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**Zawartość białka i innych związków ninhydrinododatnich
rozpuszczonych w wodzie stawu nawożonego ściekami
cukrowniczymi***

**The content of protein and other ninhydrin-positive compounds
dissolved in the water of a pond fertilized with beet sugar
factory wastes**

Wpłynęło 5 lipca 1976 r.

Abstract — The content of α -amino nitrogen was determined in condensed water, after hydrolysis of dry residue soluble (a) and insoluble (b) in phosphate buffer.

a) The compounds of molecular weight over 4000, from 4000—250, and below 250 were separated,

During the processes of production of organic matter in the pond the amount of dissolved α -amino nitrogen increases. This results both from the extracellular secretion of algae and from the autolysis of their cells, preceded by the appearance of bacteria. The amount of α -amino nitrogen in the water depends on the numbers of algae and of physiological groups of bacteria. The succession of phyto- and zooplankton influences the formation of relations between the compounds containing α -amino nitrogen of various molecular weight (protein, peptides, and amino acids).

Organic matter dissolved in the water is an important factor in aquatic systems. Above all, it is important as food for heterotrophic organisms; moreover, it acts biotically, inhibiting or stimulating the growth of organisms. A great part of this substance, consists of protein.

An investigation on the content of protein and ninhydrin-positive compounds in the accumulative pond Zimowy Wielki was carried out in autumn 1972, since this period was most characteristic for biochemical changes.

* Praca wykonana w problemie węzłowym 09.1.7.

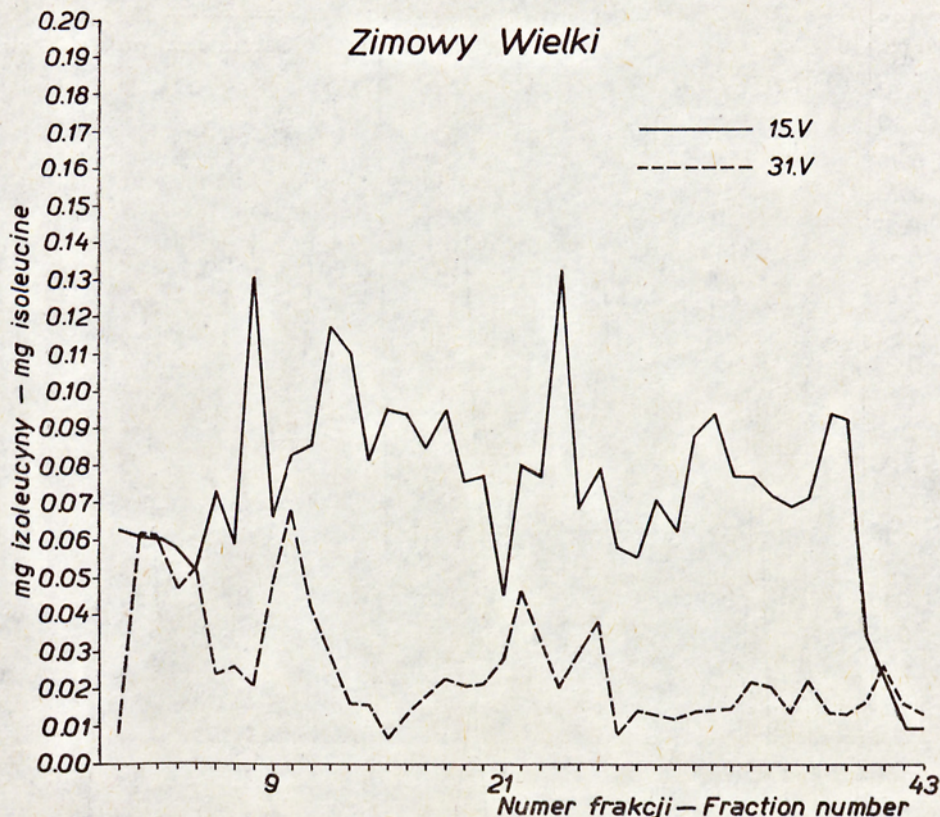
In the period preceding the investigation, from December 1971 to February 1972, beet sugar factory wastes were fed to the accumulative pond to the amount of 720 000 m³ per 8 ha × 0.9 m of mean depth. According to Lewkowicz (1973), as the process of self-purification of wastes advanced, the content of organic nitrogen in water increased. The allochthonic organic matter occurring in the water was undergoing a change under the influence of heterotrophic organisms dominating at that time (Kyselowa 1973), this being also shown in the narrow C:N ratio (Lewkowicz 1973). At the next stage, on a partially mineralized substratum, autotrophic organisms began to develop. They favourably influenced the oxygen relations, thus changing the pond biocenosis.

On the basis of the results obtained by the above-mentioned authors, an attempt was made to determine the degree to which the allochthonic organic matter (sugar factory wastes) and the organic matter produced by synthesizing algae affect the quantity of protein substances dissolved in the water.

Material and method

Samples from the waste pond Zimowy Wielki were collected from 15th May to 12th October 1972, a total of 9 samplings from this pond and 2 additional ones from the control pond Gorol being carried out. The content of total nitrogen in the water was determined using the Nessler method in samples combusted according to the Kjedahl method.

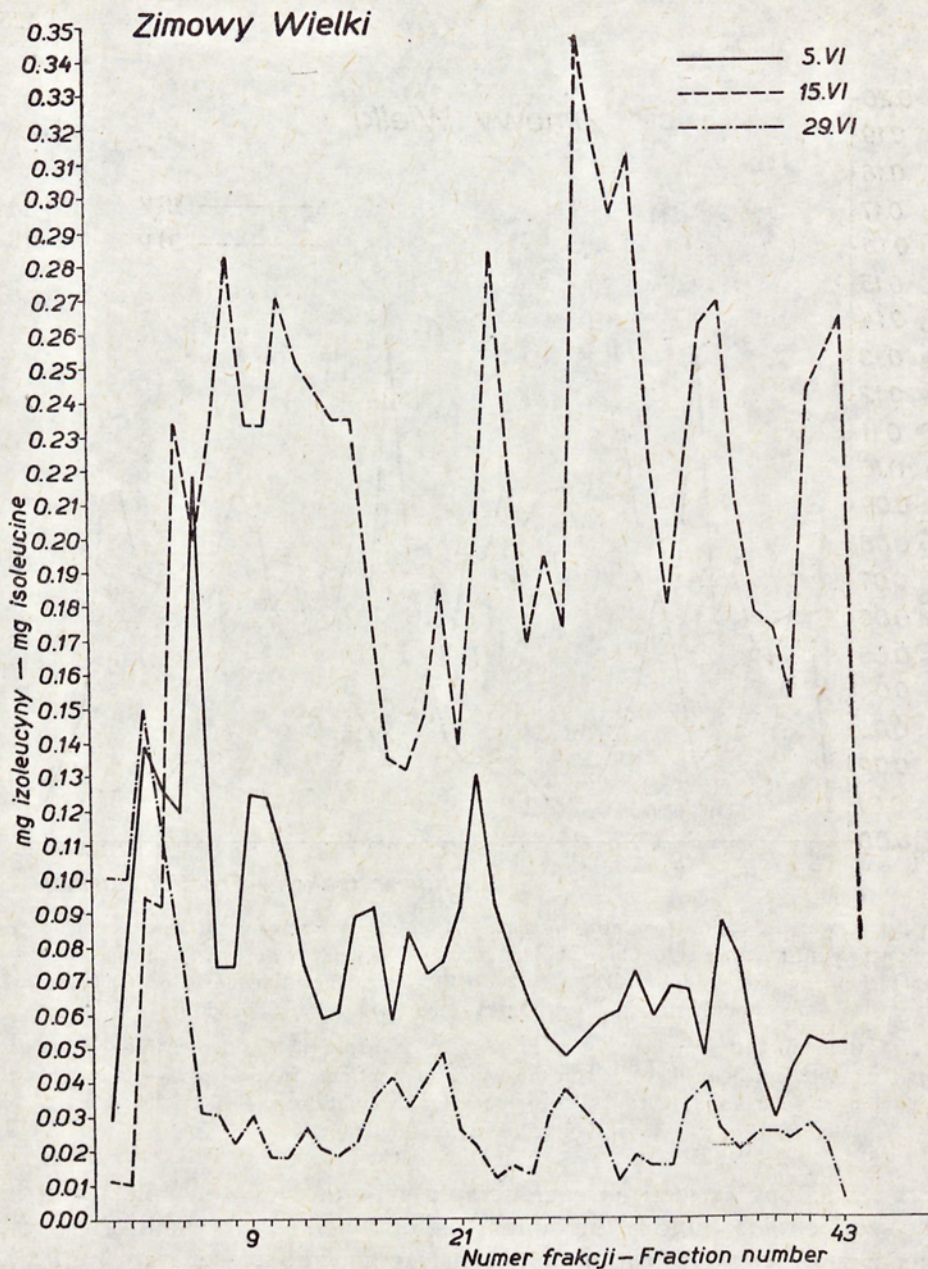
For the determination of dissolved α -amino nitrogen the water was filtered through glass GFC Whatman filters. The filtrate was evaporated at 45°C, the dry residue of about 4 mg of total nitrogen dissolved (with the exception of samples from 12th October, containing 2 mg of total nitrogen dissolved) being treated with phosphate buffer of pH = 7.6. The centrifuged supernatant containing soluble nitrogen compounds was fractionated on a column filled with Sephadex G 25 fine, 60 cm in height and 2.6 cm in diameter. The column was prepared and calibrated according to the method given by Ford (1965) and adopted by Buraczewski (1970) and Żebrowska (1971). The void volume of the column was 140 ml and the flow rate 110 ml/hr. Phosphate buffer of pH = 7.6 was used for separation. Soluble protein and peptides of molecule weight over 4000 were chiefly collected in 100—154 ml fractions, peptides of molecule weight from 4000—250 in 160—232 ml fractions, and free amino-acids in 238—370 ml fractions (figs 1—4). After an acid hydrolysis carried out in sealed glass tubes, the content of α -amino nitrogen was determined using the Moore and Stein (1954) method, in the



Ryc. 1. Zawartość azotu α -aminowego z maja 1972 r., wyrażona równoważnikową ilością izoleucyny na 1 mg azotu ogólnego rozpuszczonego w wodzie we frakcjach: od 0 do 9 — cząsteczki o masie powyżej 4000, od 10 do 21 — cząsteczki o masie od 4000 do 250, od 22 do 43 — cząsteczki o masie mniejszej niż 250

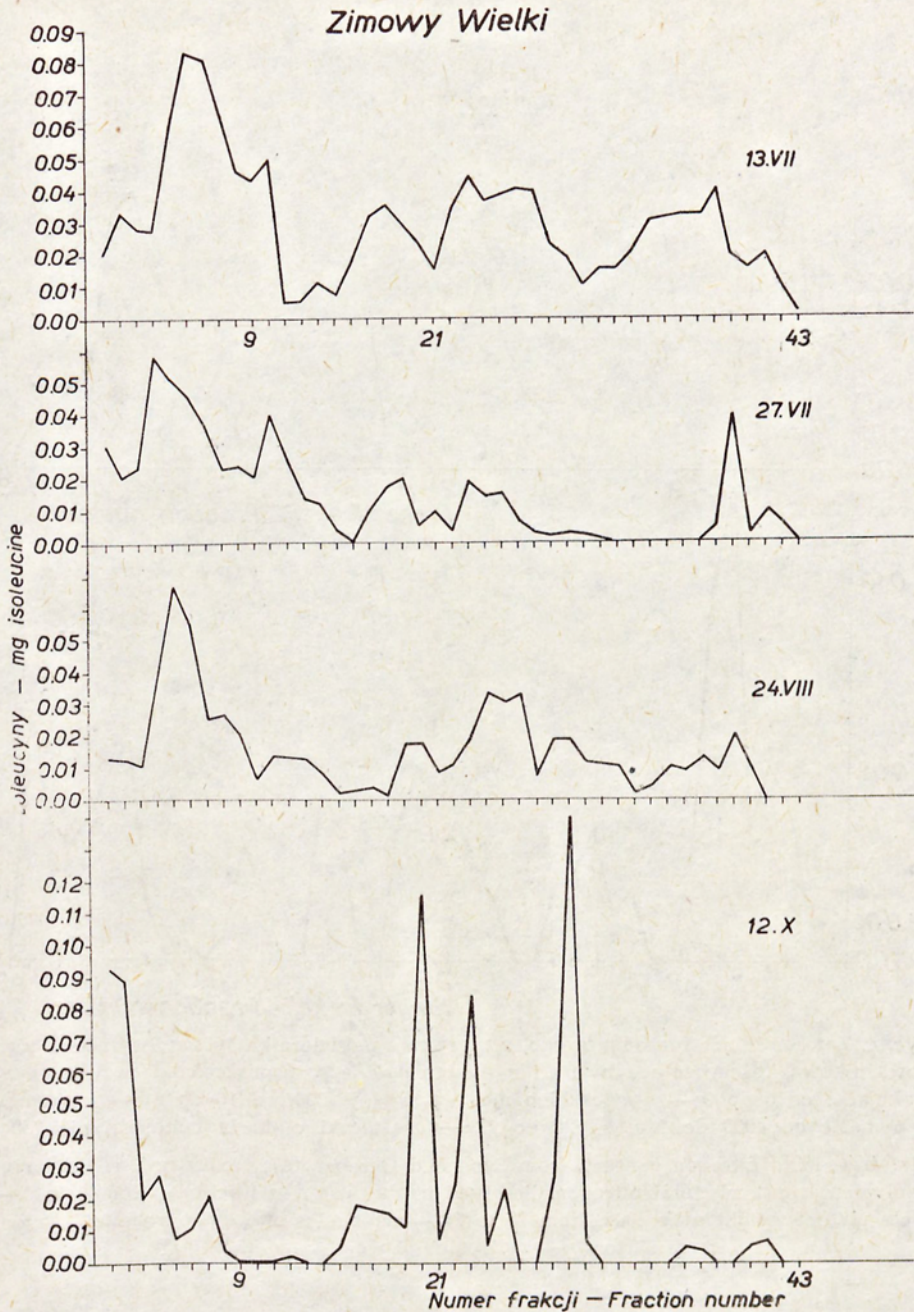
Fig. 1. α -amino nitrogen content, from May 1972, expressed as isoleucine equivalent per 1 mg total nitrogen dissolved in the water in fractions: from 0 to 9 — molecules of weight over 4000, from 10 to 21 — molecules of weight from 4000 to 250, from 22 to 43 — molecules of weight under 250

three fractions as well as each sample separately (43 samples) and in the sediment insoluble in the buffer. The obtained results are arithmetical means from two replications of each fraction and of separate outflow. They are expressed in milligrams of isoleucine, used as standard.



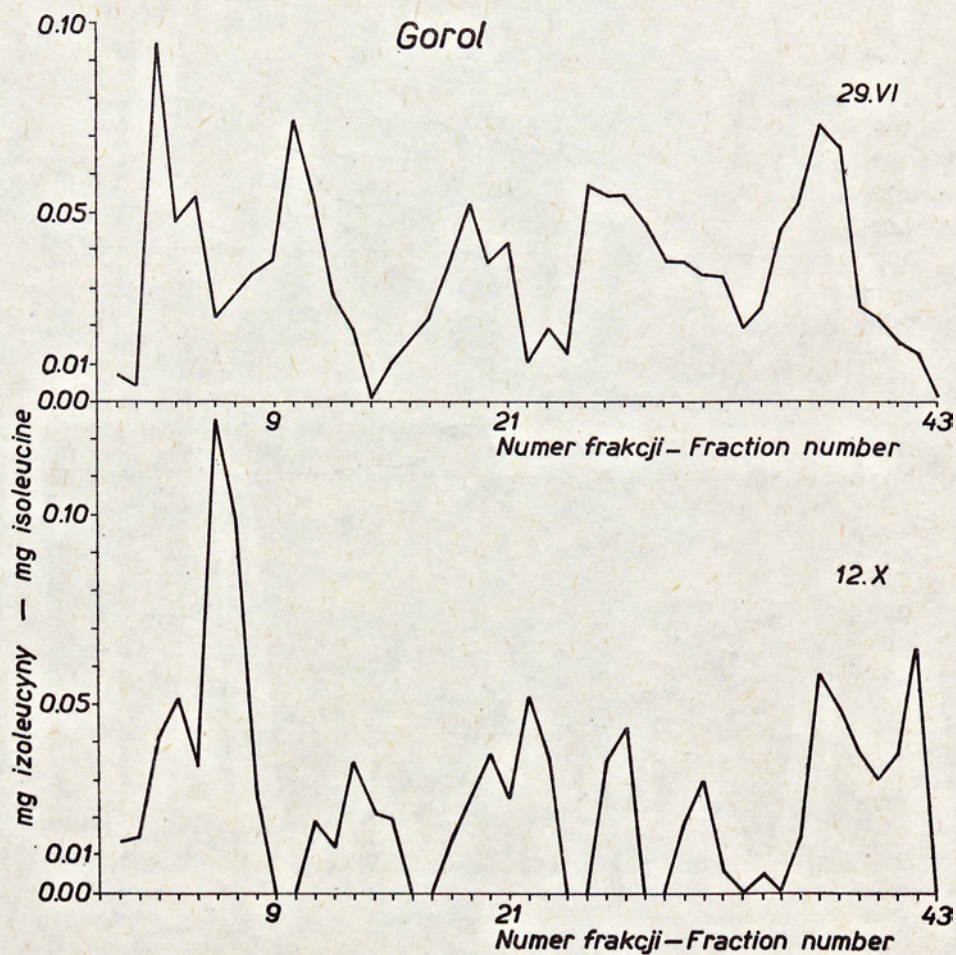
Ryc. 2. Zawartość azotu α -aminowego z czerwca 1972 r., wyrażona równoważnikową ilością izoleucyny na 1 mg azotu ogólnego rozpuszczonego w wodzie we frakcjach: od 0 do 9 — cząsteczki o masie powyżej 4000, od 10 do 21 — cząsteczki o masie od 4000 do 250, od 22 do 43 — cząsteczki o masie mniejszej niż 250

Fig. 2. α -amino nitrogen content, from June 1972, expressed as isoleucine equivalent per 1 mg total nitrogen dissolved in the water in fractions: from 0 to 9 — molecules of weight over 4000, from 10 to 21 — molecules of weight from 4000 to 250, from 22 to 43 — molecules of weight under 250



Ryc. 3. Zawartość azotu α -aminowego, z lipca, sierpnia i października 1972 r., wyrażona równoważnikową ilością izoleucyny na 1 mg azotu ogólnego rozpuszczonego w wodzie we frakcjach: od 0 do 9 — cząsteczki o masie powyżej 4000, od 10 do 21 — cząsteczki o masie od 4000 do 250, od 22 do 43 — cząsteczki o masie mniejszej niż 250

Fig. 3. α -amino nitrogen content, from July, August, and October 1972, expressed as isoleucine equivalent per 1 mg total nitrogen dissolved in the water in fractions: from 0 to 9 — molecules of weight over 4000, from 10 to 21 — molecules of weight from 4000 to 250, from 22 to 43 — molecules of weight under 250



Ryc. 4. Zawartość azotu α -aminowego, z czerwca i października 1972 r., wyrażona równoważnikową ilością izoleucyny na 1 mg azotu ogólnego rozpuszczonego w wodzie we frakcjach: od 0 do 9 — cząsteczki o masie powyżej 4000, od 10 do 21 — cząsteczki o masie od 4000 do 250, od 22 do 43 — cząsteczki o masie mniejszej niż 250

Fig. 4. α -amino nitrogen content, from June and October 1972, expressed as isoleucine equivalent per 1 mg total nitrogen dissolved in the water in fractions: from 0 to 9 — molecules of weight over 4000, from 10 to 21 — molecules of weight from 4000 to 250, from 22 to 43 — molecules of weight under 250

Results

a) Changes in the content of protein and ninhydrin-positive compounds soluble in phosphate buffer (pH = 7.6), under the influence of the environment

The investigation in the accumulative waste pond was begun in the period of intense decomposition of organic matter. Great quantities of total nitrogen (19.25 mg/l) and of dissolved α -amino nitrogen (37.46 mg/l) occurred here (Table I, fig. 1). The intensity of the decomposition processes of organic matter was also shown in the oxygen deficit, in spite of its release by synthesizing algae (M. Lewkowicz, S. Lewkowicz 1975), chlorococcous algae and euglenins appearing at this time (Krzczykowska-Wołoszyn 1977).

However, after 2 weeks when the water temperature increased by 4°C and the oxygen content was high (12.85 mg/l, this being 135% saturation), a decrease in the content of protein substances was several times noted as compared with the previous condition (M. Lewkowicz, S. Lewkowicz 1975). The above data suggest that these two factors favoured further mineralization of protein (Rjabov et al. 1974). The most intense development of algae was also observed in this period (Krzczykowska-Wołoszyn 1977), though at this stage of their development extracellular products did not bring about any increase in the amount of dissolved protein substances (Fogg 1971). Perhaps this was connected with the growth of heterotrophic bacteria assimilating food from the protein substratum (Starzecka, Ronchetti 1977).

Tabela I. Azot ogólny, azot rozpuszczony całkowity i \mathcal{L} -aminowy rozpuszczalny w buforze
Table I. Total nitrogen, total dissolved nitrogen and \mathcal{L} -amino nitrogen soluble in buffer

Staw Pond	Data Date	Azot ogólny Total nitrogen N-mg/l	Azot całkowity rozpuszczony Total dissolved nitrogen N-mg/l	Azot \mathcal{L} -aminowy rozpuszczalny w buforze \mathcal{L} -amino nitrogen soluble in buffer mg isoleucine/l	Relatywne wartości poszczególnych frakcji Relative values of particular fractions		
					4000	4000-250	250
Ziarny Wielki	15.V.	19.25	11.80	37.46	0.6	1.0	1.4
	31.V.	12.60	9.5	10.53	1.1	1.0	1.4
	5.VI.	10.60	6.90	23.33	1.0	1.0	1.4
	15.VI.	15.10	9.60	84.80	0.6	1.0	2.1
	29.VI.	9.00	5.83	8.72	1.9	1.0	1.4
	13.VII.	7.90	4.00	5.25	1.6	1.0	2.1
	27.VII.	10.10	6.91	4.32	1.7	1.0	0.7
	24.VIII.	6.30	4.62	3.07	2.5	1.0	2.3
	12.X.	4.48	3.08	2.46	1.4	1.0	1.7
Gorel	29.VI.	2.79	1.78	2.69	0.8	1.0	1.9
	12.X.	2.05	1.45	1.67	2.0	1.0	2.5

At the beginning of June when the water in the pond was 300% saturated with oxygen, a decrease in total and dissolved nitrogen, as at the earlier date, and a double content of α -amino nitrogen, soluble in the buffer, was observed (Table I, fig. 2). This resulted from the final stage of a mass development of algae which released considerable amounts of protein substances to the environment (F o g g 1971), while heterotrophic bacteria, very numerous at this time, greatly contributed to this condition, bringing about the autolysis of alga cells.

In the middle of June an increase in both total and dissolved nitrogen was noted, the content of α -amino buffer soluble nitrogen being doubled as compared with the values at the beginning of the investigation (Table I, fig. 2).

Taking into consideration that at the first sampling time the great concentration of these compounds was found at the end of the heterotrophic stage, thus chiefly originating from the allochthonic organic matter, the double concentration of these compounds observed at this time was the product of algae growth. The amount of protein and ninhydrin-positive compounds considerably increased, small molecules being most numerous. This greatly increased the amount of protein substances dissolved in the water was accompanied by a corresponding decrease in the number of algae and a rapid disappearance of heterotrophic bacteria, while at the same time the cladoceran *Moina rectirostris* occurred in masses after the rotifer *Brachionus calyciflorus* disappeared (K r z e c z k o w s k a - W o ł o s z y n 1977, M. L e w k o w i c z, S. L e w k o w i c z 1975, S t a r z e c k a, R o n c h e t t i 1977).

The very considerable consumption of dissolved oxygen noted in this period (on 5th June its content was 26.62 mg O₂/l and on 15th June only 0.53 mg O₂/l) suggested processes of destruction and respiration of the zooplankton and also contributed to the release of large amounts of free amino-acids. At this time favourable living conditions for *Moina rectirostris* prevailed in the pond, demonstrated in the highest fertility of females observed during the whole investigation period (M. L e w k o w i c z, S. L e w k o w i c z 1975). The results of an axenic culture of *Moina macropa* showed that for their proper development cladocerans need vitamins of the B group (M u r p h y, D a v i d o f f 1972). Their occurrence in the water of the pond Zimowy Wielki is manifested both by the great vitality of the species *Moina rectirostris* and by the large quantities of protein substances dissolved in the water, since it is known that vitamins of the B group take part as co-enzymes in protein metabolism (S k a r z y ń s k i et al. 1955).

In an aquatic environment bacteria and phytoplankton are the vitamin producers. As was mentioned above, before the cladoceran *Moina* appeared at the end of May, maximum amounts of phytoplankton with green-algae as dominants and the greatest share of euglenins were

observed, while on the next date the greatest development of heterotrophic bacteria was found. Daisley (1970) claims that it is *Euglena* that releases to the environment high-molecular protein substances, binding B₁₂ vitamin. Thus this genus as well as heterotrophic bacteria may enrich the water with vitamins and stimulate the development of *Moina*.

At the end of June, a distinct decrease in the content of α -amino buffer-soluble nitrogen occurred as a result of the decomposition and mineralization of the organic matter. Favourable thermic and oxygenic conditions prevailed then (M. Lewkowicz, S. Lewkowicz 1975), this coinciding with a certain advancement of phytoplankton development (Krzczykowska-Wołoszyn 1977). It should be stressed that in the control pond the content of α -amino soluble nitrogen in the parallelly collected samples was three times smaller (Table I, figs 2 and 4).

In the further course of the vegetation season, from July to October, the quantities of ninhydrin-positive buffer-soluble compounds gradually decreased and in late autumn they were similar to the values of this constituent in the control pond (Table I, figs 3 and 4). In order to explain the changes observed, it may be assumed that the processes of assimilation of proteins formed or dissolved by plant or animal organisms dominated over the release of protein substances either by way of metabolic excretion or of autolysis.

At this stage, phytoplankton receded while aquatic plants developed, e.g. *Lemna minor* and other vascular plants (Kuflikowski 1977). Aquatic plants, as autotrophs, assimilate negligible amounts of amino nitrogen dissolved in the water (Smirnova 1975) while, according to the opinion of Wetzel (1972), parallelly to the photosynthetic processes, they release protein-type substances to the environment. It is probable that the release of these constituents occurs by diffusion. The surface of contact of the cell membranes with the water is greater in algae than in higher plants. Besides, the algae have a shorter life cycle than vascular plants, this being important for the enrichment of the water with protein. Therefore, it may be accepted that the phytoplankton enriches the water with extracellular products containing amino nitrogen in greater measure than do the vascular plants.

The occurrence of zooplankton surely affected the amount of protein substances dissolved in the water, since zooplankton feeds both on the protein of organisms (bacteria, phytoplankton, detritus) and on that dissolved and formed by bacteria into aggregates (Chajlov, Finenko 1968, Chien et al. 1972). Probably, fresh water zooplankton, similarly to that in the sea (Chien et al. 1972, Webb et al. 1967), releases or excretes certain amounts of protein substances into the environment. However, it is certain that the protein balance of the growing zooplankton is positive, this in the described case resulting in a decrease in the amount of protein substances dissolved in the water.

While the content of dissolved amino nitrogen was decreasing in the water (Table I, fig. 3), the appearance of blue-green algae at the end of July and August seems fairly significant. They constituted 18%, while dominated diatoms (K r z e c z k o w s k a - W o ł o s z y n 1977). Although the main characteristic of blue-green algae is the release of protein substances to the environment, even amounting to 50% of the assimilated nitrogen (F o g g, W e s t l a k e 1955), nevertheless as compared with the previous date, the total number of phytoplankton failed to bring about an increased concentration of amino nitrogen dissolved in the water. Thus, it seems that after the domination of phytoplankton ended, extra-cellular protein substances were supplied by *Lemna minor* (which covered the water surface 50%), other vascular plants (K u f l i k o w s k i 1977), and, additionally, by blue-green algae.

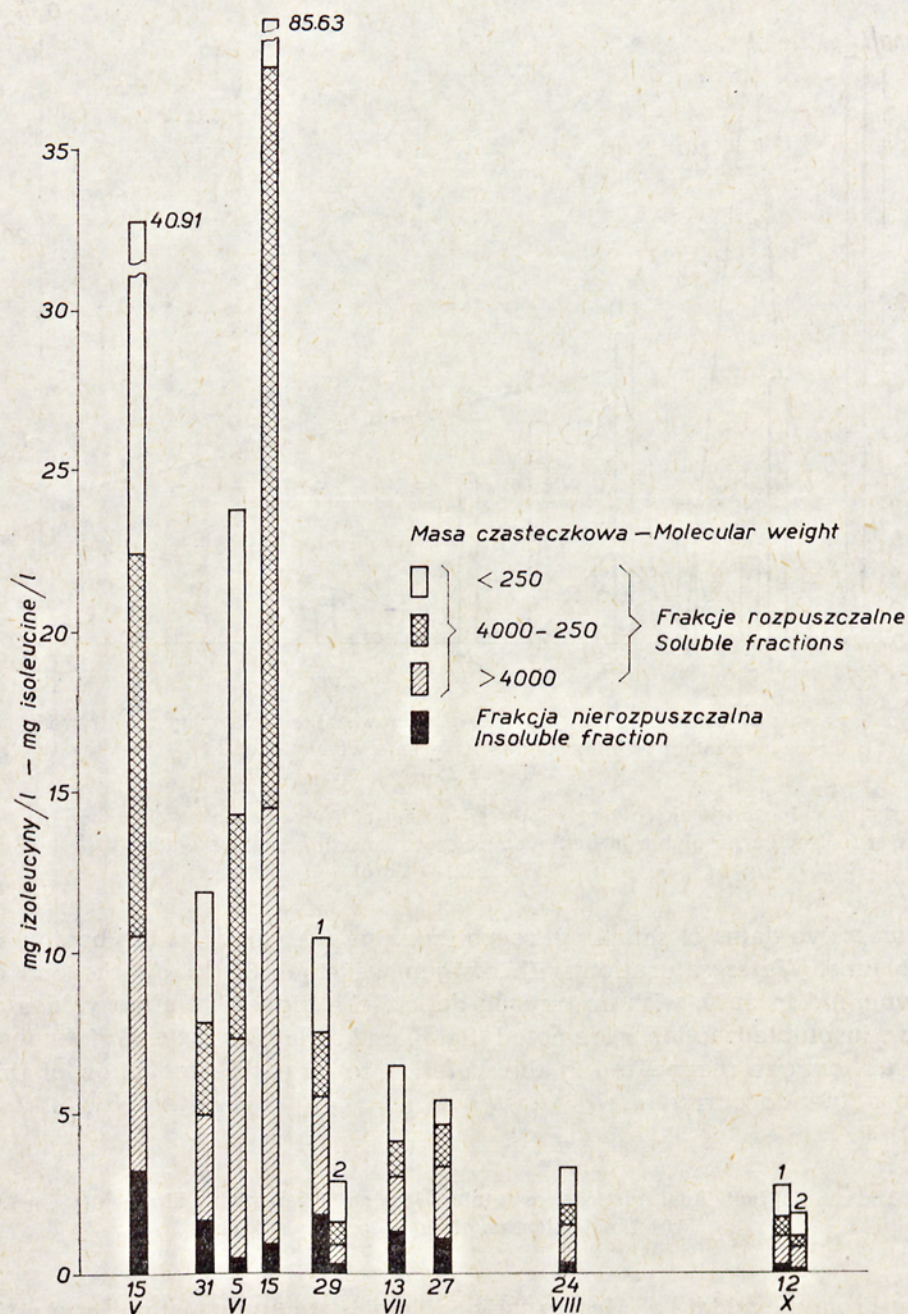
A further consequence of the intensified development of zooplankton was the appearance of ammonifiers (S t a r z e c k a, R o n c h e t t i 1977).

The desamination processes due to their occurrence were manifested in decreasing quantities of α -amino nitrogen and an increased amount of dissolved ammonia. The average content of the latter was 4 mg/l in the waste pond and 0.48 mg/l in the control one (M. L e w k o w i c z, S. L e w k o w i c z 1975).

b) Content of ninhydrin-positive substances, insoluble in phosphate buffer (pH = 7.6) and other factors of the environment

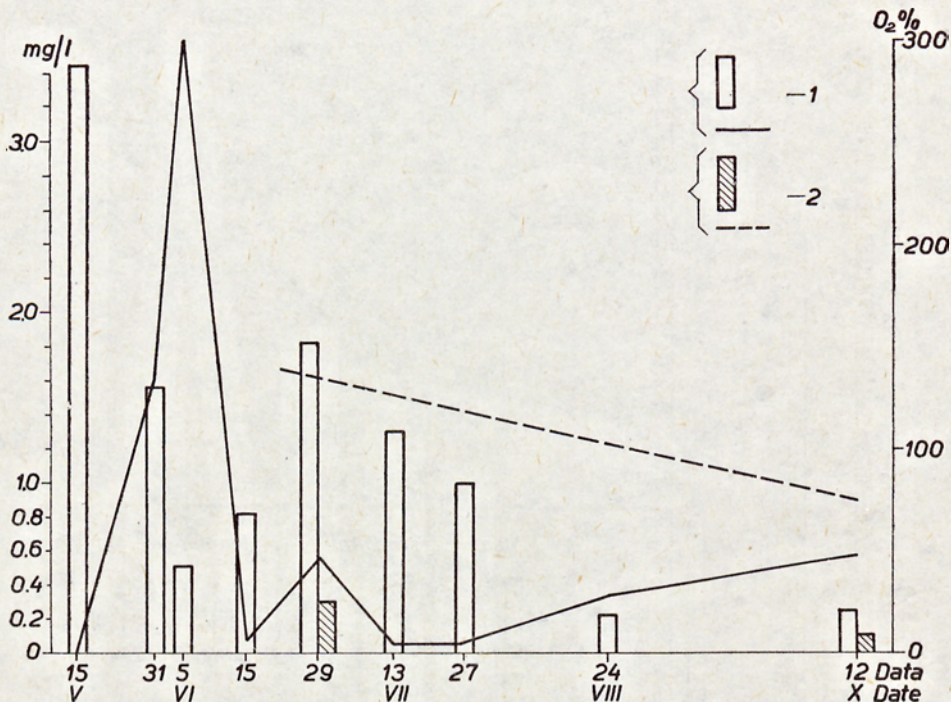
This fraction was composed of protein derivatives connected with other organic compounds. The content of α -amino nitrogen in the fraction was characteristically variable throughout the vegetation season. Its relatively greatest amount was found at the end of June (figs 5 and 6), a rapid fall in the content of α -amino buffer-soluble nitrogen being observed at the same time. Mucopeptides were probably among the constituents of this fraction, since they originated from the decomposition of heterotrophic bacteria (B r e h m 1967) whose maximum numbers were noted at that time (S t a r z e c k a, R o n c h e t t i 1977). The very dark colour of precipitate in hydrolysates of these samples showed that carbohydrates or humic substances were among them, since a certain part of the amino nitrogen dissolved in water may be among constituents of humic compounds (G e r l e t t i 1965, P o v o l e d o 1972).

Besides, a negative correlation was found between the amount of ninhydrin-positive buffer-insoluble compounds and the content of oxygen (fig. 6). From great quantities of these compounds at the beginning of the investigation, at the end of the heterotrophy and beginning of autotrophy, the content of these compounds gradually decreased during the



Ryc. 5. Związki ninhydryno-dodatnie w wodzie sączonej po zadaniu buforem. 1 — Zimowy Wielki; 2 — Gorol

Fig. 5. Ninhydrin-positive compounds in filtered water after treatment with buffer. 1 — Zimowy Wielki; 2 — Gorol



Ryc. 6. Zawartość azotu α -aminowego w mg równoważnika izoleucyny na litr wody we frakcji nierozpuszczalnej w buforze oraz nasycenie wody tlenem. 1 — Zimowy Wielki; 2 — Gorol

Fig. 6. α -amino nitrogen content expressed as mg of isoleucine equivalent per litre of water in fraction insoluble in buffer and oxygen saturation of the water. 1 — Zimowy Wielki; 2 — Gorol

successive dates of sampling, reaching minimum values at the beginning of June. Water saturation with oxygen was then 300%. At the end of June and in July, with an oxygen deficit, the highest relative values of this insoluble fraction were noted (fig. 6) and as late as the end of August, when oxygen reappeared in the water, a considerable reduction of this constituent occurred.

c) The ratio of individual fractions, containing α -amino buffer-soluble nitrogen (pH = 7.6) with the domination of phyto- or zooplankton

From the middle of May to the middle of June an almost uniform ratio of protein, peptide, and amino acid molecules was maintained (Table I), the largest number of small molecules being always observed. The beginning and end of this period coincided with two peaks of destruction of organic matter (15th May, the end of heterotrophy; 15th June, the stage

after a very intense blooming of phytoplankton) and only then was a trend towards a decreasing share of large molecules noted.

The next stage, from the end of June to October, was characterized by decreased numbers of phytoplankton in favour of zooplankton, and, as was already mentioned, by frequent oxygen deficits and great amounts of ammonia. As compared with the previous period, the number of large molecules, containing α -amino nitrogen, increased in this time. At the end of June they constituted 36%, of July 40%, and of August 39% of the total α -amino nitrogen, dissolved in the water.

It was only in the middle of July, when the phytoplankton numbers increased again, that the share of large molecules amounted to 27% of all ninhydrin-positive compounds dissolved in the water (Table I).

This increase in the content of the largest molecules probably arose from the faeces of the filtering zooplankton then prevailing (M. Lewkowitz, S. Lewkowitz 1975) and releasing 20—30% of consumed food to the environment (Johanes, quoted according to Hilbricht-Ilkowska 1974). Non-assimilated food contains easily washed out organic substances, probably composed of molecules of great weight. The dependences presented above suggest a differentiation in the molecule composition of ninhydrin-positive substances dissolved in the water, under the influence of the phyto- or zooplankton.

Conclusions

The following conclusions may be drawn on the basis of the investigation on α -amino nitrogen dissolved in the waste pond:

1) in the process of production of organic matter in the pond the quantity of this constituent distinctly increases owing both to the extracellular secretion of synthesizing algae and to the autolysis of decaying cells and their decomposition under the interaction of bacteria;

2) the amounts of dissolved protein, peptides, and amino acids depend in great measure on the numbers of algae and the duration of their occurrence as well as on the domination of physiological groups of bacteria;

3) the succession of phyto- and zooplankton is decisive for the formation of ratios between the ninhydrin-positive components of different molecular weight (protein, peptides, and amino acids) dissolved in the water.

STRESZCZENIE

Pracę podjęto w celu zbadania, w jakim stopniu materia organiczna allochtoniczna (ścieki cukrownicze) i materia organiczna wyprodukowana przez syntetyzujące glony wpływa na ilość azotu α -aminowego rozpuszczonego w wodzie. Badania wykonywano od maja do października 1972 r. Analizowano głównie zawartość azotu α -aminowego po hydrolizie osadu wytrąconego buforem fosforanowym i azotu α -aminowego rozpuszczonego w buforze fosforanowym, w związkach o masie cząsteczkowej: większe niż 4000, od 4000 do 250 i mniejsze niż 250.

Wyróżniono dwa maksima koncentracji azotu α -aminowego rozpuszczonego w wodzie. Pierwsze miało miejsce w okresie beztlenowym, odpowiadającym heterotrofii, na początku badań (15. V), przypadającym pod koniec rozkładu i transformacji allochtonicznej materii organicznej; drugie w okresie tlenowym (15. VI), związane z ustępowaniem masowego pojawu glonów chlorokokkowych. To ostatnie dostarczyło jednak dwukrotnie większych ilości związków rozpuszczonych, zawierających azot α -aminowy. Na tak dużą zawartość rozpuszczonego białka, peptydów, a szczególnie aminokwasów wpłynęły, poza produkcją ekstracelularną glonów, również procesy autolizy ich komórek, poprzedzone masowym wystąpieniem bakterii heterotroficznych (tabela I, ryc. 1, 2 i 5). W obu tych terminach przeważały rozpuszczone w wodzie aminokwasy nad peptydami i białkiem.

W dalszym ciągu sezonu od lipca do października zawartość azotu α -aminowego rozpuszczonego w wodzie stopniowo malała, tak że w jesieni była zbliżona do ilości tego składnika w stawie kontrolnym (tabela I, ryc. 3, 4 i 5). Wśród składników zawierających azot α -aminowy rozpuszczony w wodzie było na ogół najwięcej dużych cząsteczek białkowych, a najmniej cząsteczek małych, to jest aminokwasów. Prawdopodobnie wpłynął na to zooplankton filtrujący, występujący wówczas w przewodzie, który wydalał nie strawione resztki pokarmu, zawierające duże cząstki białkowe, jak również bakterie amonifikujące, uwalniające amoniak w procesach dezaminacji.

Ilość związków zawierających azot α -aminowy nie rozpuszczalny w buforze fosforanowym skorelowana była ujemnie z zawartością tlenu (ryc. 6). Rzutowały na nią także procesy rozkładu materii organicznej, gdyż stosunkowo najwięcej było ich pod koniec heterotrofii i u schyłku zakwitów glonów.

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