicity [10-13].

The comet assay or single cell gel electrophoresis (SCGE) assay is a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells [14]. The assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells. The modification of the assay, such as alkali conditions or combination with certain enzymes (e.g. endonucleases), enables detection of the DNA single strand breaks (strand breaks and incomplete excision repair sites), alkali labile sites and cross-linking [15,16].

Since 1998, when the comet assay was first performed on *Mytilus* sp., there is a steady and continuous interest each year in application of the comet assay on this mussel species. However, there are issues related to the inter-laboratory differences in the comet assay procedure. The factors that are varying the most are the preparation of cells suspensions, the conditions of the denaturation and electrophoresis and the determination of the shape, size and amount of DNA within comets. To make the assay more robust, several approaches have also evolved to quantify the extent of damage more reliably, reproducibly and meaningfully. Such quantification includes both visual examinations (i.e., photographic, occulometer or non-specific image analysis systems) and the usage of commercially available (or public domain specific) image analysis software packages. Such specific software packages also facilitate statistical analyses, plotting and documentation of the data [16]. Besides that automated system provides an advantage over manual, not only for easier management, but also because of diminishing the observer subjectivity.

As there are more parameters for the selection (Olive tail moment, tail length, and tail intensity), it leads to controversy among researchers, which is the most suitable parameter for assessing the damage of DNA. Similarly, which tissue (of the mussels) is suitable for a comet assay at best is an important aspect to know.

The objective of this study was to find out which estimated parameters are the most reliable for the *in situ* assessment of genotoxicity by sampling from sites with different anthropogenic impacts in the Boka Kotorska Bay in southern Adriatic Sea (Montenegro). Mediterranean mussel *(Mytilus galloprovincialis*) was selected as bioindicator organism and the data were obtained from comet assays performed on haemolymph, gills and digestive glands. The ranked measurement parameters were tail length, tail intensity and Olive tail moment. Moreover, we wanted to reveal, which type of scaling is appropriate at best for such type of data.

2. Materials and methods

2.1 The specimen collection

The study was carried out on 84 specimens of *M. galloprovincialis* from the southern Adriatic Sea. The specimens shell length 35-50 mm were collected in July and December 2011 and May, July and October 2012 from 5 sites with different level of pollution in the Boka Kotorska Bay, Montenegro (Figure 1).

The Kotor site is under the impact of wastewaters originating from the town Kotor and intense ship trafficking. The Dobrota site is located approximately 2 km from the Kotor site, down the current. The Tivat site is located nearby the airport; this site is also under the impact of wastewaters originating from the Tivat town. The Bijela S site is under the impact of wastes originating from the shipyard Bijela. At the Bijela F, mussels were collected from the mussel farm located approximately 1km from the shipyard, up the current.

2.2 Haemolymph collection and gill/digestive gland cells suspension preparation

Mussels were transferred to laboratory in cooling boxes and subjected to comet assay. For each sampled group of mussels (for each site) the osmolarity of Hank's balanced saline solution (HBSS) was adjusted to correspond to the level of salinity measured at the sampling site. Haemolymph collected from the adductor muscle of 3-5 specimens was mixed with the equal volume of osmotically corrected HBSS into 1.5 mL microtubes, centrifuged for 10 min at 2000 rpm and the pellets were resuspended in 60 μL of residual supernatant.

Single-cell suspensions of gills and digestive gland tissue were prepared by method of Coughlan *et al*. [17]. Tissue was excised and chopped separately in 0.2 mL of osmotically corrected HBSS by using two fresh scalpel blades in a scissor-like movement on a petri dish, washed off gently into a 15 mL centrifuge tube with a further 2.8 mL osmotically corrected HBSS and 0.03 mL of trypsin (0.5 %). The suspensions were gently rocked for 10 min at room temperature, after which 10 mL of osmotically corrected HBSS was added and the suspension was passed through a sieve to remove any large fragments that remained. After centrifugation (2000 rpm for 5 min), the supernatant was discarded and the pellets were carefully suspended in 1 mL of osmotically corrected

HBSS. The suspension was then centrifuged for 10 min at 2000 rpm and the cell suspension was made in 60 μL of residual supernatant.

2.3 Comet assay

The alkaline comet assay procedure was performed under yellow light, basically as described by Singh et al. [14]. Microscopic slides were coated with 1 % normal melting point agarose (NMP) and air dried for 24 h. To form a second, supportive layer, 80 μL of 1 % NMP was gently placed on top of the 1 % NMP layer and spread over the slide using coverslip. The slide was placed on ice for 5 min to allow complete polymerization of agarose. After the coverslips were removed, 30 μL of cells pellet suspension, gently mixed with 70 μl of 1 % low melting point agarose (37 ºC) agarose, was pipetted on the supportive layer of 1 % NMP and covered with a coverslip. After 5 min on ice the coverslips were removed and the slides were lowered into freshly made cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton X-100, pH 10) for 1 h. To allow DNA unwinding slides were put in an electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. Electrophoresis was performed by setting the power supply at 0.5 V/cm and adjusting the current to 300 mA for 20 min. After electrophoresis, the slides were placed into freshly made neutralizing buffer (0.4 M Tris, pH 7.5) for 15 min. Staining was performed with 20 μL per slide of EtBr (2 μg mL^{-1}). The slides were examined with a fluorescence microscope (Leica, DMLS, Austria, magnification 400×, excitation filter 510-560 nm, barrier filter 590 nm). Microscopic images of comets were scored using Comet IV Computer Software

(Perceptive Instruments, UK). Images of 50 cells were collected from each slide per sample and among the parameters available for analyses; tail length, tail intensity and Olive tail moment were chosen as the measure of DNA damage.

Eighty-four specimens of *M. galloprovincialis* were investigated. Precisely three tissues were analyzed from each specimen: haemolymph (h), digestive gland (d) and gills (g). As shown in Table 1, results are presented for each site per tissue and for all three evaluation method for fluorescence measurements: tail length (l), tail intensity (i) and Olive tail moment (m).

All measurement values for sampling places (and dates) were averaged: a hypothetical average specimen was defined such a way (five sampling site in summer and winter in 2011 as well as spring, summer and autumn in 2012, altogether 25 averages).

2.4 Calculations, modeling

Sum of ranking differences (SRD) has recently been introduced for method and model comparison [18-20]. The rank numbers of the actual and a reference (benchmark) ranking are subtracted and the absolute values of rank differences are calculated and summed for each method. Such a way all three tissues and three evaluation methods could be compared in all combinations (the nine methods denoted by lh, mh, ih, ld, md, id, lg, mg, ig); all of them receives an SRD value. As the various methods were measured on different scales, data pretreatment has been carried out column-wise ~~is necessary and~~ as follows: Numerical scales were transferred into ranks (rnk); range scaling between 0 and 100 (scl), standardization (autoscaling, std) and normalization to unit length (nor) were carried out. During rank transformation the numerical values of each column of supplementary Table 1 were arranged in increasing order, the smallest value received rank number one, the second smallest number two and so on till the largest received rank number *n* (the number of rows).

Four orderings were completed (by SRD) according to data pretreatments. Row-medians have been used as reference (benchmark). Uncertainty values were assigned to SRD values using a bootstrap like validation technique (cross-validation) as follows: Approximately 1/7th of the samples were removed seven times. In each step, the ranking of methods were completed on the remaining (6/7th) of the samples, i.e. on the training set(s), and the left out part was simply ignored. As the number of samples during cross-validation is smaller, the variance is slightly overestimated (a conservative estimation). Seven-fold cross-validation multiplied the SRD values seven times: such a way 3(tissues) * 3(evaluation methods) * 4(pretreatment methods) * 7(repetitions) = 256 SRD values were calculated and later subjected to variance analysis (ANOVA).

ANOVA is a technique used to assess effects of the categorical factors and their interactions [21]. The following model was considered:

$$\mathrm{SRD} = b_0 + b_1{*}I1 + b_2{*}I2 + b_3{*}I3 + b_{12}{*}I1{*}I2 + b_{13}{*}I1{*}I3 + b_{23}{*}I2{*}I3 + b_{123}{*}I1{*}I2{*}I3 \quad (1)$$

where SRD stands for the sum of absolute ranking differences, $I1$ is the type of evaluation for fluorescence measurements (3 levels denoted by l, m, i), $I2$ is the tissue (organ) studied (3 levels: h, d, g), $I3$ is the type of data pretreatment (4 levels: rnk, scl, nor, std). Seven repetitions allow us to test the significance of factors and their interactions.

The main advantage of SRD procedure is its simplicity and the easy way to assess the results: the smaller the SRD the better.

Rescaling the data and ordering them by SRD make possible to reveal one more effect by ANOVA (the effect of data pretreatment, scl, nor, rnk and std), whereas the classical ANOVA would provide four different, contradictory two-way ANOVA results (just on the effects of two factors: type of tissues and evaluation methods for fluorescence measurements).

Mann Whitney U-test and ANOVA calculations have been carried out by STATISTICA (data analysis software system), version 7.1. StatSoft, Inc. (2005) www.statsoft.com.

A computer code for method and model comparison (ranking and grouping, as well), i.e. a Visual Basic Application program for MS Excel was applied for SRD ranking; it can be downloaded from the homepage:

http://aki.ttk.mta.hu/srd

and it is called: Compare Ranks with Random Numbers (CRRN) without ties. This program was used for all SRD calculations.

3. Results and Discussion

The results indicated variations in the level of DNA damage at different sites (Supplementary Table 1). Excluding the spring season, the Dobrota site can be set aside as the site with the lowest level of DNA damage and the lowest variations in DNA damage throughout different seasons.

Seasonal variations in the level of DNA damage were observed for all three tissues alike. For haemolymph and digestive gland, the level of DNA damage was significantly higher during summer in comparison with the winter/autumn of the corresponding year (Mann Whitney U-test, $p<0.05$). Observed differences were especially evident for Olive tail moment and tail intensity. The significant increase in DNA damage in gills and digestive glands was detected in spring 2012, comparing to winter 2011.

Table 2 summarizes the ANOVA results. All three factors (type of tissue; evaluation method, pretreatment method) are significant alone (separately). Their cross coupling ($I1*I2*I3$) is not significant and the interaction term (“type of tissue” * “pretreatment method”) is not significant, either. The other two interaction terms are significant at the predefined 5 % level (c.f. last column in Table 2).

Figure 2 shows the effect of all factors in an easily perceivable way. As SRD the smaller the better, ANOVA SRD evaluation provides an easy selection of best measurement methods: Olive tail moment is the best (produces the smallest SRD) for all tissues (green line, rhombuses in Figure 2) except perhaps for normalized data and digestive gland, when it is equivalent with (not significantly better or worse than) tail intensity (normalized data pretreatment and for digestive gland). Tail intensity is better for gills and heamolymph, but this is not the case for digestive gland. Considering the data pretreatment methods a relatively stable pattern can be observed a minimum at the rank transformation: i.e. the latter is the best treatment. Even the exceptions have shown a pattern, scaling is the best in one particular case (scaling & gills & tail moment); or scaling is the second best showing a zigzag pattern (three additional case see Figure 2).

Rearranging the same information Figure 3 shows a different pattern with the same conclusions.

All the three line plots for tail moment (right part of Figure 3) do locate with smaller SRD values than the remaining line plots. The SRDs for the three tissues in case of tail length are close to each other the error bars are overlapping. Somewhat larger differences (among the line plots for different tissues) can be observed in case of tail intensity. Then, the digestive gland produces the best result for all data pretreatment methods. However, the main conclusions are that tail moment is the best evaluation method for all organs, and rank transformation is the (far) best data pretreatment method. Accordingly this combination can be recommended for further studies.

There are some more additional proofs to select (validate) the best data pretreatment method. Three techniques are at our disposal.

(i) The SRD values can be arranged so that the columns (methods to be compared) contain the three methods for pretreatment. Such a way 63 rows are built corresponding the seven repetitions by cross-validation and nine combination of tissues and evaluation methods (denoted by lh, mh, ih, ld, md, id, lg, mg, ig). Then a new SRD ranking has been carried out using row-minimums as reference. The results can be seen on Figure 4.

There is no doubt that rank transformation is the best method, standardization and normalization is practically (and statistically) indistinguishable and range scaling is the worst method for these type of data.

(ii) Cross-validation (in this case seven-fold plus SRD values for all *n*, where *n* is the number of rows, 63) is able to render uncertainties to the SRD ranking. A box and whisker plot shows the uncertainties: suitable test (*t*-test with the assumption of normality), sign test and Wilcoxon's matched pair test unambiguously shows the equivalence of the standardization and normalization. All other comparisons are significantly different at the 5 % error level (Figure 5).

(iii) ANOVA result shows significance for the data pretreatment factor (c.f. Table 2). However, the individual comparisons suggest that only the rank transformation is different from all others.

Few precautions were taken to optimize the comet assay procedure in order to enable the outcomes of this study to be applicable on the results of the other laboratories. Taking into account that cell suspension preparation and manipulation with cells can affect the background level of DNA damage, we used osmotically corrected solutions for cell dissociations as recommended by many authors [17,22,23]. Considering that conditions of denaturation and electrophoresis differ among the laboratories, we used conditions that we found suitable for the cells of the most animals, i.e. freshwater mussels, freshwater fish, mammals etc. [24-27].

We have observed variations in the level of DNA damage in mussels collected at different sites probably caused by the difference in origins of pollution. The sites Kotor, Dobrota and Tivat are mainly under the impact of municipal wastewaters, which are disposed immediately at the coastal line [28]. Influence of shipyard in Bijela has been emphasized in a study of Da Ros *et al*. [29] employing various bioassays on *M. galloprovincialis* such as lysosomal response and metalthione induction. In the same study, significantly higher levels of pollution pressure in comparison with Dobrota were detected at the sites Tivat and Bijela, which is in compliance with our study.

Also, we have detected seasonal variations in the level of DNA damage. There are few possible explanations for the variations. First of all, selected sites are under different pollution pressure during different seasons. The sites Kotor and Tivat are centers of tourism. Regularly, they are inhabited by 20,000 citizens. However, during summer season, the number of visitors is several times higher. Also, shipyard in Bijela operates more actively in summer season because of the weather conditions.

Secondly, variations could be linked to seasonal variations of water temperature. We assume that the decrease in filtration rate during months with a lower water temperature may be the one of the reasons for the decrease in DNA damage due to lower exposure to genotoxic substances in water [30-32]. Our results are in compliance with the study of Pavlica *et al*. [33] performed on the same species of marine mussel, in northern Adriatic, which shows the existence of seasonal variation in the level of DNA damage, assessed by micronucleus test, correlated with water temperature. Also, Sokolova and Lannig [34] emphasized the impact of environmental temperature on the modulation of the toxicity of waterborne pollutants in ectotherms such as mollusks, through changes in uptake and accumulation rates, and through modulation of the intrinsic sensitivity of intracellular targets to pollutants.

Intense rainfall during spring 2012 caused decrease in salinity at the sampling sites, which could be an explanation for the observed increase in DNA damage. The salinity is recognized as abiotic factor: it could influence the baseline DNA damage levels according to numerous studies [35-37]. Annual average values for salinity at the sites Dobrota and Kotor are 31 ± 2 ‰ and 30 ± 6 ‰ respectively. Salinity values were 8 ‰ for Dobrota and 4 ‰ for Kotor in spring 2012.

Regarding selection of the tissue or the analyses, three tissues were selected, following previous research, haemolymph, digestive gland and gills. Majority of the genotoxicity studies on *Mytilus* sp. are performed on the haemolymph [13]. The main reason is that haemolymph can be easy collected without sacrificing of the specimens which gives high advantage to this tissue (repeatable usage of the same specimens). Comparing to the other tissues, preparation of the haemolymph for the comet assay procedure requires less handling which results in the lower level of DNA back-ground damage in this tissue [23]. Although the majority of the studies employ haemolymph, the study of Hartl *et al*. [22] showed that the level of DNA damage in haemocytes should not be used to predict the level of DNA damage in cells of other organs. Reason for this can be higher cell turnover rate in haemocytes comparing to other tissues [38] or the differences in enzymatic and

DNA repair activities in different tissues [23]. Therefore, we suggest that haemolymph should be used as a biomarker only for acute contaminations while for chronic exposures, which are common for in situ studies, we recommend gills and digestive gland. Gills are favored tissue for assessment of genotoxicity by many authors because of the direct contact with medium and higher concentrations of oxygen during [11] while digestive gland is often used because it is the main organ of metabolism of organic compounds and the main site of biotransformation activities [39]. Moreover gills and digestive gland show similar genotoxic response and often higher response in comparison with other tissues i.e. haemolyph and gonads [40,41]. Three measures of DNA migration are commonly used: tail length, tail intensity and Olive tail moment [42]. So far, the most used parameter in studies performed on *Mytilus* sp. was tail intensity, which is understandable considering that by many authors it is advisable to use this parameter for inter-laboratory comparisons [42,43]. The second most used is tail moment, which by Kumaravel and Jha [44] is as reliable as tail intensity.

Our results suggest that selection of tissue and measurement of DNA damage does matter and that assessment of genotoxicity differs significantly based on selected data set. Selecting Olive tail moment and digestive glands (as suggested measurement/tissue by SRD), variations between the sites/seasons are easily noticed while selecting other sets of data, such as tail length and gills, differences between sites/seasons were not so evident or completely lacking. Although our results suggest usage of tail moment, it should be emphasized that our results imply usage of the Comet assay IV software. Among the different software packages, there is a variation in algorithms used to define the center of gravity of DNA distribution of the heat and tail, which is essential in Olive tail moment calculation.

4. Conclusion

Sum of ranking differences (SRD) and analysis of variance (ANOVA) provide a unique and unambiguous way of decomposing the effects and determine the best combination of factors.

The rank transformation is far better than any other way of scaling. This has also been proven by ordering the SRD values by SRD, ANOVA (and cross validation).

Tail moment is the best for all data treatments and for all tissues; second best is tail length, and tail intensity is the third (except for digestive gland).

Whereas rankings for gills and heamolymph cells do not differ significantly, cells of digestive gland are much better for genotoxicity estimation.

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Table 1 Comet assay results represent three parameters: tail length (l), tail intensity (i), tail moment (m) of *M. galloprovincialis* sampled at 5 sites in the Boka Kotorska Bay, Montenegro during 2011-2012

Sampling	Site	Specimen	hemolymph			digestive gland			gills		
			l	m	i	l	m	i	l	m	i
Summer 2011	Dobrota	1	18.92	0.80	5.98	15.10	1.00	12.19	17.50	0.87	8.48
		2	19.68	0.94	6.12	16.32	1.02	12.42	18.14	0.79	7.60
		3	24.90	1.23	7.53	18.00	1.37	11.36	17.44	1.05	10.90
	Kotor	1	46.66	2.04	13.37	18.32	1.79	19.63	28.40	2.31	18.50
		2	44.12	2.89	16.58	17.80	2.16	22.98	36.14	4.20	23.13
		3	37.58	2.21	12.81	37.90	4.11	21.04	36.90	3.34	19.64
		4	46.24	2.39	15.03	33.54	2.88	17.49	28.58	2.62	18.19
	Tivat	1	26.04	1.05	7.29	17.64	2.08	18.06	25.08	2.81	19.37
		2	19.86	1.16	9.23	21.24	2.44	18.47	21.64	2.19	15.45
		3	24.54	1.44	9.55	20.40	2.30	20.42	28.16	3.30	23.49
	Bijela S.	1	25.96	1.83	14.53	35.58	3.26	17.83	35.50	3.27	17.67
		2	49.82	2.41	18.03	12.56	1.03	18.64	24.68	2.30	15.53
	Bijela F.	1	16.12	0.80	8.40	27.12	3.32	18.64	23.64	1.99	15.44
		2	16.46	1.20	9.74	27.22	2.61	17.46	22.56	2.68	18.58
Winter	Dobrota	1	13.38	0.28	2.99	27.08	1.41	8.31	22.08	1.52	11.62
		2	12.16	0.36	3.99	25.90	1.24	8.02	23.74	1.33	9.03

2011		3	10.60	0.30	4.02	20.44	0.74	6.93	28.64	2.14	11.47
		4	12.62	0.48	4.61	17.80	0.90	8.57	19.70	0.89	7.92
	Kotor	1	16.76	0.38	3.54	18.46	0.68	7.17	25.58	1.04	8.00
		2	24.50	1.06	7.20	34.78	2.26	11.38	52.62	6.56	25.18
		3	30.88	1.93	10.82	37.32	2.10	10.78	46.64	4.22	18.37
		4	26.36	0.89	5.52	29.12	1.34	8.54	33.82	1.85	10.43
	Tivat	1	26.24	1.12	6.78	24.82	1.66	9.21	24.42	1.43	10.48
		2	25.34	1.06	6.83	33.28	2.34	13.04	20.22	0.86	7.43
		3	22.20	0.81	5.71	36.04	2.58	14.27	28.84	1.44	9.87
		4	19.80	0.77	6.05	17.62	0.66	6.46	28.84	2.14	14.32
	Bijela S.	1	30.90	1.85	11.68	14.02	0.73	8.01	26.56	1.69	11.15
		2	24.96	1.61	11.00	32.00	1.45	8.79	26.30	2.61	15.21
		3	23.92	1.37	9.78	31.54	1.32	8.49	22.68	1.42	22.47
		4	23.42	1.29	9.15	18.86	0.97	8.70	22.04	1.65	11.76
	Bijela F.	1	20.56	1.06	8.84	18.98	0.93	7.95	17.50	1.05	10.57
		2	19.72	0.67	5.99	19.92	1.15	9.66	20.32	1.60	13.26
		3	16.88	0.74	6.74	20.31	0.95	9.16	35.36	4.31	10.12
		4	17.28	0.63	6.04	23.62	1.48	11.42	23.00	2.18	14.96

Table 1 (cont.)

Sampling	Site	Specimen	hemolymph			digestive gland			gills		
			l	m	i	l	m	i	l	m	i
Spring 2012	Dobrota	1	28.46	1.42	9.16	30.27	3.27	15.63	23.27	1.93	21.70
		2	24.44	1.44	9.29	46.68	4.25	16.74	39.74	3.81	17.40
		3	23.96	1.18	7.32	68.58	11.60	35.89	54.36	6.06	21.83
	Kotor	1	24.40	0.81	5.98	35.22	2.52	14.76	41.90	5.63	25.85
		2	21.52	0.66	5.31	50.55	7.79	27.40	48.58	9.83	39.52
		3	28.08	1.76	9.93	44.04	4.76	21.04	42.24	6.13	23.48
		4	19.32	0.71	5.60	49.40	5.03	22.68	49.78	6.19	20.67
	Tivat	1	41.88	3.42	14.92	35.80	2.60	14.44	33.64	2.13	21.80
		2	25.38	1.38	7.89	41.62	3.13	13.83	34.48	1.10	10.73
		3	17.64	0.84	7.40	28.00	1.20	6.09	58.68	5.53	21.80
	Bijela S.	1	25.50	0.96	6.42	69.06	8.03	23.99	55.62	3.77	19.90
		2	32.36	1.97	9.14	50.08	3.22	13.94	30.20	1.55	10.95
	Bijela F.	1	29.56	1.34	7.98	47.66	2.97	13.16	58.84	5.72	18.52
		2	27.38	1.33	8.39	49.80	3.86	14.12	30.58	1.74	11.87

		3	22.30	1.51	10.37	34.00	2.11	12.81	43.64	3.20	18.00
		4	35.40	1.71	6.93	34.98	2.45	11.74	29.86	2.47	17.54
Summer 2012	Dobrota	1	48.26	2.19	8.91	61.30	3.79	13.60	48.96	3.75	12.96
		2	47.84	1.91	8.06	59.52	3.10	11.53	54.46	2.89	10.56
		3	51.40	3.77	13.56	49.90	3.15	13.07	67.72	4.89	15.75
	Kotor	1	51.32	2.39	9.85	60.58	5.28	17.25	62.72	5.03	15.61
		2	49.24	2.03	9.14	66.42	7.73	23.65	59.64	3.69	12.96
		3	62.28	5.36	19.79	57.78	4.01	14.65	71.12	7.93	24.30
		4	60.60	4.39	16.34	51.78	5.10	17.79	84.48	19.03	46.40
	Tivat	1	65.30	4.77	18.12	54.52	5.61	22.63	78.94	9.42	28.17
		2	66.92	5.74	21.76	49.96	4.38	18.85	87.34	10.20	28.82
		3	64.18	5.42	20.56	73.76	6.51	20.99	67.12	4.41	14.80
		4	56.12	4.36	18.41	78.14	7.50	24.47	61.68	4.53	17.27
	Bijela S.	1	32.28	2.33	13.37	37.76	2.60	14.52	50.62	3.86	16.23
		2	54.46	3.71	14.76	37.48	2.27	10.37	57.88	3.50	12.79
	Bijela F.	1	53.42	2.80	11.01	51.12	2.93	11.38	52.70	2.92	11.94
		2	46.66	2.32	8.35	38.92	1.82	9.09	53.50	2.18	8.57
		3	52.76	2.36	7.89	58.80	5.10	17.65	66.88	4.45	14.74
		4	56.18	2.98	10.41	52.74	5.86	21.05	54.72	2.58	9.99
Autumn 2012	Dobrota	1	34.32	0.50	2.20	24.48	0.43	2.50	43.94	2.78	10.68
		2	34.06	0.95	5.03	28.12	0.44	2.55	42.82	2.30	9.34
	Kotor	1	30.22	0.76	4.16	37.94	3.30	14.22	40.24	2.59	12.04
		2	32.66	0.77	3.91	48.72	2.71	9.46	31.10	1.77	8.37
		3	36.02	0.73	3.32	23.56	0.87	5.35	40.60	0.74	3.16
		4	20.24	0.12	1.08	16.58	0.24	2.37	25.60	1.00	6.26
	Tivat	1	16.68	0.88	7.11	30.08	1.91	10.67	33.74	1.61	8.52
		2	27.70	0.60	3.62	33.42	1.39	7.54	24.54	1.40	8.59
		3	29.06	0.92	4.57	24.76	1.05	5.81	27.20	1.43	9.04
	Bijela S.	1	28.18	1.34	6.58	47.52	2.96	11.09	33.44	1.19	6.06
		2	37.94	2.73	12.36	48.66	4.70	16.29	28.94	1.07	5.10
		3	29.12	1.19	7.00	43.86	2.72	11.26	58.18	5.37	16.76
		4	39.33	1.07	4.39	48.12	4.93	18.03	46.48	5.09	18.34
	Bijela F.	1	36.80	1.44	6.01	23.38	0.55	3.95	40.84	2.40	9.70
		2	53.46	3.55	13.53	52.00	3.35	11.99	27.48	1.73	10.17
		3	31.84	1.06	5.27	32.60	1.73	8.34	37.80	2.40	11.61
		4	32.12	0.95	4.89	55.00	5.67	20.83	40.32	2.56	11.28

Table 2

Univariate tests of significance for 252 SRD values (Over-parameterized model, Type III decomposition) *I*1 – evaluation methods: tail length, tail intensity and Olive tail moment; *I*2 – tissues: haemolymph, gills and digestive gland; *I*3 – pretreatment methods: rank transformation, range scaling, standardization, normalization to unit length. Significant factors are indicated by bold.

	Sum of squares	Degree of freedom	MS	*F*	*p*
Intercept	**1051481**	**1**	**1051481**	**14771.07**	**0.000000**
***I*1**	**16108**	**2**	**8054**	**113.14**	**0.000000**
***I*2**	**1086**	**2**	**543**	**7.63**	**0.000629**
***I*3**	**4631**	**3**	**1544**	**21.69**	**0.000000**
***I*1*I*2**	**2961**	**4**	**740**	**10.4**	**0.000000**
*I*1**I*3	695	6	116	1.63	0.140912
***I*2*I*3**	**1883**	**6**	**314**	**4.41**	**0.000316**
*I*1**I*2**I*3	944	12	79	1.1	0.357599
Error	15376	216	71		

Figure 1

Sampling sites at the Boka Kotorska Bay

Figure 2

Effect of factors by variance analysis for seven-fold cross-validation of SRD values. (The median was used for reference in ranking.) Raw SRD values were plotted on the y – axis. Vertical bars denote 0.95 confidence intervals.

Figure 3

Effect of factors (differently grouped) by variance analysis for seven-fold cross-validation of SRD values. (The median was used for reference in ranking.) Raw SRD values were plotted on the y – axis. Vertical bars denote 0.95 confidence intervals.

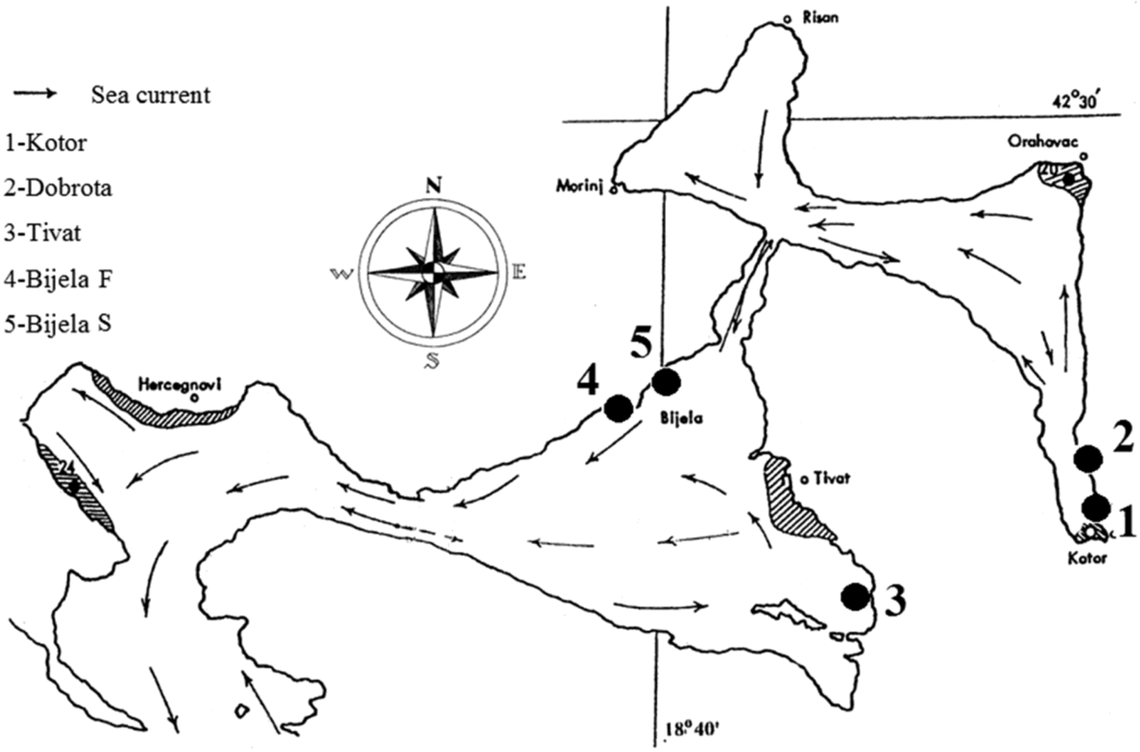
Figure 4

Ordering of data pretreatment methods using sum of ranking differences. Row-minimums were used as benchmark. Scaled SRD values (between zero and hundred) are plotted on x axis and left y axis (the smaller the better). Right y axis shows the relative frequencies (only for black Gaussian curve). Parameters of the fit are m=66,67 s=5.39. Probability levels 5% (XX1), Median (Med), and 95% (XX19) are also given.

Figure 5

Box and whisker plot for seven-fold cross-validation for four data pretreatment methods. SRD values are plotted on the y axis.

Figure 1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Hercegnovi
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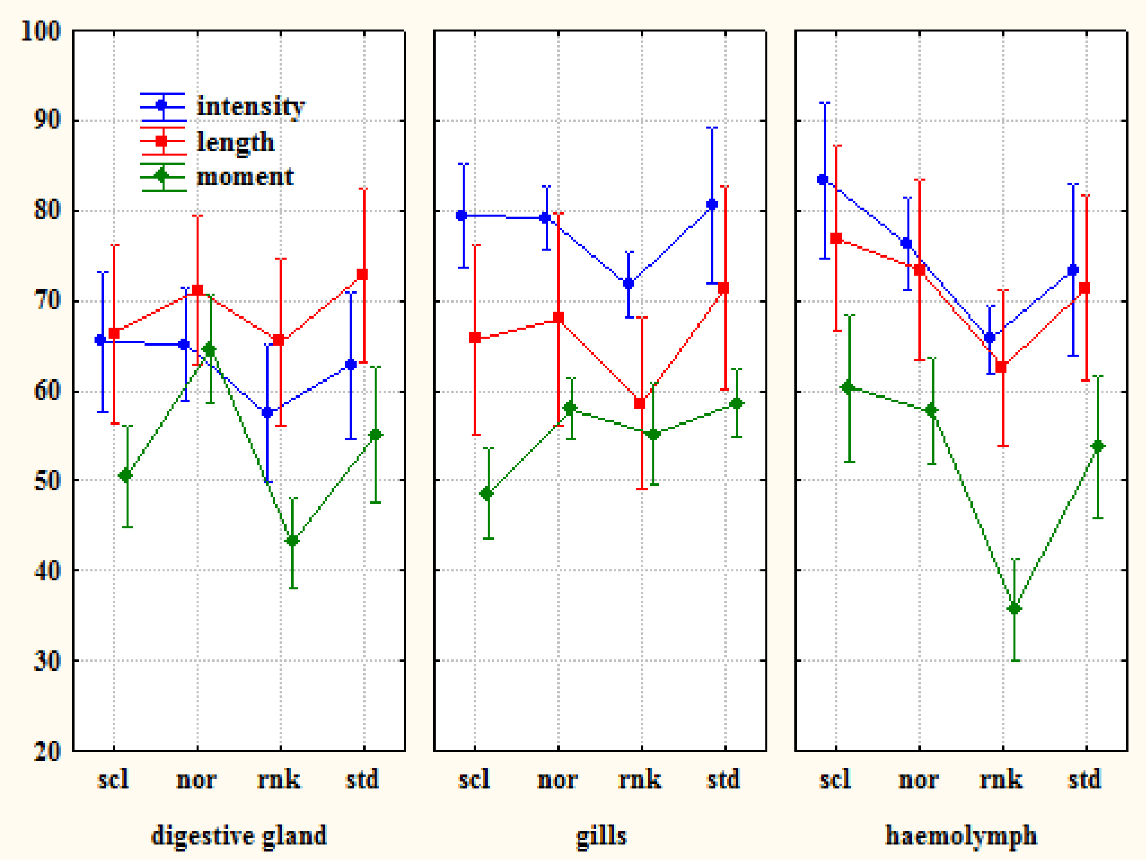
Figure 2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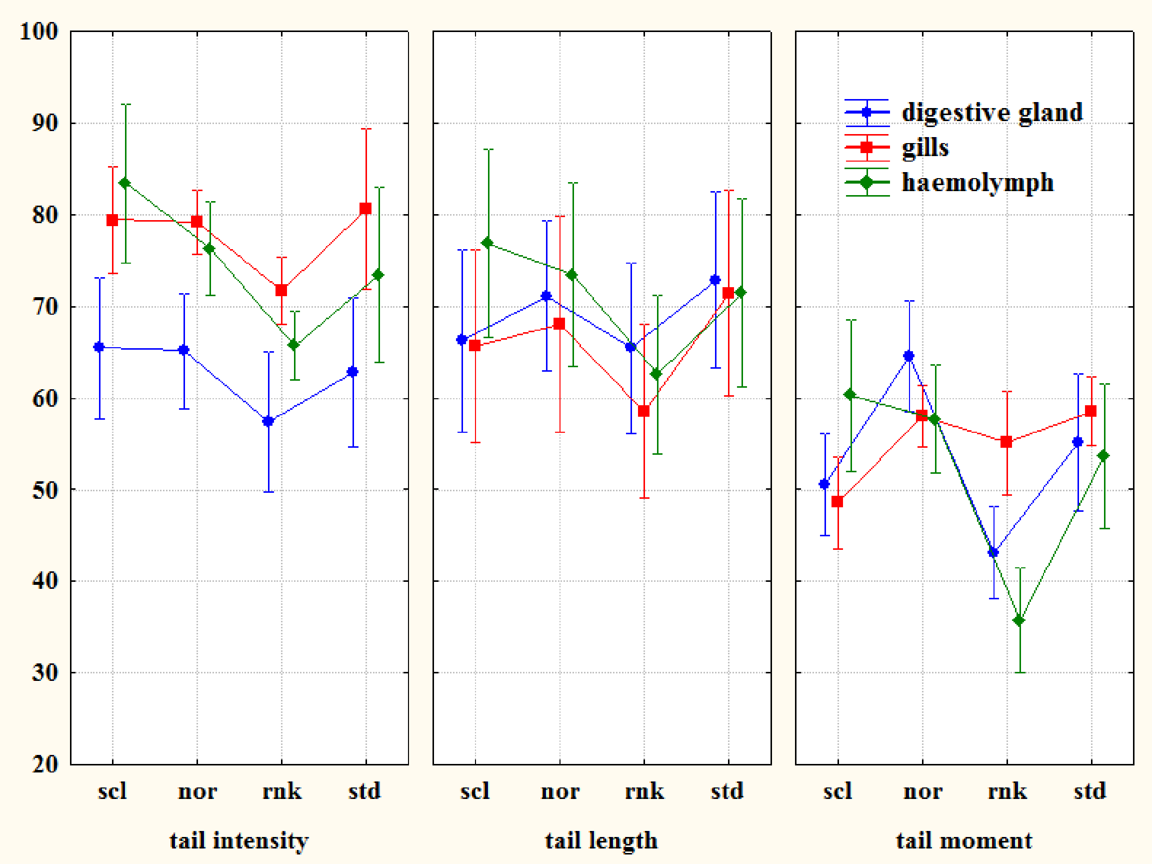
545 Figure 3



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548 Figure 4

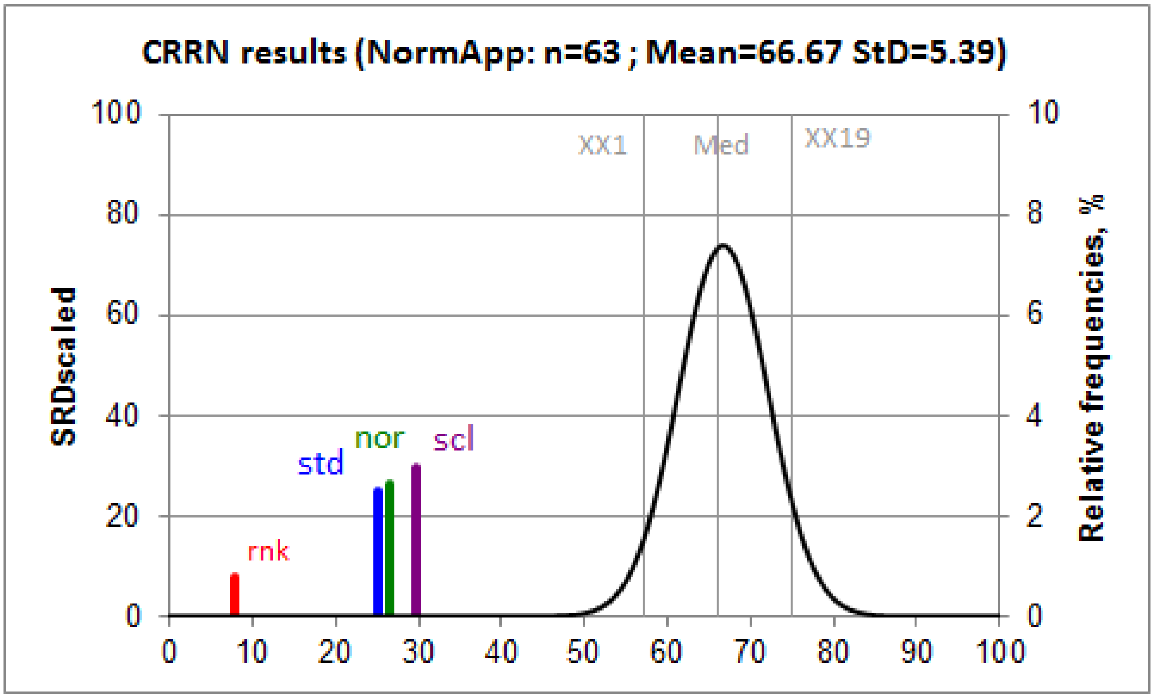

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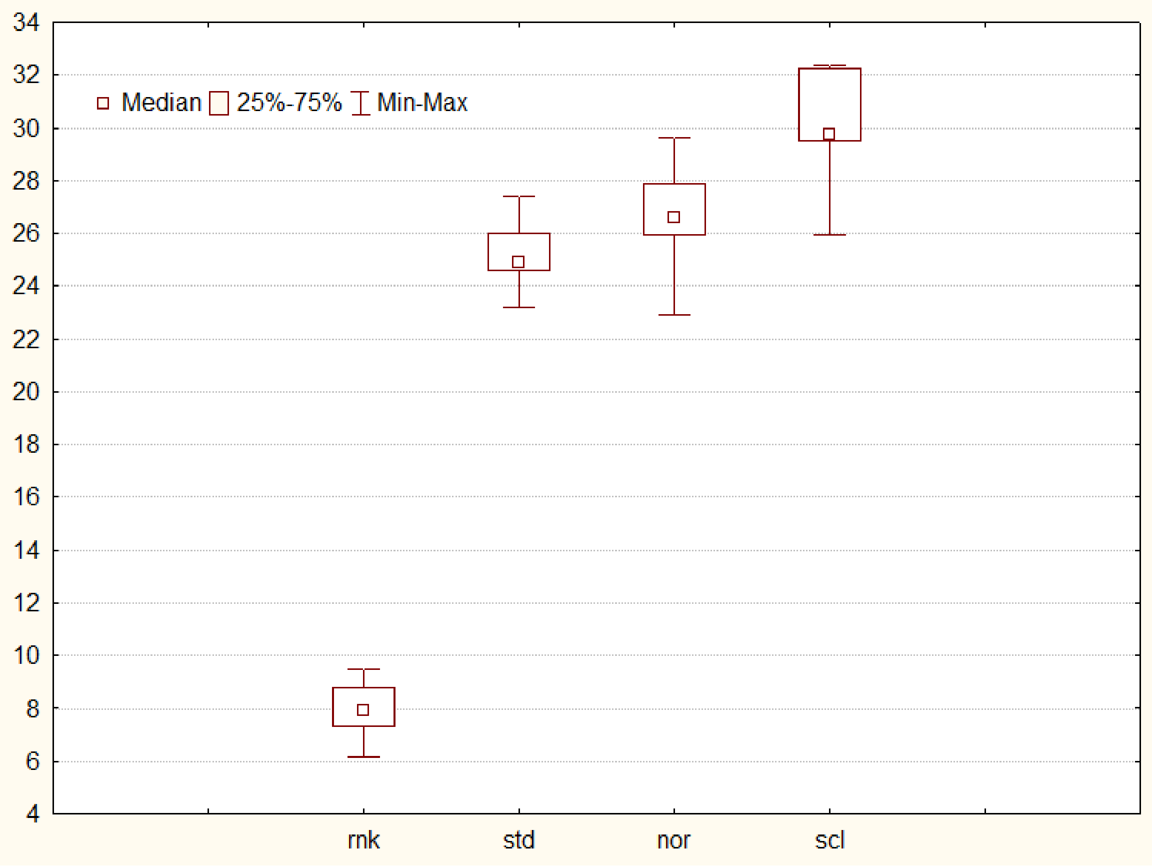
Figure 5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