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OF ARTS AND SCIENCES

PROGRESS REPORT 1973

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NETHERLANDS CENTRAL INSTITUTE FOR BRAIN RESEARCH AMSTERDAM

PROGRESS REPORT 1973

History and function of the institute

In 1905, the Royal Netherlands Academy of Arts and Sciences applied to the Dutch Government for permission to found an institute for brain research. The government consented and on June 8th, 1909, the Netherlands Central Institute for Brain Research was opened. The institute was seated in a wing of the then newly erected Department of Anatomy and Embryology of the Municipal University of Amsterdam, built under the direction of L. Bolk, professor of anatomy and embryology, who, together with the neurologist professor C. Winkler, was one of the most important supporters of the institute. The first director was Dr. C. U. Ariëns Kappers who became world-famous as a comparative neuroanatomist. After some years he was appointed professor of neuroanatomy at the University of Amsterdam. After his death, in 1946, Kappers was succeeded by professor B. Brouwer, who previously had held the chair of neurology at the University of Amsterdam. Brouwer was primarily interested in neuropathology. During his management Dr. J. Drooglever Fortuyn, at present professor of neurology at the University of Groningen, introduced electrophysiology at the institute.

After the untimely death of Brouwer, in 1949, the Dutch government agreed that the institute should be reorganized and extended. Thus it became possible to found some new divisions enabling the institute to perform multidisciplinary research in the broad field of neurosciences, which was in accordance with its original aim.

In 1952, professor S. T. Bok, who previously had held the chair of histology at the University of Leiden, was appointed director. His merit has been to further multidisciplinary research at the institute on a large scale. He was one of the first researchers in the quantitative analysis of the brain, especially of the cerebral cortex, who earned great fame. After his retirement he was succeeded by J. Ariëns Kappers, previous professor of anatomy at the University of Groningen, who at the same time was appointed professor of neuroanatomy at the University of Amsterdam.

In former times comparative neuroanatomy was the only field of research at the institute. The scientific and the technical staffs were very restricted in number. As mentioned above professor Bok changed this situation. Since then the number of staff members and of research projects has increased progressively. At the end of 1973, the total number of staff members was 81, 38 of whom were either full-time or part-time research workers.

In 1964, the institute moved to a provisional, but much larger building and some additional barracks have since been built. Plans are now ready for a final large Institute for Brain Research on the site of the new Medical Centre of the University of Amsterdam.

The institute is a governmental institution, as its financial support is exclusively supplied by the government. It is managed by a director under the supervision of a board of professors of various disciplines at Dutch universities. Members of the board are appointed by the Royal Netherlands Academy of Arts and Sciences.

At present, there are 4 main research divisions at the institute: morphology, physiology, neurochemistry and neuropharmacology. The divisions of morphology and physiology comprise several working teams such as the teams for histology and neuroendocrinology, comparative and quantitative neuroanatomy, histo- and cytochemistry, electron microscopy, experimental physiology, comparative and develop-

mental neurophysiology, physiology of behaviour, and system analysis. The institute has also a number of workshops and is provided with excellent technical equipment including some computers. A close contact between the members of the various divisions and special teams contributes considerably to the integration of neurobiological research. During the past years relations have been established between some teams and clinicians of the University.

Although the institute has no special funds available to enable guestworkers to work at the institute, some non-Dutch residents could do research work during the last year, subsidized by special funds or fellowships such as offered by the International Brain Research Organization. Moreover, the institute offers an opportunity to Dutch students in medicine, psychology, biology, etc. of all Dutch universities to do work on special subjects connected with their study.

Scientific Staff (as from December 31st, 1973)

J. Ariëns Kappers, Director

DIVISION OF PHYSIOLOGY

Section of Experimental Neurology

Head: J. P. Schadé

J. C. de Valois

W. van Emde Boas

B. M. F. v. Cranenburgh

C. V. de Blécourt

J. P. Muizelaar

P. de Groot

Section of Comparative Physiology

Head: M. A. Corner

K. Boer

H. A. A. de Jong

W. L. Bakhuis

R. Baker

J. du Pont

Section of Behaviour Physiology

Head: H. van Dis

N. E. van de Poll

A. P. van der Meché

J. P. C. de Bruin

B. Bermond

M. Roest

J. Röthengatter

Computer Section

J. Smith

DIVISION OF MORPHOLOGY

Section of Histology and Cytology

Head: J. Ariëns Kappers

R. A. C. de Vries

A. R. Smith

P. Pevet

Section of Histochemistry and Cytochemistry

Head: D. F. Swaab
G. J. Boer

Section of Comparative and Quantitative Neuroanatomy

Head: G. J. Smit
K. C. Hodde
P. Kenemans
C. J. van der Sloot
H. Uylings

Section of Electron Microscopy

Head: H. R. Romijn
J. Varela

DIVISION OF NEUROBIOCHEMISTRY

Head: B. Oestreicher
C. van Leeuwen

DIVISION OF NEUROPHARMACOLOGY

Head: J. J. Meisch
M. van Wijk

Division of Morphology

1. SECTION OF HISTOLOGY AND CYTOLOGY

Prof. Dr. J. Ariëns Kappers:

Assistance was given to Dr. A. R. Smith, P. Pevet and Dr. H. J. Romijn (Section of Electron Microscopy) in their investigations on the rat and rabbit pineal gland (see below).

Together with K. C. Hodde (Section of Comparative and Quantitative Neuroanatomy), the parietal eye in the lizard, *Lacerta viridis*, was extirpated in order to investigate the exact course of the nerve fibres originating in this organ using the degeneration techniques of Nauta-Gygax and Fink-Heimer. A number of brains are now embedded and ready for processing.

Dr. A. R. Smith:

The investigation concerning the function of the epiphyseo-hypothalamo-hypophyseal-gonadal axis has been continued. The results will be published in two papers which are now in press. The investigation brings histological evidence of an influence, exerted by the pineal on the arcuate and ventromedial nuclei forming part of the hypothalamic hypophyseotropic area. On the ground of the presented and literature data, the hypothesis is

put forward that the serotonergic neurones inhibit, in the same nuclei, the production of the hypothalamic LH regulating factor. As yet, the exact function of an autofluorescent substance, observed in pineal cells as well as in neurones of the hypothalamic arcuate and ventromedial nuclei, is not yet known.

In addition, an investigation was started into the influence of pregnyl (PMSG), gestyl (HCG), LH and FSH injections on the 5HT-containing neurones and the autofluorescent cells in both, the pineal gland and the arcuate-ventromedial hypothalamic region. Preliminary results indicate that after injection during 4 days a maximal decrease in number of autofluorescent cells appears in the pineal, while after injection during 7 days a recovery to above the control value ($\pm 300\%$) could be observed. It was, moreover, found that no further increase could be induced even after injection during 4 weeks. It appeared that injections of pregnyl and LH produce a maximal effect. As yet, no data are available about the influence of these substances on the 5HT-containing cells in the pineal gland and the hypothalamic nuclei. The effect of gestyl and LH injections on the pineal gland and the parvocellular hypothalamic nuclei after gonadectomy and pinealectomy is now under investigation.

P. Pevet:

Together with Dr. A. R. Smith, a study was started concerning the functional relationship between the pineal gland and the adenohypophysis, and vice versa, during different experimental conditions, such as gonadectomy, pinealectomy and hypophysectomy, etc., using microchemical, fluorescence microscopical and immuno-fluorescence histochemical methods. Changes in the biochemistry of the pineal gland due to the above-mentioned hormone injections are now investigated.

Dr. R. A. C. de Vries:

The visit of Dr. R. A. C. de Vries to the Department of Physiology, director Prof. McCann, of the University of Texas at Dallas, U.S.A., to learn the radio-immuno-assay technique, especially as the gonadotropic hormones are concerned, has been prolonged to the end of this year. This visit is subsidized by an IBRO/Unesco grant.

2. SECTION OF COMPARATIVE AND QUANTITATIVE NEUROANATOMY

H. B. M. Uylings and Dr. G. J. Smit:

In continuation of the description of topological and metrical aspects of the branching pattern of the basal dendrites of pyramidal cells in the cortex, in 1973 a quantitative insight was obtained into the accuracy of the measuring procedure used. This is important for the interpretation

of the large variation in, *e.g.*, the segment lengths and the bifurcation angles of the basal dendrites. It was proved that the large variation found in the material is indeed a real biological phenomenon. A paper on the spatial configuration of the dendrites of cortical pyramidal cells will be completed in 1973.

The functional anatomical model for bifurcations was also extended. This model was developed to solve the question whether there is any functional connection between cytoplasmic transport within the dendrites and the shape of the bifurcation angles. This optimization-model was shown to be applicable for symmetrical bifurcations. This model has been further developed for asymmetrical bifurcations.

On the basis of this model the plausible conclusion was drawn that cytoplasmic movement is a form-related factor, perhaps even a form-regulating factor. In the future it is hoped that more physical form-functionals can be considered. This shall be done in order to examine which physiological factors are important in the determination of the shape of the dendritic tree. This project is necessarily deferred, however, until completion of a project, which is performed in collaboration with Prof. M. C. Diamond (University of California, Berkeley, U.S.A.).

The literature dealing with anatomical alterations in the mammalian C.N.S. in response to the alteration of the environmental conditions was examined. Special attention was given to the articles of Rosenzweig, Diamond and Bennett. This was done in order to formulate the research program undertaken in collaboration with Dr. Diamond concerning the capability of dendrites of adult mammals to adapt their dendritic structure to functional demands (in the present case: environmental alterations). At this moment, the project is in the histological phase. This research project is a follow-up of the results of the analysis published in 1972. An insight into the adaptive possibilities of the C.N.S. in response to functional demands, with their implications for learning processes and memory, is of fundamental importance for a full insight into the function of the C.N.S.

P. Kenemans:

After completing the descriptive and quantitative analysis of the cranial nuclei in the brain stem of *Polypterus*, a primitive bony fish, the cells of the reticular formation and the cerebellum, and also of the ascending and descending fibre systems, much attention was given to the pattern of ventricular sulci in the brain stem.

The longitudinal grooves in the ependymal ventricular surface of the brain stem are considered to be important parameters for describing areas of morphological and functional significance.

The classical concept regarding the fundamental morphological structure of the brain stem of vertebrates, *i.e.*, four longitudinally arranged cell

zones, separated by three ventricular sulci, moreover, implicates a consistent pattern of grooves.

In an additional investigation, this pattern of ventricular grooves present in the mesencephalon and rhombencephalon has also been examined in some other carefully selected fishes (*Scylliorhinus*, *Calamoichthys*, *Amia* and *Protopterus*) and the postulated idea of consistency in this pattern was thoroughly checked.

The results do not indicate any constancy in the pattern of these grooves. After evaluation of the method, and by means of a statistical investigation, it became clear that variations in the pattern, caused by graphics (photographs, drawings), observers (different researchers), and object (different animals of the same species; intraspecies variation) were less important than the interspecies variations. The non-interspecies variation was of the same order as the variation between both symmetric brain stem-halves (left-right variation).

Grooves which occur with some consistency, regardless of the specimen, the species or the investigator, were established. These sulci allow a qualitative description of the ependymal surface of the ventricles of the brain stem. From this surface any neuron present in the brain stem has started its migration in a peripheralward direction during ontogenetic development. This surface is, therefore, of special significance to the comparative neurologist.

Moreover, a method was developed which topologically transforms the total ventricular surface of any brain stem into a surface of standardized length and width. This offers the possibility for direct quantitative intra- and interspecies comparison. Such comparisons have been made this year, indicating the value of this method. Next year, the results will be published in a doctor thesis.

K. C. Hodde:

In the first half of the year the results of a microscopical inventory of the spinal cord and brain stem of the spotted dogfish, *Scylliorhinus canicula*, were reviewed. The results were categorized for use in a topological analysis of the brain stem of, in total, five species of fish.

In the second half of 1973 the experimental results of spinal hemisections in *Scylliorhinus canicula* and *Ginglystoma cerratum*, two species of sharks, were worked out in cooperation with Dr. Ebbeson of the University of Virginia, Charlottesville, U.S.A. This material is now being prepared for publication.

3. SECTION OF ELECTRON MICROSCOPY

Dr. H. J. Romijn:

Research on the electron microscopy of the rabbit pineal gland consisted of three parts: (a) the autonomic innervation of the organ, (b) the

subcellular localization of serotonin/melatonin synthesis, and (c) pineal function. By electron microscopy, intrapineal neurones were observed showing clear synaptic structures on their cell body and dendrites. To trace the origin of the preganglionic (parasympathetic) nerve fibres forming these synapses, the facial nerves (at present their presumed origin) were coagulated by stereotactic methods in anaesthetized rabbits. The material obtained is now in study. In order to localize serotonin/melatonin synthesis on the subcellular level, as well as to investigate the influence of the (para)sympathetic innervation on pineal ultrastructure, pharmacological experiments were performed. Rabbits were acutely or chronically treated with specific drugs either in combination or not with surgical denervation of the pineal. Fourteen different drugs were used.

In connection with the antigonadotropic action of the pineal gland the possible presence of a feed-back system was looked for. For this purpose male and female rabbits were gonadectomized. In addition, females in different stages of pregnancy were sacrificed for electron microscopic investigation. The material obtained from all experiments mentioned will be studied next year.

On the initiative of Dr. M. A. Corner (Section of Developmental and Comparative Physiology) and in collaboration with Ir. J. Smith (System Analysis) and members of the Section of Quantitative Anatomy, a multi-disciplinary investigation was started on the morpho-physiological backgrounds of the normal and epileptic EEG in the chicken cortex during ontogenetic development.

Aid was given to P. Pevet concerning his study on the functional relationship of the mammalian pineal gland with the adeno-hypophysial-gonadal axis.

Service was rendered to the Division of Neurochemistry and the Section of Histochemistry.

P. Pevet:

A light and electron microscopic investigation was performed into the relationship of the mammalian pineal gland with the hypophyseo-gonadal axis. Some mammals show a seasonal sexual cycle, characterized by alternating periods of sexual activity and – quiescence. Based on the antigonadotropic action of the pineal gland, the possible presence of a feed-back system was studied. For this purpose the pineal gland of the hedgehog and the mole was investigated during the sexual cycle by electron microscopy and by fluorescence microscopy. In the hedgehog pinealocytes, reticular formations of lamellae and vesicles were observed. These reticular formations were shown to transform into annular structures during the period of sexual quiescence. On the other hand, during the period of maximal sexual activity, just after the first mating period, numerous so-called “cytoplasmic condensations” could be observed.

Investigation of the pineal gland of the mole revealed the presence of many paracrystalline structures, so far never found in other mammals. These structures were shown to be of reticular origin.

In addition, the autonomic innervation of the hedgehog pineal was studied. Although fluorescence microscopy revealed the presence of a strong sympathetic innervation, nerve fibres and endings could, so far, not be detected by electron microscopy. This investigation will be continued with the aid of false neurotransmitters.

Dr. J. M. Varela:

It was shown that there are 4 acetylcholinesterase (AChE) isoenzymes in both the muscle and the brain of rat, and that there is an AChE isoenzyme predominating in brain and a different one abounding in muscle. These differences in isoenzymic populations might explain the fact that brain and muscle AChE of rat differ in several kinetic properties. Indirect evidence for the implication of the isoenzymes 1 and 3 in K^+ and Na^+ permeabilities, respectively, has been collected. These findings were the subject of two communications. Full reports of the results obtained are now in press.

Purification of the multiple molecular species of AChE has been started in order to obtain, through *in vitro* binding experiments, more direct evidence for the involvement of the above isoenzymes in such ionic fluxes and to disclose actual implication of the isoenzymes 2 and 4 in Cl^- and Ca^{++} conductances. Purification of these isoenzymes will permit, moreover, studies of their localization at the subcellular level (ultrastructural immunohistochemistry) and, thus, a final insight into their relevance for synaptic transmission. Much attention is also being paid in these investigations to the significance of the occurrence of AChE in both free and bound forms.

4. SECTION OF NEUROHISTOCHEMISTRY AND NEUROCYTOCHEMISTRY

Dr. J. F. Jongkind:

The experiments on energy consumption in whole embryonal hemispheres were pursued by analysing the local glucose and ATP consumption in different parts of the cerebral hemispheres of 19 days old chicken embryo's. With quantitative histochemical techniques, the cell-rich ectostriatum could be easily identified and isolated from frozen-dried cryostate sections. The adjacent dorsal and ventral cell-poor areas were taken as a control. Using the closed system approach of Lowry, the glucose and ATP content were determined in microgram samples at different times after decapitation of the embryo's.

Two substrate determination methods were employed:

- a. The resazurine method of Guilbault (Guilbault *et al.*, *Annal. Biochem.* 31, 91, 1969). This method was discontinued due to lack of sensitivity.
- b. A direct fluorimetric method using microfluorimetry on microdroplets (see abstract Proceedings of the 14th Federation Meeting of the Dutch Medical Biological Association, Groningen 1973, p. 202).

With method b, we were able to measure glucose and ATP levels in microgram samples of embryonic brain.

In the ectostriatum the level of glucose was 2.5 times lower than that of the control area as measured on a dry weight base. In addition there was a lower glycolytic breakdown of glucose in the ectostriatum. The ATP content of the ectostriatum was also 2.5 times lower than that of the adjacent control area. Virtually no decrease in ATP level occurred in both areas up till 2 minutes after decapitation.

The results indicate that, in the developing chicken brain, at least one area (ectostriatum) is present which has a deviating behaviour concerning glucose and ATP level and glucose utilization. It is, therefore, not permissible to study embryonic brain metabolism using whole hemispheres without taking into account the local differences in energy consumption.

Together with Dr. L. Szegedy, Budapest, Hungary (4 months research visit) earlier experiments already published were extended. With the help of an automatic fluorimetric equipment which was developed in our laboratory, it was possible to study in more detail than before the influence of kidney insufficiency on the metabolism of mouse brain. The results of the experiments now performed indicate that during ureter ligation (for periods up till 30 hours) the glycogen and glucose content of the brain steadily increases. By using the closed system approach of Lowry it could be established that the ATP-utilization decreases.

Dr. D. F. Swaab:

In continuation of last year's research, the main part of the work performed was related to a multidisciplinary investigation of the physiology and pathology of hypothalamo-hypophyseal systems. This is a project of the Fungo-group: "Regulation of hypophyseal functions" and will form part of the theses of G. J. Boer, K. Boer and W. J. Honnebier. In the present project, neuroendocrine systems were studied during the normal physiology and pathophysiology of diuresis, intrauterine growth and reproduction. Reviews on the present knowledge of these subjects were published.

Three projects stood in the centre of this investigation: (1) The normal physiology of the hypothalamo-neurohypophyseal system (HNS); (2) The pathophysiology of the HNS; (3) The role of the fetal hypothalamus in intrauterine growth and parturition.

(1) An important part of this subject was the development of an immunofluorescence technique for neurohypophyseal hormones in collaboration with Dr. Tauber (Amsterdam University Hospital), Dr. Feltkamp and Mr. Van Rossum (Central Service for Bloodtransfusion). Rabbits, injected with vasopressin, appeared to develop a severe diabetes insipidus. Since their HNS was found to be intact and even appeared to be histochemically and histologically activated, the formation of antibodies against vasopressin became probable. Later investigations showed that these animals produced indeed very potent antibodies against vasopressin. These antibodies are used by Tauber's group to develop a radio-immunoassay for vasopressin and by us to develop an immunofluorescence technique for vasopressin. Immuno-fluorescence techniques were learned at the department of Dr. Feltkamp. The immuno-fluorescence technique for vasopressin makes it now possible to localize this hormone specifically in the cell bodies of the supraoptic and paraventricular nucleus, in their axons as well as in the neurohypophysis. This technique will be used in collaboration with G. J. Boer for the separation of pituicytes and axonal endings of the neurohypophysis.

For the collaboration with G. J. Boer concerning the microchemical investigation of the neurohypophysis and with K. Boer on the role of the HNS in parturition and lactation reference is made to the progress report of these investigations (see below).

(2) In addition to the investigation on the activity stage of the HNS in the vasopressin-injected rabbits of Dr. Tauber (see project (1)), the HNS of the hereditary, hypothalamic form of diabetes insipidus of the "Brattleboro rat" was studied. Histochemically and microchemically, the HNS of the homozygous Brattleboro rat appeared to be strongly activated, while, in this respect, the heterozygous animals were intermediate between the normal Wistars and heterozygous Brattleboro rats. The results of these findings have been reported. Also parturition of homozygous animals was abnormal.

(3) This investigation was performed in collaboration with W. J. Honnebier, gynaecologist (Amsterdam University Hospital). As appeared from a study of 147 human anencephalics, the fetal brain would have a stimulating influence on intrauterine growth of the fetus and the placenta. In contrast to what could be expected from literature data, the human fetal brain did not appear to play an essential role in the initiation of labour. A model, that was developed to study these data in animal experiments, confirmed these findings in rat. By direct injection of various hypothalamic extracts and hypophyseal compounds, it was shown that some hypophyseal hormones could indeed stimulate the intrauterine growth of the fetus and placenta. The results have been published.

Injections of hypophyseal hormones directly into human anencephalics confirmed the negative conclusion on the role of the fetal hypophysis

in the onset of parturition and showed the sensitivity of the human fetal adrenal for ACTH. This finding has also been published.

In collaboration with Prof. Dr. A. Querido (Leyden University) a survey has been composed from literature on the development of human brain. These data concerning hyperplasia (neuroblasts and glia), hypertrophia and myelination will be used as an argument to supply children in iodine deficient areas also after birth, in order to protect their still developing brains.

On invitation of Dr. P. W. Nathanielsz, work has been done for two weeks at the physiological laboratory in Cambridge, England. The main purpose was helping Nathanielsz with some problems concerning our technique of intrauterine aspiration of fetal brains. Much time was left, however, to practice some techniques that were important for ourselves. The same operation was made possible for rabbits and a technique was started for permanent infusion of various compounds into the rat by means of chronic aortic-cannulas. In Dr. Fitzsimons' department the technique of implanting chronic hypothalamic cannulas was learned. A procedure for the implantation of pressure sensitive "radio-pills" into the rat uterus was also developed. The place (Richmond) where these radio-pills and receivers are produced, was visited to obtain additional information. These radio-pills are used now in collaboration with K. Boer from our Institute for measuring uterine contractions and milk ejection. In addition, this technique is used by G. J. Boer as a sensitive bioassay for oxytocin for the separation of axons and pituicytes of the rat neurohypophysis.

G. J. Boer:

Investigations were performed on (1) the role of the pituicytes in the hormone release phenomena of the neuroendocrine hypothalamo-neurohypophyseal system (HNS); (2) the influence of alcohol and water-load on the production and release of neurohypophyseal hormones, in collaboration with F. van Leeuwen (student) and Dr. D. F. Swaab; (3) the state of activation of the HNS of the Brattleboro rat, in collaboration with Dr. D. F. Swaab, and (4) the influence of different conditions of the HNS on duration of pregnancy and parturition in the rat, in collaboration with Dr. D. F. Swaab and K. Boer.

(1) Previous work had shown that with section histochemistry it was impossible to localize, in either the axonal or the pituicytic compartment, a microbiochemically measurable increase in lysosomal enzyme activity of the neural lobe after enhanced release of oxytocin and vasopressin. This year, therefore, procedures were started aiming to isolate both axonal endings and pituicytes from the neural lobe, introducing the possibility for an enzyme assay within both elements separately. Because of the low

weight of the rat neural lobe (0.5 mg), this had to be a micro-isolation. Dispersion of the cellular elements of the neural lobe was obtained by incubating in a trypsin medium and subsequent sieving in a micro-syringe through a sieve of 200 and 50 μ pore size. The second stage, the micro-gradient centrifugation to accumulate the different elements on the base of their density, is still under investigation. Ficoll- and Ludox-gradients of 200–300 μ l were characterized by means of micro-assays for protein, DNA (marker of pituicytes), oxytocin (involved in the milk-ejection reflex and as a marker of axonal endings; with K. Boer) and lactate dehydrogenase (marker of cell injury). Furthermore, microscopical observation was used for the vasopressin immuno-fluorescence (marker of axonal endings; with Dr. D. F. Swaab) and for studying the morphological structure while also electron microscopy has been applied. Preliminary results have been communicated in some lectures.

(2) Alcohol treatment and water-load of the rat were applied as factors possibly inhibiting the function of the HNS. Using our enzymatic parameters for the synthesis and release of neurohypophyseal hormones, however, the generally supposed direct effect of alcohol on the HNS could not be confirmed. The response after water-load agreed with the increased diuresis, measurable at the same time.

(3) The enzymatic findings in the HNS of the Brattleboro rats, having a hereditary hypothalamic diabetes insipidus, supported the hypothesis that the HNS of these rats produces large amounts of posterior lobe hormones, from which vasopressin will be defective. This result has been published.

(4) For the results obtained in this investigation reference is made to the report of K. Boer (see below).

Division of Physiology

1. SECTION OF EXPERIMENTAL NEUROLOGY

Dr. B. van Cranenburgh, Dr. W. van Emde Boas and Dr. J. P. Schadé:

The physiological concomitants of experimental atherosclerosis have been studied and a statistical analysis of firing patterns was performed. In order to induce atherosclerosis experimentally, rabbits were fed on a high cholesterol diet. In this way, vascular lesions were predominantly obtained in the large vessels, which resembled closely the vascular atheromata observed in man.

As a first approach to the analysis of the functional cerebral changes it was decided to study spontaneous neuronal activity in the course of the experimental procedure. Several statistical methods for neuronal firing patterns were tried out and led to the following conclusions:

1. Measurement of the mean spontaneous firing rate is not sufficient to characterize the neuronal firing pattern.
2. Additional information can be given by calculation of the interval histogram which could be adequately expressed in: a shape-characteristic, the mean interspike interval, the coefficient of variation and the modal value.
3. Several circumscribed brain areas were characterized by the occurrence of particular percentages of interval histograms.
4. In cortical areas as well as in the caudate nucleus, a low firing rate and a rather irregular firing pattern were found. These areas are thought to be under rather strong inhibitory influence.
5. The globus pallidus and adjacent areas are characterized by a rather high spontaneous firing rate and a large regularity of the firing pattern.
6. In 55% of the neurones, the intervals between the spikes exhibited no serial dependency (renewal). A rather small part of the firing pattern could be described by a Poisson process.
7. To describe the non-renewal properties of the spike train, *i.e.*, various kinds of mutual dependency yielding periodicity or other special features in the microstructure of the spike train, the serial correlogram proved to be most useful.
8. A strong correlation was found between the firing rate and the variability of the signal. Populations of cortical neurones were characterized by a steep slope in the mean interval-standard deviation graph.

In the course of experimental atherosclerosis the following changes were observed:

9. Increase in the number of spontaneously active neurones.
10. Increase in firing rate and regularity in the cortical areas and the caudate nucleus.
11. Disappearance of the association between brain area and type of histogram.
12. Continuous increase of regularity during the 6 months of the diet, and a tendency of the firing rate to decrease again after 6 months,

The changes mentioned are thought to be caused by:

- a. loss of inhibitory influence, which is supported by the fact that areas with low activity show significant alterations;
- b. degeneration of the dendritic tree in a later stage, causing the excitatory influences on the neuron to decrease.

The results of this investigation have been published by Van Cranenburgh and Van Emde Boas in a doctor thesis.

Dr. J. C. de Valois, Dr. J. P. C. Peperkamp, C. V. de Blécourt and P. A. de Groot:

Earlier, cerebral blood flow (CBF) was measured in the rabbit under various experimental conditions using the 85-Krypton intra-arterial injection method. The results of these measurements have been published and resulted in the thesis of J. P. C. Peperkamp. In 1973, further research was initiated to study the influence of different isotopes (85-Krypton, 133-Xenon and 14-C-Antipyrine) on flow measurement. Preliminary results indicate that diffusion limitations play an important role, especially at high flow rates.

A 14-C-Antipyrine technique was developed to regionalize the flow measurements to small (*i.e.*, about 100 mg) areas of the brain. This technique is presently in use to investigate flow patterns in experimental ischaemia (induced vasospasm and atherosclerosis).

Another project in the group is the correlation between CBF and EEG. In a small series of experiments a positive correlation was found in animals in which electrical brain activity was artificially altered by means of photic stimulation. Further research will be necessary to evaluate this correlation in statistical terms.

Finally, in the course of 1973, clinical flow measurements were started in some cases of subarachnoid haemorrhage. The results seem to be promising.

J. P. Muizelaar:

The investigation of neuronal sensitivity to iontophoretically applied neurotransmitters in both normal and cholesterol-fed rabbit was continued.

The results obtained with the application of acetylcholine and noradrenaline are indicative of a difference between normal and cholesterol-fed rabbits. These substances were also selected for a second series of experiments in order to compare the effects of iontophoretically applied atropine and glutamate and intravenously administered atropine. The most prominent finding is a markedly increased neuronal sensitivity for the excitatory action of acetylcholine in the cholesterol-fed rabbit. Since the sensitivity to atropine remains unchanged, it is proposed that the increased sensitivity to acetylcholine should be ascribed, not to a molecular change in the postsynaptic membrane receptors for acetylcholine, but to a insufficient antagonism of degenerated monoaminergic inhibitory systems.

In co-operation with J. H. M. de Groen a study was undertaken to determine the levels of the dopamine and serotonin metabolites, HVA and 5-HIAA respectively, in cerebrospinal fluid. The amount of these substances appeared to be decreased in the cholesterol-fed rabbit.

2. SECTION OF COMPARATIVE AND DEVELOPMENTAL PHYSIOLOGY

Dr. M. A. Corner:

The project on prenatal brain function and its expression as spontaneous behaviour aims at understanding on the one hand, the neuronal organization underlying stereotyped bioelectric discharges in the cerebrum (and its developmental stages) and, on the other hand, the relationship of cerebral functional patterns to the overall state(s) of the developing organism.

With respect to the former aspect, the year was spent largely on collaborative efforts with the computer and electron microscope divisions. EEG recordings were subjected to a number of quantitative procedures which confirmed the existence of a 'critical period' at 17 days *in ovo*. The stereotyped waveforms which then appear have now been characterized better than previously, and a striking similarity demonstrated with waveforms induced by glutamate or methionine sulfoximine in earlier stages. Furthermore, these waveforms show strong resemblance with, but also certain differences from the discharge patterns elicited by a variety of 'epileptica'. Synaptic development proved to follow closely the time-course established for the dendrites, becoming mature only considerably after the cerebral bioelectric pattern had attained its definitive appearance. The next steps projected are: (1) studies of unit firing patterns correlated with the stereotyped gross potentials, and (2) quantitative studies at the cyto-morphological level (Golgi and EM preparations).

The latter aspect of the project has been pursued with W. L. Bakhuis, and involves a polygraphic study of sleep in infant rats. Striking behavioural similarities with the hatching behaviour pattern in birds have been demonstrated, and the correlation with different phases of the sleep cycle is in the process of being investigated. Cortical EEG recordings together with several peripheral physiological variables are the necessary basis for such correlations, and the technical problems in such registrations have been solved.

W. L. Bakhuis:

The first 6 months of this year have been spent at the Caribbean Marine Biological Institute in Curaçao, with the financial aid of the Ministry of Culture and Education of the Netherlands Antilles and the WOTRO (Netherlands Foundation for the Advancement of Tropical Research). As has already been stated in the Progress Report of 1972, the intention was to study the nervous regulation of stereotype colour behaviour in the squid *Sepioteuthis sepioidea* (Decapods). Due to an unexpected cold stream flowing alongside the island this year, these squids were not available in sufficient quantities. Therefore, for the same project an easily available fish was chosen, namely the spotted goatfish, *Pseudupeneus maculatus*

(Mullidae). As this had never been done before, the colour behaviour (the different liveries) of this species was studied thoroughly, as the necessary first step in this project. A report of the results has been finished and a paper is in preparation.

Due to technical and organizational reasons this project will not be continued in the nearby future. Instead, the study of the nervous regulation of hatching behaviour in chicks has been reassumed (see Progress Report of 1971 for the preliminary results of this project). In the meantime, an article on hatching movement in the chick has been prepared and accepted for publication. Since earlier results indicated that young immobilized rats show similar rhythmic activity bursts, though less regular than in the chick, this species was included in the project. Both species appear to be deeply asleep (paradoxical sleep) during these total body bursts. This has been established using EEG, EMG and behavioural criteria. Future research will include the repeating of experiments with rats already done with chicks, precise neuro-physiological localization of the centres of the central nervous system responsible for the rhythmic behaviour concerned and establishment of the relation of these centres to sleep and arousal mechanisms. The investigation will be performed with the aid of stimulation and lesion experiments using depth electrodes.

K. Boer:

In collaboration with Dr. D. F. Swaab and G. J. Boer the influence upon gestation length and duration of parturition by water deprivation, alcohol and hereditary diabetes insipidus was studied in the rat. It appeared, that alcohol cannot be considered a specific inhibitor of the hypothalamo-neurohypophyseal system (HNS) as has been supposed by some authors. Furthermore, the results of this study were more in conflict with than in support of the idea, that the posterior lobe is involved in the start of parturition in rat.

Together with Dr. Lincoln (University of Bristol, Department of Anatomy), who stayed with us in July and August, unit activity was recorded from neurosecretory cells in the supraoptic nucleus of anaesthetized rats. Identification of the cells was achieved by antidromic activation from the posterior pituitary and tests of constant latency, frequency following and collision were applied. A 'new' dorsal approach for the stimulation of the pituitary was perfected and antidromic activation, and orthodromic release of oxytocin, was obtained with currents as low as 60–150 μ A. This is one-tenth of the current commonly employed. The use of such low currents prevented the problem of stimulus spreading to the oculomotor and trigeminal nerves. As a result it was proved that neurosecretory cells can conduct impulses throughout their length at the high frequencies observed in the neurosecretory nuclei (50–100 pulses/sec) at the time of oxytocin release.

Intramammary pressure was recorded in lactating rats for the 'on-line' assay of oxytocin released by electrical stimulation through the electrodes implanted or during the suckling of a litter of pups. Teat-ducts were cannulated and the pressures measured by the adaptation of a pressure-sensitive radio-pill. This assay is now used by G. Boer (Section of Histochemistry and Cytochemistry) in his attempts to separate by ultracentrifugation the pituicytes and nerve endings of the posterior pituitary. For conscious work the telemetric system was miniaturized and implanted subcutaneously along the abdomen. The first measurements of intramammary pressure in the conscious rat were thereby obtained.

Labour was induced in conscious rats by electrical stimulation of the posterior pituitary on day 21 of gestation. The parameters of stimulation were those earlier determined for obtaining release of oxytocin. Platinum/stainless steel concentric electrodes were constructed and implanted by the route earlier mentioned and controlled by the assay procedure. The induced labour paralleled closely spontaneous delivery and as such differed much from the delivery in the rabbit, the only species in which labour had previously been evoked by electrical stimulation. Pressure-sensitive radio-pills were implanted into the uterus (one fetus being removed) on day 19-21 of gestation and pilot studies were conducted to explore the pattern of uterine activity associated with spontaneous labour and that induced by electrical stimulation or exogenous oxytocin.

H. A. A. de Jong:

The study on statocyst-evoked neuronal activity in the central nervous system of *Helix* and *Aplysia* during 1973 was focussed upon the analyses of the experimental data collected in September 1972 at the "Stazione Zoologica", Naples. In order to learn more about the intergration of sensory information in the CNS of Molluscs, the input from the 10-13 sensory cells in the statocyst, demonstrated in the static nerve, was analysed and compared with the postganglionic responses.

Separation of "unit activity" in the static nerve was based upon amplitude discrimination and detection of propagation velocity of the action potentials. In this way a complete picture of the behaviour of the sense cells, depending on spatial and temporal stimulation has been realized.

Efferent propagated action potentials in the static nerve as described in the literature are not obvious in *Aplysia*. In *Helix* this phenomenon is still obscure.

Frequency responses of the overall activity in the static nerve and cerebropedal connectives (cpc), evoked by tilting of the preparation, show a good correlation with the behavioural responses of the animals as described by DIJKGRAAF (1967) and by WOLFF (1969).

A relationship between one particular unit of the statocyst and a few detectable units in the cpc is obvious during the first 3 seconds of tilting the preparation.

Preliminary investigations of intracellular recordings in the cerebral ganglion of *Aplysia* point to four distinct regions of neurones having a specific function in relation to gravital perception.

3. SECTION OF BEHAVIOURAL PHYSIOLOGY

Research has been centred on three main projects: (1) neural regulation of sexual behaviour, (2) neural regulation of aggressive behaviour, and (3) learning and memory, in fish and rat.

J. P. C. de Bruin (1):

In the Siamese fighting fish (*Betta splendens*), the study of the function of the telencephalon in the regulation of sexual and parental behaviour has been continued. Besides high frequency lesions, surgical ablations were performed. The results demonstrate the importance of the forebrain for the coordination of reproductive behaviour patterns. A detailed analysis of the results is carried out, while additional experiments are designed to contribute to the present findings.

The interaction between sexual and aggressive behaviour is a striking phenomenon in the display of the male Siamese fighting fish. A separate study was undertaken to see whether the sexual behaviour occurring during the initial stages of the aggressive display will predict the level of sexual motivation towards a responsive female. No positive correlation could be demonstrated.

B. Bermond, H. van Dis and N. E. van de Poll (1):

Last year's research was focussed upon various aspects of psychosexual differentiation of male and female rats, and attention was given to the neural and hormonal determinants of these differentiated behaviours.

Studies on the effect of neonatal androgen manipulation in male and female rats upon both masculine and feminine mating patterns were completed. In neonatally and adultly castrated males and in neonatally androgenized and control female rats the effect of oestrogen treatment and of testosterone treatment in adulthood upon masculine and feminine sexual behaviour was studied. It was concluded that organizational factors are more important for the readiness to display masculine or feminine mating patterns than the kind of hormone administered in adulthood. The role of the C.N.S. was further studied by making lesions in the preoptic area and anterior hypothalamus in male rats. It was shown that the regulating systems for masculine and feminine mating patterns show a different neuroanatomical representation. This is further explored by implantations of crystalline testosterone.

The excessive mounting behaviour in pCPA-treated female rats was

further investigated. Evidence was found to exclude the involvement of the adrenal cortex.

Attention has been given to the phenomenon of 'sexual exhaustion'. Following unlimited mating of male rats, absolute and relative refractory periods of several days are seen. The role of androgens in both the exhaustion process itself as well as in recovery was studied. Furthermore, a new series of preoptic self-stimulation was started in which this sexual exhaustion was manipulated.

J. P. C. de Bruin (2):

The main object of interest is the regulatory role of the telencephalon of the Siamese fighting fish for the co-ordination of the reproductive behaviour patterns (see project (1)). In addition, attention was given to some methodological and technical aspects. The present experimental design necessitates frequent aggression tests of a rather long duration. Since, under these conditions, the response towards dummies or living opponents shows a high degree of waning, a separate study was undertaken to measure the habituation in a mirror-image-stimulation situation. The results have been analysed and a publication is in preparation.

The workshop of electronics developed a counter-unit which is connected with the observation keyboard. This has reduced the time involved with analysing the behavioural records. Furthermore, in co-operation with the workshop of electronics and the Section of System Analysis, a system is under development, which will enable the recording and transcription of behavioural data in a computer-compatible form.

B. Bermond, H. van Dis and N. E. van de Poll (2):

Further studies on rat intermale aggressive behaviour were done with the S-3-strain. The role of androgens and of the preoptic anterior hypothalamic continuum was investigated in the regulation of these behaviour patterns.

T. P. van der Meché, M. Roest and T. Röthengatter (3):

The study of the memory formation in goldfish (*Carassius auratus* L.) using learning experiments in a shuttle-box has been continued. The hypothesis, formulated in the 1972-Progress Report was tested, and it was demonstrated that the punishment during the conditioning exhibits an inhibitory influence on the barrier-crossing during the intertrial intervals.

Experiments, in which the telencephalon was completely removed, showed that this ablation resulted in a complete disappearance of the spontaneous barrier-crossing in the shuttle-box. The influence on retention and acquisition of an active avoidance response was only slight. There

is some evidence that this slight effect can be attributed to the disappearance of 'false' avoidance, *i.e.*, barrier-crossings that occur during the CS-US interval due to the spontaneous activity in the shuttle-box, and that the 'true' conditioned avoidances are not influenced. It seems that goldfish in which the telencephalon has been removed can learn this active avoidance task. The extent of the ablations has been studied with the use of macrophotographs, while a more precise histological control is in progress.

An activity meter has been developed in co-operation with the workshop of electronics. This will enable experiments to determine if telencephalon extirpation lowers the total locomotory activities or only abolishes the spontaneous barrier-crossings in the shuttle-box.

A possible effect of ACTH and dexamethasone on active avoidance conditioning could not be demonstrated.

B. Bermond, H. van Dis, T. P. van der Meché, N. E. van de Poll, M. Roest and T. Röthengatter:

Preliminary work was undertaken to study sex differences and psycho-sexual differentiation in some learning behaviours in rat.

4. SECTION OF NEUROPHYSIOLOGICAL SYSTEM ANALYSIS

Ir. J. Smith:

Systematic analysis of synaptic mechanisms shed light on the mechanisms of action potential generation within the observed cell, on the synaptic input of the cell and on the mechanisms by which the latter is transformed into a postsynaptic output (MOORE, G. P. *et al.*, 1966; PERKEL, D. H. *et al.*, 1967 and VAN WILGENBURG, H. and SMITH, J., 1970). In an attempt to classify the patterns of activity belonging to particular cells involved in neuronal networks, single neuronal systems were systematically described in statistical terms of output of the system. This multidisciplinary research was done for different parts of the rabbit brain, in collaboration with the Section of Experimental Neurology, resulting in a thesis of W. VAN EMDE BOAS and B. VAN CRANENBURGH. This approach furnishes the basis for theoretical models and is necessary for any quantitative theory of information processing in the central nervous system.

As part of our main research program to examine some likely causal factors regulating functional development in the cerebrum, the following subjects have been investigated:

1. the relationship of cerebral functional patterns to the overall states of the developing brain.

2. the neuronal organization underlying basic cerebral electrical activity.

In the past year typical computer approaches were developed to characterize quantitatively the EEG of the chick embryo.

- a. *Spectral analysis.* The first and most conventional method used was to obtain the power spectrum for the developmental EEG (stage 42-45). This was computed every 10.24 sec for 13.6 min over the range 0.5-30 Hz, which proved the existence of a "critical period" at 17 days *in ovo*, *i.e.*, stage 43. The smoothed power spectrum proved to be unimodal, skewed to the right and localized in the delta range (80% of the total power) at all stages of development, with a peak frequency between 0.9 and 1.0 Hz, and a "dominant" frequency being consistently somewhat higher than this (1.2-1.3 Hz) (SMITH, J., 1973).
- b. *Amplitude-interval analysis.* This aperiodic analysis method computes the intervals between successive peak-to-peak deflections above a predetermined amplitude, or "hysteresis" level (SMITH, J. *et al.*, 1973). As a result, the following distributions have been calculated: the interval amplitude histogram (2-dimensional distribution); the interval histogram and the extreme-amplitude histogram.
- c. *Pattern recognition analysis.* Finally, in order to attack more directly the question of cerebral EEG organization in time, a detection algorithm was developed using a non-recursive digital filtering method, in order to decide between two hypotheses, "pattern" and "no-pattern" (SMITH, J. and KUYPERS, K., 1973). The circuitry underlying these patterns, is completely unknown. The systematic quantitative analysis might reveal functional relationships between pathological and normal physiological mechanisms, *i.e.*, neuronal organization underlying the basic cerebral electrical activity.

As part of our program to examine further some likely causal factors regulating functional development in the cerebrum, and the role played by neuronal electrical activity in the development of functional properties of the nervous system, a dynamic neuronal network computer model was developed (KUYPERS, K. and SMITH, J., 1973).

In collaboration with the Section of Experimental Neurology, cerebral bloodflow (CBF) has been studied in correlation with the EEG. The bicompartamental model of the brain, determining the CBF of this tissue, is used routinely as a measure of the functional relationship between the CBF and the EEG (DE BLÉCOURT, C. *et al.*, 1973; DE VALOIS, J. C. *et al.*, 1973). Such an analysis might also furnish a basis for theoretical models, concerning mechanisms of neuronal features and brain function as a whole. The clinical significance of the bicompartamental model is now under investigation with Dr. J. C. de Valois and Dr. J. P. C. Peperkamp (Amsterdam University Hospital).

In a collaborative effort with the Section of Histology and Cytology,

simple methods have been introduced in computerized automation of the scanning and measuring techniques of neuronal processes in fluorescence microscopical sections.

An unique modular system of optical, mechanical and electrical units enables rapid tracking and accurate measurements of neuronal processes in fluorescence microscopical sections. The system for doing this was a frontend digital mini-computer, which controls stepping motors in a closed loop, attached to the stage of a Leitz Orthoplan microscope.

Division of Neurochemistry

Dr. A. B. Oestreicher and C. van Leeuwen:

The investigations about structural components of nerve cells obtained by subcellular fractionation of the central nervous system were continued. Since some aspects of the development of electrical activity in growing embryos and in post-hatched chicks are being studied by Dr. Corner and coworkers (Section of Comparative and Developmental Physiology), it was thought that an insight into the macromolecular structure of synaptic membranes isolated from the brain of chicks could form a basis for studying correlations between neurophysiological phenomena and synaptic structures.

The procedures worked out during subcellular fractionation of whole rat brain (MORGAN *et al.*, Biochem. Biophys. Acta 241: 737 (1971)) were applied to the isolation of 4 subfractions from a crude mitochondrial pellet separated from a homogenate of whole brains or of hemispheres, optical lobes and cerebellum of 3 to 6 day old chicks. Analyses with the electron microscope demonstrated that these 4 fractions are respectively:

1. enriched in myelin, containing various membrane fragments,
2. enriched in synaptosomes, containing synaptic vesicles and mitochondria,
3. enriched in synaptosomes and free mitochondria,
4. enriched in free mitochondria of which this fraction is mainly composed.

The yield of protein in these 4 subfractions is, respectively, about 17, 6.5, 4.5 and 5 mg protein per gram wet weight.

The measurement of the distribution of marker enzymes for the various subcellular structures in these fractions, such as lactate dehydrogenase, acetylcholinesterase, monoamine oxidase, acid phosphatase and rotenone-sensitive and -insensitive NADH-cytochrome-C reductase, correlate well with the observations obtained with the electron microscope. Analyses of some chemical markers, *i.e.*, glycoprotein-bound N-acetylneuraminic acid (NANA) and total phospholipids, showed that the fractions enriched in synaptosomes contain about twice the amount of glycoprotein-NANA and phospholipids as does the purified mitochondrial fraction, 4.

Dr. A. B. Oestreicher and J. Moen:

Most of the glycoproteins in the CNS (about 80% of the total) are membrane-bound. Many studies suggest that the biosynthesis of glycoproteins takes place mainly on the endoplasmatic reticulum and in the rough and smooth membranes of the Golgi-apparatus in the neuronal perikarya. The glycoproteins are transported to the nerve endings by axoplasmic flow. It has been shown that concanavalin A binds with glycoproteins present at the surface of the membranes of various cells and subcellular organelles. Concanavalin A has been used to separate and to isolate specific glycoproteins from a mixture of other proteins. In a first approach to the study of the origin of glycoproteins found in the synaptic membranes of the CNS, it was thought useful to detect and separate some of the soluble glycoproteins, since they may be precursors of, and related to, the membrane-bound glycoproteins.

When whole chick brain is homogenized in hypotonic buffer, about 24% of the protein and about 15% of the NANA present can be isolated in the soluble fraction.

About 5% of the protein of the soluble fraction has been separated by means of affinity chromatography over a column filled with Sepharose to which Con A is attached covalently. This protein fraction is characterized by the property that the proteins are bound and retarded by the Concanavalin A-Sepharose column and eluted by a specific competitor for binding, methyl α -D-glucopyranoside. The fractions obtained by this separation method will be analysed according both to various polypeptides, by means of polyacrylamide gel electrophoresis, and to their sugar composition.

C. van Leeuwen:

During a Traineeship of the European Training Programm in Brain and Behaviour Research, at Strasbourg from November 1972 till August 1973, a similar approach as mentioned above has been used to isolate a population of glycoproteins, enriched in galactose from the soluble fraction of whole rat brain. This was done by affinity chromatography over a column of Sepharose, to which the lectin isolated from *Ricinus sanguinis* has been coupled covalently. This lectin binds preferentially to glycoproteins, in which galactose residues are located in an exposed position of the polysaccharide side chains.

It turned out that in order to recover most of the protein, which was bound with a high affinity to the lectin, a prolonged and recirculating elution with hapten was required. However, still another protein fraction remained on the column. This fraction could be eluted only with denaturing agents. The so-called Ricinus-positive glycoproteins have been characterized by polyacrylamide gel electrophoresis followed by staining for protein and for sugar. The sugar and amino acid compositions have been estimated by gas liquid chromatography.

Division of Neuropharmacology

Dr. J.-J. Meisch and M. van Wijk:

The pharmacodynamics of false transmitters was further investigated. An extensive study with H 75/12 (4-methyl- α -ethyl-metatyramine) was completed and will be published in the near future. H 75/12 was injected i.p. into mice and rats and the false transmitter as well as 5 HT were analysed in brain, heart and blood. Time course and dose response curves were obtained. In some rats transverse cerebral hemisections were performed in order to produce a degeneration of the monoamine pathways prior to the administration of H 75/12. These experiments showed a pronounced extra-neuronal binding. The effect of various tricyclic antidepressants on the neuronal uptake of H 75/12 was studied in the two animal species. Finally, some starvation experiments suggest that the metabolism of H 75/12 is modified under these conditions.

The work on tricyclic antidepressants developed into a separate branch of our research. For orientation, time curves for imipramine and desimipramine were obtained. The turnover of 5 HT under the influence of imipramine, desimipramine, chlorimipramine and protriptyline was also studied, using the probenecid method. At the same time, the antidepressants and their relevant metabolites were determined in brain and plasma, in so far as the technical facilities were already available. The results were roughly in agreement with those in the literature. In all experiments the drugs were administered acutely.

A study was started on the results of an acute versus a chronic treatment with the drugs mentioned. The first series of experiments has just been finished. The results seem to be striking, but, so far, the number of data is too small to draw definite conclusions, so that the work will be partly repeated.

It is also planned to tackle the turnover problem in another way, namely by means of decarboxylase inhibitors. However, a study of the membrane pump blocking action of the antidepressants under acute versus chronic administration is intended.

Besides the turnover work, some experiments concerning the metabolism of imipramine in fasting animals were performed. This seemed to be changed as was the case with the false transmitters.

J. ARIËNS KAPPERS

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HUBRECHT LABORATORY

International Embryological Institute – Utrecht

PROGRESS REPORT 1973

History and objectives of the Institute

The Hubrecht Laboratory was founded in 1916 in memory of the Utrecht zoologist and embryologist Prof. A. A. W. Hubrecht. It is a semi-governmental institution operating under the supervision of the Royal Netherlands Academy of Arts and Sciences.

The objective of the Laboratory is to function as an *international research and service centre for developmental biology*. To ensure a multidisciplinary approach to the many problems of development eight research units applying a variety of experimental approaches have been established (see under Scientific Staff below).

The Laboratory aims at stimulating international co-operation and understanding by, among other things, organizing International Research Groups in Developmental Biology at more or less regular intervals, and by the biennial publication of an international directory of investigators active in developmental biology (General Embryological Information Service).

The Laboratory houses the Central Embryological Library (collection of reprints covering the field of developmental biology) and the Central Embryological Collection (microscope slides and material preserved in alcohol).

Individual guest workers are welcome at the Laboratory. Partial financial support is available in special cases only.

Management and Scientific Staff

P. D. Nieuwkoop, Ph.D.—Director, Prof. of Experimental Embryology, University of Utrecht

J. Faber, Ph.D.—Deputy Director

B. Cool—Laboratory Manager

B. Z. Salomé, M.Sc.—Chief Librarian

Romee Verhoeff-de Fremery, M.Sc.—Supervisor of animal care

J. G. Bluemink, Ph.D.—Research unit of ultrastructural research

Elze C. Boterenbrood, Ph.D.—Research unit of experimental morphology; Curator of the Central Embryological Collection

K. Hara, Ph.D.—Research unit of experimental morphology

Vacancy—Research unit of developmental physiology

S. W. de Laat, M.Sc.—Research unit of biophysics

Kirstie A. Lawson, Ph.D.—Research unit of tissue and organ culture

W. J. Ouweneel, Ph.D.—Research unit of developmental genetics

P. T. M. van der Saag, M.Sc.—Research unit of biochemistry

Geertje A. Ubbels, Ph.D.—Research unit of histo- and cytochemistry

Visiting Scientists – 1973

B. M. Carlson, Ph.D. (Ann Arbor, Michigan)

Rosine Chandebouis, Ph.D. (Marseille, France)

R. O. Kelley, Ph.D. (Albuquerque, New Mexico)

W. P. Luckett, Ph.D. (New York, N.Y.)

V. Nanjundiah, Ph.D. (Chicago, Ill.)
Alina Sutasurja (Bandung, Indonesia)
Marianne Veini (Athens, Greece)

Temporary Research Assistant – 1973

R. T. M. Hengst, M.Sc.

Ph.D. student (University of Utrecht) – 1973

R. A. F. Dekker, M.Sc.

Graduate students (University of Utrecht) – 1973

Marianne I. Bakker, B.Sc.
P. W. J. A. Barts, B.Sc.
G. T. Blanken, B.Sc.
R. J. A. Buwalda, B.Sc.
A. M. M. C. Habets, B.Sc.

(For Dutch workers B.Sc. and M.Sc. are used as the approximate equivalents of the Dutch degrees of Biol. Cand. and Biol. Drs.).

INTRODUCTION

This year an important decision was taken with regard to the research policy of the Hubrecht Laboratory. The desire had been felt for some time to bring more unity to the research programme by choosing projects that would converge upon *a limited number of major areas of research*. These areas have now been defined for an approximate period of five years (not excluding the possibility of changes over the years). They are *numbered I-IV* in the following report. As a consequence the arrangement of the individual sections of the report is quite different from previous years. Sections I-III deal with investigations at the broadly organismal and organ levels in amphibians, insects and mammals, while section IV treats the work carried out at the more strictly cellular and molecular levels in amphibians.

There are still a number of research projects in progress which are peripheral to the four major areas. These are grouped together under heading nr. V, which follows the same order from the broadly organismal to the molecular level and includes several research projects carried out by guest workers. In so far as staff members are explicitly or implicitly involved in these projects it has been decided that they will gradually be terminated.

I. Origin and establishment of embryonic axes in amphibians

Following the discovery of mesoderm induction in the amphibian blastula in this Laboratory about seven years ago (see previous reports) it soon became apparent that the establishment of the future antero-posterior and dorso-ventral axes prior to gastrulation is intimately

connected with the inductive interactions between the "endodermal" and "ectodermal" moieties of the blastula. This led to a tentative re-appraisal of the role of the grey crescent and to a renewed interest in the blastula stages generally.

A. MORPHOLOGICAL AND CYTOCHEMICAL CLEAVAGE STUDIES

1. *Cinematographical study of the cleavage pattern (Discoglossus pictus)* (K. Hara)

One pair of films was taken of a cleaving *Discoglossus* egg, using the "double-camera" time-lapse method previously applied to eggs of *Ambystoma mexicanum* (see report of 1969, section I.a.5 and later reports). In general the cleavage pattern was similar to that of *Ambystoma*. The main differences noted in *Discoglossus* were: (1) there is one extra strictly synchronous cleavage cycle in the animal pole area before the onset of asynchrony; (2) there is no ingression of vegetative cells prior to gastrulation. In the area of the vegetative pole the 11th cleavage divisions occur at about the same time as the 12th divisions at the animal pole. At the time of incipient gastrulation the "fastest" cells in the animal pole area are already going through their 16th division, while most of the cells near the vegetative pole are only at the end of the 14th division. Thus, the *Discoglossus* egg by the beginning of gastrulation has gone through at least one more cleavage cycle than the *Ambystoma* egg. The study is being extended.

2. *A possible correlation between the initiation of 4th-cleavage furrows and nuclear position or phase prior to 4th cleavage (Ambystoma mexicanum)* (V. Nanjundiah, G. A. Ubbels)

The fourth cleavage furrows appear earlier in the micromeres than in the macromeres. It has been suggested that the cleavage furrows may be initiated by a stimulus emanating from the nuclei. A study has been started covering the period between the 3rd and 4th cleavages to see whether during this time the nuclei of the micro- and macromeres are at the same or different distances from the cell surface, and whether or not they pass through their premitotic and mitotic phases in synchrony.

3. *Cytoplasmic differentiation near the base of the first cleavage furrow (Ambystoma mexicanum)* (G. A. Ubbels)

Numerous tiny granules (1 μm) of lipoprotein character are present in the uncleaved egg, both in the region of the diastema (where the new cell membrane will appear) and in a glycogen and pigment-rich region of yolk-free cytoplasm found immediately around the base of the cleavage furrow. The hypothesis has been put forward that the granules in the latter region might represent either mitochondria, lysosomes, or residual bodies of yolk granules (see reports for 1971 and 1972, sect. I.C.1a).

The enzymes acid phosphatase and tetrazolium reductase were chosen as markers for lysosomes and mitochondria, respectively. Both enzymes withstand freeze-drying followed by embedding in low-melting-point (39° C) paraffin wax—this was shown in liver tissue stained on paraffin ribbons. In eggs, acid phosphatase was demonstrated only incidentally in yolk granules and never in the small granules near the base of the furrow—these therefore probably are not lysosomes, although the definitive proof can only be provided by electron microscopy.

The results of the tetrazolium reductase reaction were inconsistent owing to the dissolution of the reaction product in the xylene used for deparaffination. Therefore, a cryostat method for sectioning amphibian eggs is being elaborated.

The cytoplasm around the furrow-base stains neither for ribonucleo-protein (Brachet's method) nor for protein-SH (DDD method); the latter observation probably indicates the absence of microtubules.

4. *Cytochemistry of blastulae and early gastrulae (Ambystoma mexicanum)* (G. A. Ubbels, R. T. M. Hengst)

Variations in staining intensity of the cytoplasm of different blastomeres in blastulae and early gastrulae of *Xenopus laevis* and gastrulae of *Ambystoma mexicanum*, upon application of Motomura's (1967) mucopolysaccharide fixation and staining technique, suggested the possibility of local cytochemical differences (see reports for 1968, sect. I.C. 2 and 1969, sect. I.C.1). Most of the stained material is glycogen, although acid mucopolysaccharides are also present (see report for 1970, sect. I.C.1).

The cytochemical heterogeneity of the cells could have been only apparent, since the specificity of the method used was doubtful. Therefore a procedure for freeze-drying followed by paraffin-wax embedding was worked out. On the basis of PAS staining intensity the amounts of (muco)polysaccharides present in the individual blastomeres in freeze-dried *Ambystoma* blastulae appeared to be rather uniform. The cytochemical heterogeneity demonstrated with Motomura's method could be due to: 1) unsuitability of the technique, 2) differential distribution of substances during cell division, or 3) fluctuations in the concentration of particular cytoplasmic components during the cell cycle.

Therefore Mr. Hengst studied the distribution of (muco)polysaccharides in freeze-dried 16 to 128-cell blastulae and early gastrulae of *Ambystoma*, with the following results: 1) there is no mosaic-like pattern of PAS-stained blastomeres. However, from the 32-cell stage onwards the tips of the cells directed towards the blastocoelic cavity often contain largely yolk-free cytoplasm. The cytoplasm here and elsewhere is rich in glycogen and neutral mucopolysaccharides, but contains neither sulfated mucopolysaccharides nor hyaluronic acid-containing sugars; 2) a series of stages fixed at 15-min. intervals from the 16 till the 32-cell stage shows no variations in staining intensity which could reflect events in the cell

cycle. The conclusion is that the originally observed cytochemical heterogeneity in blastulae and gastrulae of *Ambystoma* and *Xenopus* is due to the unsuitability of Motomura's fixation method.

The staining obtained in early gastrulae after freeze-drying demonstrates that the material stained is glycogen, and not mucosubstances as suggested by Motomura. In this case the occurrence of differential cell divisions cannot be excluded. The blastoporal region is characterized by the presence of large amounts of intercellular glycogen.

B. MESODERM INDUCTION IN THE BLASTULA

1. *Further analysis of embryonic axis formation in induced mesoderm in recombinates of reaggregated Ambystoma mexicanum endoderm and intact Triturus alpestris ectodermal cap material* (P. D. Nieuwkoop)

Because last year's recombinates, in which both the animal ("ectodermal") and vegetative ("endodermal") material from blastulae had been disaggregated, yielded such poor results (see previous report, sect. I.A.2), a different experimental procedure was adopted. It was assumed that the animal (ectodermal) cap possesses no regional differences in mesodermal competence (see Nieuwkoop, 1969b), so that it could be used intact. Only the endoderm was disaggregated and the cells thoroughly mixed to abolish any regional polarization as regards mesoderm-inducing capacity. The reaggregated endodermal material was then recombined with an intact ectodermal cap.

The outcome of the experiments was encouraging. The recombinates formed either single or multiple embryonic axes whose orientation seemed not to bear any preferential relationship to the visible dorso-ventral orientation (grey crescent) of the ectodermal cap. This suggests that the assumption of the equipotentiality of the ectodermal cap was correct.

When ectodermal caps of different developmental stages (H. st. 7-9) were recombined with reaggregated stage 8⁺ endoderm, the impression was obtained that the percentage of recombinates forming head structures (prechordal mesoderm and endoderm, and anterior brain) increased markedly when using progressively younger ectoderm. This suggests that the longer the endoderm is able to exert its inductive effect (within the period of mesodermal competence), the stronger is the vegetalization of the ectoderm. However, a definitive experimental series will still have to be made next year.

At present deductions as to the character of the process of axis formation are being attempted from the frequencies and spatial distributions of single and multiple axis formation.

2. *The regional distribution of mesoderm-inducing capacity in the endodermal yolk mass of the blastula (Ambystoma mexicanum)* (G. T. Blanken)

In the intact blastula only the peripheral part of the vegetative yolk

mass is continuous with the hollow animal "ectodermal" moiety; the central part is separated from it by the blastocoelic cavity. A study was started in which the mesoderm-inducing capacity of peripheral portions of the yolk mass was compared with that of central portions of varying sizes by recombining them with animal, ectodermal material. The results are being analysed.

3. *The regional capacity for primordial germ cell formation in the animal, "ectodermal" moiety of the urodelan blastula (Ambystoma mexicanum, Triturus spp., Pleurodeles waltlii) (A. Sutasurja)*

This study is a continuation of that reported last year (see report of 1972, sect. I.A.4).

3a. Origin of primordial germ cells

Xenoplastic recombination experiments involving "ectodermal" caps and ventral portions of the vegetative yolk mass of Harrison stage $8\frac{1}{2}$ – $8\frac{3}{4}$ blastulae of *Ambystoma mexicanum* on the one hand, and of *Triturus alpestris*, *Tr. cristatus carnifex* or *Pleurodeles waltlii* on the other, have now convincingly shown that in the urodeles the primordial germ cells (PGCs) originate *exclusively* from the totipotent animal, "ectodermal" moiety of the blastula and not from the vegetative, "endodermal" yolk mass (as they do in the anurans).

A similar study involving homoplastic recombinates consisting of "ectodermal" caps labelled with ^3H -thymidine and unlabelled "endoderm", and conversely, has led to the same conclusion.

3b. Induction of primordial germ cells

A definitive series of experiments were carried out in which the entire animal, ectodermal hemisphere of st. $8\frac{1}{4}$ blastulae was isolated and subdivided into seven regions: four peripheral (d, v, l, r), two intermediate (d, v), and one central region. Each of these regions was recombined with a left or right ventral portion of the vegetative yolk mass of a st. $8\frac{1}{2}$ – $8\frac{3}{4}$ blastula. The results show that PGCs may arise from any part of the ectodermal hemisphere under the inductive influence of the ventral endoderm. The number of PGCs formed is maximal in the ventral-peripheral region and falls off both towards the dorsal periphery and from the periphery towards the animal pole. This decline is not correlated with the area or volume of the regions tested and must therefore find its basis in regional differences in competence within the ectodermal hemisphere.

The equatorial-animal gradient of decrease in PGC formation reflects true differences in competence. In contrast, the ventro-dorsal gradient is due to a gradient in notochord formation in the opposite direction, notochord being a differentiation incompatible with PGC formation. The dorso-ventral decrease in notochord formation in the peripheral

“ectodermal” region in its turn is due to the inductive action of the dorsal vegetative yolk material which had already taken place prior to isolation and recombination.

The differences in competence for PGC formation are not due to a progressive loss of competence in the central and intermediate regions during development: st. 7 central ectoderm, upon recombination with st. $8\frac{1}{2}$ – $8\frac{3}{4}$ endoderm, does not form more PGCs than similar ectoderm from stage 8 or 9.

Although it was not possible to measure the exact amount of ventro-caudal mesoderm induced in the ectoderm in these recombinates (which had to be cultured for 12 days), it seems most likely that the number of PGCs formed is correlated with the amount of induced ventro-caudal mesoderm. This would mean that in the urodeles PGCs arise from *common* ectodermal cells which only acquire their specificity in the course of development under the inductive action of the ventral endoderm.

The general conclusion is that an uninterrupted “germ line” such as is found in anurans does not exist in the urodeles.

C. THE ORIGIN OF BILATERAL SYMMETRY IN AMPHIBIAN EGGS

1. *Regional morphological and cytochemical differentiation in anuran eggs from oviposition till first cleavage*

Preliminary observations made by R. W. Glade (Burlington, Vt., U.S.A.) in this Laboratory had shown a characteristic distribution of yolk platelets of different sizes in newly laid eggs of *Xenopus laevis*. This distribution changes in the fertilized egg, suggesting that the entrance of the sperm initiates a cytoplasmic streaming movement resulting in a dorso-animalward displacement of the originally equatorially located medium-sized yolk platelets (see report for 1969, sect. I.C.3).

1a. *Discoglossus pictus* (J. Klag, G. A. Ubbels)

This work is a continuation of that described in the previous report (sect. I.C.1c). The eggs of *Discoglossus pictus* show a distinct grey crescent and are therefore very suitable for the study of the origin of bilaterality in anurans. The grey crescent starts to appear at ca. 75 mins. after oviposition (p.o.) and is very clear at 90 mins. p.o. During the first 75 mins. of development two kinds of cytoplasmic localization become apparent: 1) a vertical cytoplasmic column poor in yolk granules, extending between the animal cortex and the centre of the egg; 2) a number of yolk-free cytoplasmic islands in the subcortical region, which disappear after 90 mins. p.o. (cf. report for 1971, sect. I.C.1a). At that time yolk granules are virtually absent from the cytoplasm in the centre of the egg. This “light cytoplasm” is continuous with the vertical cytoplasmic column; during the next 45 mins. it extends progressively towards the cortical

region of the grey crescent area, i.e. towards the future dorsal side. The vertical column remains connected with the central cytoplasm. Its upper end shifts in a ventral direction along the animal cortex. Finally, about 10–15 mins. before first cleavage, only a very thin layer of yolk granules and pigment separates the "light cytoplasm" from the dorsal cortex. The cytoplasm both in the vertical column and in the central region stains intensely for glycogen and ribonucleoprotein. In unfertilized eggs a vertical column of cytoplasm also develops, but no central "light cytoplasm" appears and the position of the vertical column does not change with time.

The phenomena observed in the fertilized egg can be explained by postulating a cytoplasmic streaming movement which is initiated by the entering sperm. Certain components of the "light cytoplasm" might play a part in the formation and/or activation of the grey crescent and in the dorso-ventral polarization of the endoderm. Cytochemical analysis is in progress.

1b. *Xenopus laevis* (G. A. Ubbels, R. T. M. Hengst)

Eggs of *Xenopus laevis* often but not always show a less distinct grey crescent than those of *Discoglossus pictus*. About 10 mins. p.o. a region of cytoplasm devoid of yolk granules is present in the centre of the egg. At 45 mins. p.o. the grey crescent starts to appear. Ca. 60 mins. p.o. the yolk-free region is found in the dorsal-animal part of the egg; the fusion of the pronuclei takes place in its neighbourhood. The grey crescent is then maximally formed. At 90 mins. p.o., i.e. after first cleavage, the yolk-free region is usually found in one of the two blastomeres. The development of the unfertilized egg parallels that of the fertilized egg in some features, but a central yolk-free region is absent and no displacement of medium-sized yolk granules towards the dorsal side occurs.

Small yolk-free cytoplasmic areas in the subcortical region are only observed during the first 10 mins. of development. Small islands re-appear in this region ca. 30 mins. p.o. and grow progressively. They probably have something to do with furrow formation.

The cytochemical analysis of the cytoplasm in the yolk-free region is in progress. Positive reactions were found for glycogen, neutral mucopolysaccharides, and ribonucleoprotein, which are all found in the cytoplasm generally. Sulfated mucopolysaccharides as well as hyaluronic acid-containing sugars and also protein-SH and protein-SS are absent from the cytoplasm.

As in *Discoglossus*, the morphological findings can be explained on the basis of a dorso-animalward cytoplasmic streaming movement. Therefore, the possible factors involved in cytoplasmic movements in fertilized anuran eggs will be further analysed, with special reference to biogenic amines such as serotonin (5-HT) and related compounds (fluorescence microscopy).

2. *Experimental analysis of the morphogenetic role of the yolk-free cytoplasm in the fertilized egg (Xenopus laevis)* (R. T. M. Hengst)

A technique is being worked out for the extirpation and (possibly) transplantation of the yolk-free cytoplasm from eggs in which this cytoplasm is known to be located dorsally (from 45 mins. p.o. till first cleavage; see 1b above). It then lies ca. 150–200 μm below the dorso-animal cortex. Up till now, upon introduction of a micropipette with a tip diameter of 15 μm to this depth only 26% of the eggs survived till hatching. Introduction of the pipette from the vegetative side yielded a still lower survival rate. This was probably due to the larger distance which the tapering pipette had to traverse, as a result of which the hole in the cortex also was larger. Narrower pipettes are being tried. (The pipettes are connected to a micro-injection device developed in this Laboratory, see previous report, sect. I.4.)

D. A POSSIBLE ROLE FOR CYCLIC AMP

1. *Amphibian embryonic cells attract slime mould amoebae (Ambystoma mexicanum)* (V. Nanjundiah)

A basic question in amphibian development is: how early can spatial variations in well-characterized biochemical properties be demonstrated which correlate with different (presumptive) regions of the adult? The present study (made possible by the assistance of Dr. Th. M. Konijn, Leiden) hints at one possible answer. Already at Harrison stages 9–10⁻, cells of the axolotl blastula are able to differentially attract slime mould amoeba via chemotaxis. The strength of attraction seems to decrease in the following order: dorsal vegetal \rightarrow dorsal animal \rightarrow ventral animal \rightarrow ventral vegetal. These results are based on the "Konijn test", in which the material to be assayed is placed a small distance ($< 500 \mu\text{m}$) away from a drop of chemotactically sensitive amoebae on hydrophobic agar (< 1000 amoebae in a drop of ≤ 1 mm diameter) and the response of the amoebae is observed. (See sect. VII, ref. 21.)

Previous work with slime moulds (by Konijn and others) has shown that amoebae at the stage used in this experiment possess a strong and specific chemotactic sensitivity for cyclic AMP. However, more data will be needed if non-trivial conclusions are to be drawn from the present study—for instance, a direct biochemical assay of regional levels of cyclic AMP and phosphodiesterase during early embryonic stages is essential.

II. Pattern formation in insects

Insect imaginal discs are eminently suited for the study of problems of pattern formation. In this Laboratory at present both experimental-morphological and genetic methods are being used.

1. *Duplication and regeneration in the wild-type haltere disc (Drosophila melanogaster)* (W. J. Ouweneel, J. M. van der Meer)

As mentioned in the previous report (sect. V.1.1b), anterior and posterior fragments of mature haltere discs were given time to proliferate by culturing them in adults, and subsequently carried through metamorphosis in host larvae. This year the results were definitively analysed. Anterior fragments are able to regenerate missing anlagen, while posterior fragments are only capable of duplicating the anlagen present in them. Beside polarized mirror-image duplication, local multiplication of units or size increase of structures also occurs.

An attempt was made to interpret these results, together with literature data, in terms of a monotonic gradient of "positional value" (Wolpert) running through the disc in antero-posterior direction, with its high point (arbitrarily) located anteriorly. Fragments containing higher parts of the gradient would be able to regenerate lower parts of the gradient during growth, whereas in the case of proliferating fragments containing lower gradient parts a zero boundary would be established at the tip of the blastema, where a new gradient would be set up with reversed polarity (see ref. 14).

2. *Genetic pattern alterations in the haltere disc (Drosophila melanogaster)* (W. J. Ouweneel)

2a. Replacement patterns of homoeotic wing structures in halteres

It had been shown earlier that the haltere disc has an organ map homologous to that of the wing disc (see ref. 15). This led us to make a morphological analysis of all cuticular structures of the halteres of two mutants: *halteroptera*, in which the anterior part of the haltere is replaced by anterior wing structures, and *postbithorax*, which replaces the posterior haltere by posterior wing parts. Because of these partial replacements it is possible at each level in the haltere to determine which of its structures corresponds to a particular wing structure, and thus to establish homologies. Roughly, the scabellum corresponds to the wing base with the proximal costa, the pedicel is homologous to the medial costa with the adjacent posterior structures, while the capitellum is convertible into the distal costa, the more distal wing borders, and the wing spread. It is possible to relate these alteration patterns to the organ maps and to determine with considerable accuracy which part of the haltere disc is changed by either *halteroptera* or *postbithorax*. By substituting the homologous wing structures in the haltere-disc organ map, a fairly accurate organ map for the wing disc could be proposed. These results are being extended by morphological and histological examination of the homoeotic haltere disc. (See refs. 13, 21.)

2b. Genetic duplication patterns in halteres

The recently discovered mutation *Costal* brings about duplications of the anterior borders of the wing and haltere. A study of the pattern of these duplications in the haltere has just been started, in order to compare them with the duplications occurring in our culturing experiments, both in the haltere disc and in the adult haltere (see 1 above). Other mutations are being assembled for similar studies.

III. Epithelial-mesenchymal interactions in organogenesis

1. *Tissue interactions in branching morphogenesis (Mus musculus, Rattus norvegicus)* (K. A. Lawson)

The pattern in which a branching epithelium develops, such as that in salivary gland, lung or kidney, is characteristic for each organ and can be described in terms of points of branching and growth of the epithelium. It has long been known from organ culture experiments that the investing mesenchyme is essential for both growth and morphogenesis of these epithelia. Recombinates of epithelium and mesenchyme are being used to investigate branching morphogenesis *in vitro*.

1a. Mesenchyme specificity in salivary gland development

Contrary to published reports mouse submandibular epithelium is able to develop in lung mesenchyme (see previous report, sect. IV.A.1). Several factors contribute to this contradiction: 1) lung mesenchyme is quantitatively less effective than submandibular mesenchyme for supporting submandibular morphogenesis; this is thought to be due to the reciprocal requirement of lung mesenchyme for lung epithelium (see 1b below); 2) whereas the response of submandibular epithelium to its own mesenchyme is equally good on an agar or a millipore filter substratum, that to lung mesenchyme is severely reduced or eliminated on millipore filter under otherwise identical culture conditions. Lung mesenchyme cells are closely packed on agar, but spread out on millipore filter, reducing the number of cells per unit area by about half after 6 days; submandibular mesenchyme cells are diffusely distributed on both substrata (no difference in cells/unit area). This suggests that submandibular mesenchyme can support branching morphogenesis at a lower cell density than lung mesenchyme.

1b. Reciprocal action of epithelium and mesenchyme

Comparison of homotypic submandibular and lung recombinates with the heterotypic recombinant of submandibular epithelium with lung mesenchyme showed that, after 6 days of culture: 1) the volume of submandibular epithelium in the heterotypic recombinant was about one third of that in the homotypic recombinant, and 2) the number of

mesenchyme cells in the heterotypic recombinate was about half that in the homotypic submandibular recombinate and one quarter of that in the homotypic lung recombinate. This suggests that 1) the growth rate of lung mesenchyme is influenced by lung epithelium, which cannot be completely replaced by submandibular epithelium; 2) a high growth rate of lung mesenchyme is necessary for optimal growth of submandibular epithelium. These possibilities are being more fully investigated.

2. *Electron microscopy of branching morphogenesis in the submandibular gland and the lung (Mus musculus, Rattus norvegicus)* (J. G. Bluemink, K. A. Lawson)

Tissue culture experiments have shown that lung and submandibular gland exhibit a different pattern of branching morphogenesis. Bernfield and Wessells have proposed a mechanism for submandibular morphogenesis in which material at the epithelial/mesenchymal interface (EMI) is essential for initiating clefts in the submandibular epithelium. The purpose of this study was to examine the EMI both in the region where the epithelium is budding and in the region where it has become stable, i.e. where buds are not being formed. Explants were fixed after two and four days of culture. The ultrastructural features of the EMI in homotypic recombinates (epithelium reassociated with its own mesenchyme) were compared with those in intact organ rudiments.

The main findings were as follows. Intact rudiments and recombinates of mouse submandibular gland consist of many solid buds at the periphery (i.e., the morphogenetically active epithelium) which surround a tubular epithelium (the morphogenetically inactive part). A continuous basal lamina (BL), stratified into a lamina lucida and a lamina opaca, lines the whole epithelium. The mesenchyme cells are loosely arranged with large intercellular spaces containing collagen. There is definitely more collagen in the EMI along morphogenetically inactive epithelium. Young epithelial buds exhibit narrow, deep clefts near the bottom of which lies a bundle of tightly packed collagen fibrils.

Lung epithelium of mouse and rat develops from a slightly lobulated vesicle (2 days) into a highly lobulated bronchiolar epithelium (4 days). The epithelium is ca 1-3 cells thick and is arranged around a wide lumen. Along regions of morphogenetically inactive epithelium the BL is continuous and stands out clearly, but it thins and shows breaks in the regions where new buds are forming. Here direct cell-to-cell contacts occur between the epithelium and the mesenchyme. The mesenchyme cells are tightly packed and closely apposed to the epithelium. The intercellular space of the EMI is taken up almost completely by the BL. Instead of deep clefts the epithelium shows invaginations with a low radius of curvature. Comparing branching morphogenesis in the lung and in the submandibular gland, it is clear that marked morphological differences exist along the EMI at the ultrastructural level.

3. *Electron microscopy of branching morphogenesis in heterotypic recombinates of submandibular epithelium with lung mesenchyme (Mus musculus, Rattus norvegicus)* (J. G. Bluemink, K. A. Lawson)

Tissue culture experiments by K. A. Lawson have shown that heterotypic recombinates of mouse submandibular epithelium with mouse or rat lung mesenchyme will undergo morphogenesis *in vitro*. The purpose of this study was to analyse the epithelial/mesenchymal interface (EMI) in the region of morphogenetic activity, i.e. there where epithelial buds are being formed, and in the region of the morphogenetically inactive epithelium where the epithelium has become stable. Recombinates were fixed after two and four days of culture. The main findings were as follows.

In contrast to homotypic submandibular recombinates, which have a continuous and stratified basal lamina (BL) along morphogenetically active regions, the heterotypic recombinates here showed an amorphous, more electron-dense, and discontinuous BL characteristic of homotypic lung recombinates. After four days of culture there are many solid epithelial buds. Collagen is present at the EMI and there are narrow, deep clefts. Near the bottom of young, deep clefts lies a small bundle of tightly packed collagen fibrils. Such young clefts are characteristic for the type of branching seen in the salivary gland. They have not been found in mouse or rat lung. Thus, although the structure of the BL and the organization of the mesenchyme is very similar to that seen in normal lung, the structure of the clefts is typical for branching in the salivary gland.

4. *An ultrastructural analysis of cell and matrix differentiation during early limb development (Xenopus laevis)* (R. O. Kelley, J. G. Bluemink)

The early development of the hind limb of *Xenopus* (stages 44–48 N. & F.) has been analysed at the level of ultrastructure with emphasis on the differentiation of extracellular matrix components and intercellular contacts. By stages 44–45 the mesenchyme is separated from the prospective bud epithelium by numerous adepidermal granules in a subepithelial compartment (the lamina lucida), a continuous basal lamina, and several layers of collagen (the basement lamella). Tricomplex stabilization of amphoteric phospholipid demonstrates that each adepidermal granule consists of several membrane-like layers (electron-lucent band 25–30 Å, electron-dense band 20–40 Å) which are usually parallel to the basal surface of adjacent epithelial cells. Collagen fibrils are interconnected by filaments (35 Å in diameter) which stain with ruthenium red. Mesenchymal cells predominantly exhibit close contacts (100–150 Å separation), with few focal tight junctions on various areas of their surface.

By stages 47–48, the adepidermal granules are absent beneath the bud epithelium and the layers of collagen in the basement lamella lose their filamentous cross-linking elements. Filopodia of mesenchymal cells

penetrate the disorganized matrix and abut on the basal lamina. Hemidesmosomes disappear at the basal surface of the epidermis, and mesenchymal cells immediately subjacent to epithelium exhibit focal tight junctions and gap junctions at their lateral borders.

These structural changes may be instrumental in the epithelio-mesenchymal interactions of early limb development. The degradation of oriented collagenous lamellae permits direct association of mesenchymal cell surfaces (filopodia) with surface-associated products of the epithelial cells (organized into the basal lamina). The development of structural pathways for intercellular ion and metabolite transport in the mesenchyme may facilitate the coordination of events specific to limb morphogenesis. The results have been submitted for publication (see ref. 17).

IV. Regulation of the cell cycle: role in development and differentiation

1. *An outline of the project* (S. W. de Laat, P. T. M. van der Saag, J. G. Bluemink)

In cooperation with several departments of the Universities of Utrecht, Amsterdam, and Nijmegen a working party* has been established to start coordinated and multidisciplinary investigations on the regulation of the cell cycle. The core problem of cellular differentiation coincides with those endogenous processes which make available to daughter cells genetic information which was not accessible to the mother cell. Thus there must be a relationship between the regulation of the cell cycle and differentiation. This coupling could provide the clock according to which genetic information already present in the zygote will be expressed during development. At the molecular level the mechanism of this coupling is far from clear. Even the individual processes which regulate the cell cycle via feedback mechanisms have not been unequivocally identified.

On the other hand, it is now known that processes such as RNA, protein, and phospholipid synthesis show cyclic variations in rate during the cell cycle. The same holds for changes in membrane permeability, ion metabolism, cyclic AMP metabolism, etc. Causal relationships or correlations have been established between these processes and DNA synthesis. It becomes more and more clear that changes in cell membrane properties play an essential part in the regulation of the cell cycle (Burger *et al.*, 1972; McDonald *et al.*, 1972).

This fits in with our own findings that in *Xenopus laevis* embryos ion

* This working party functions within BION, a foundation for biological research operated by the Netherlands Organization for the Advancement of Pure Research (ZWO); participants from other laboratories: W. J. C. Ames (Van 't Hoff Lab., Utrecht), S. K. Brahma (Anat.-Embryol., Utrecht), O. H. J. Destree (Anat.-Embryol., Amsterdam), J. M. Vlak (Physiol. Chem., Utrecht), B. A. M. van der Zeijst (Veter. Virology, Utrecht), Th. F. J. de Greeff (Chem. Cytol., Nijmegen).

permeability, intracellular ion distribution, and the electrical properties of both the outer and the intercellular membrane show cyclic variations during the cell cycle due to new membrane formation (see below). Preliminary experiments have shown that properties of the cell membrane are dependent on DNA synthesis and that changes in the intracellular ionic environment influence the duration of the cell cycle.

Perhaps the cell membrane can regulate the rate of DNA synthesis via changes in cAMP metabolism. DNA synthesis will induce mitosis, which in its turn alters the cell membrane properties. As a first approach to this problem the working party is attempting to refine this rough model, and to subdivide the regulatory mechanism into discrete steps. Secondly, modifications of these processes are being studied during well-defined differentiation processes. At present three different systems are being used: early development of *Xenopus laevis* (Hubrecht Laboratory), differentiation of neuroblastoma cells (Univ. of Utrecht), and differentiation in *Physarum polycephalum* (Univ. of Nijmegen).

2. *The effect of cytochalasin B (CCB) injected at the onset of cleavage (Xenopus laevis)* (D. Luchtel, J. G. Bluemink, S. W. de Laat)

The purpose of this study was to examine the ultrastructure of the filament layer in the region where the furrow had regressed as a result of injection of CCB directly underneath the cell membrane (see previous report, sect. I.B.3). A description of the changes in gross morphology has been published (see ref. 11), together with the electrophysiological data. A second manuscript describing the electron-microscopical findings is in preparation.

The findings are as follows. After DMSO-injection (control experiment) the profile of the furrow may be distorted but filaments are present in a layer both at 4 and at 10 mins. after the onset of cleavage. In CCB-injected eggs, at 4 mins. after the onset of cleavage the furrow has partially regressed but the filaments are still present. However, at 10 mins. after the onset of cleavage in such eggs the furrow has regressed completely and an unpigmented area replaces the former groove. Sections cut through the former furrow area reveal scattered groups of four to eight filaments near the midline (cleavage plane). In cross sections the filaments show up as dots. In sections cut parallel to the midline filaments can also be demonstrated, but they are more difficult to find. It is uncertain whether the structures seen represent filaments which are resistant to degradation by CCB (dispersal of the layer and disorientation of the filaments being the reason why smaller numbers are seen), or whether they are the last filaments to have escaped degradation.

Our conclusion is that injected CCB affects the cell-contractile machinery. The primary site of CCB binding cannot be elucidated by EM analysis of cell ultrastructure.

3. *New membrane formation during cytokinesis in normal and cytochalasin B-treated eggs (Xenopus laevis)*

3a. Electron microscopy (J. G. Bluemink, S. W. de Laat)

The purpose of this study and its findings were described in the previous report (sect. I.B.4). The results are discussed in a paper published in 1973 (see ref. 1). The *Xenopus* egg in certain respects constitutes favourable material for the study of new membrane formation. The application of freeze-facturing techniques is considered the most promising approach for the continuation of this project.

3b. Electrophysiology (S. W. de Laat, J. G. Bluemink)

This work is a continuation of that described in the 1971 report (sect. I.E. 2). The electrical membrane potential and electrical membrane resistance were measured during first cleavage of eggs cleaving within and outside the vitelline membrane, in the presence and absence of cytochalasin B (CCB). The results were further evaluated and correlated with measurements of the geometrical surface area of both the newly formed membrane and the pre-existing membrane (see ref. 1). Under all conditions the decrease in membrane resistance could be correlated with the area of newly formed membrane exposed to the outer medium. It was concluded that this new membrane has other ionic permeability properties than the pre-existing membrane (most probably a relatively high permeability for K^+ -ions). Its mean specific resistance is $1.5 \text{ k}\Omega\text{cm}^2$ as against $74 \text{ k}\Omega\text{cm}^2$ for the pre-existing membrane. No influence of CCB on the electrical properties of the pre-existing or new membrane could be detected. The results are in the press (see ref. 19).

4. *Ion activities and ionic permeability during first cleavage (Xenopus laevis)* (S. W. de Laat, R. J. A. Buwalda, A. M. M. C. Habets)

This work is a continuation of that described in the previous report (sect. I.E.2). The electrical membrane potential (V_m) and electrical membrane resistance (R_m) change according to a characteristic pattern during first cleavage. This is due to the formation, in the plane of cleavage, of new membrane having ionic permeability properties different from those of the pre-existing outer membrane. In eggs cleaving within the vitelline membrane a small fraction (10%) of this new membrane is inserted into the outer surface (see 3b above). It was reported before that the changes in intracellular Na^+ , K^+ and Cl^- activities cannot account for the changes in V_m . To determine the relative ionic permeability properties of the new membrane, simultaneous measurements of the intracellular Na^+ , K^+ and Cl^- activities and both V_m and R_m were carried out in eggs cleaving inside and outside the vitelline membrane. Ion-selective micro-electrodes and conventional glass micro-electrodes were used. These measurements were made in bathing media of different

ionic composition obtained by equimolar replacement of ions. Both for eggs cleaving inside and outside the vitelline membrane the dependence of the membrane potential on the external K^+ activity can be described by the classical Goldman equation with a Na^+/K^+ permeability ratio of 0.14.

The cell membrane of the uncleaved egg appears to be highly impermeable to Na^+ , K^+ and Cl^- ions. Preliminary results of influx measurements of ^{22}Na and ^{42}K confirm this conclusion. This implies that the origin of the small negative intracellular potential of the uncleaved egg remains to be clarified.

5. *Electrical properties of membranes of pregastrula embryos (Xenopus laevis)* (S. W. de Laat, P. W. J. A. Barts, M. I. Bakker)

This work is a continuation of that described in the previous report (sect. I.E.3). To obtain further insight in the possibility of exchange of ions between individual cells, the intercellular resistance (R_{ij}) and the resistance between the cytoplasm and the external medium (R_{i0}) were measured continuously during development from the first till the fifth cleavage. Measurements were made across each intercellular membrane formed within this period, distinguishing between dorsal and ventral, and animal and vegetative membranes if possible. R_{i0} shows a decrease with time which is probably related to the insertion into the outer surface of a small fraction of the new membrane formed at each cleavage. R_{ij} increases at each cleavage due to the formation of new intercellular membranes. During each cleavage cycle both resistances show cyclic variations which can be correlated with changes in intercellular surface area due to movements of peripheral parts of adjacent blastomeres during the cleavage process. A digital computer model was constructed of the electrical network formed by the individual surface and intercellular membranes, in which the surface areas of individual membranes were calculated and values of the specific surface and intercellular membrane resistances could be fitted to the measured R_{i0} and R_{ij} . Furthermore, the possible role of the blastocoel in the exchange of ions could be evaluated by means of the model.

An analogue model of R_{ij} was constructed as formed by the complex network of all intercellular membranes. Using this model, the values of the resistances of the individual intercellular membranes could be obtained for each stage of development, and by combining these findings with data on the surface areas the specific resistance as a function of time could be calculated for each intercellular membrane.

V. Other research projects

1. *Differentiation tendencies of Hensen's node (Gallus domesticus)* (M. Veini)

This work is a continuation of that described in the previous report (sect. III. A.2). Hensen's node in the avian embryo is considered as being

homologous to the amphibian dorsal blastoporal lip. Autoradiographic studies have shown that after the definitive primitive-streak stage no endoderm invaginates through the node any longer. However, there still is little information on the exact differentiation tendencies of the node during the period of endoderm ingression.

Hensen's node was isolated from blastoderms of the medium-streak and later stages, from which the endodermal layer (hypoblast) had been previously removed. Both the node and the isolated hypoblast were then cultured *in vivo* by means of the intracoelomic grafting technique.

The isolated node, consisting of epiblast and invaginating cells, was found to form endodermal structures when taken from the medium and definitive primitive-streak stages but to have completely lost its endodermal differentiation tendencies by the head-process stage. The isolated hypoblast exhibited endodermal differentiation tendencies at all stages. Neural differentiation tendencies were always present in nodes isolated (without endoderm) from the definitive primitive-streak stage onwards, but in nodes from earlier stages the incidence of neural differentiation was significantly lower. The incidence of mesonephric differentiation rose steadily throughout the stages examined.

The conclusion may be drawn that there are some distinct parallels between node cells and amphibian blastoporal lip material with respect to the sequence of determination during invagination.

2. *Ultrastructural studies on the closure of the neural tube (Xenopus laevis)*
(R. A. F. Dekker – with financial support of the Netherlands Organization for the Advancement of Pure Research)

Closure of the neural tube requires some of the neural ectoderm cells to establish contact with each other. The surface of these cells, which was originally external and did not have adhesive properties, plays an important part in the closure of the neural tube and in the reconstruction of the integrity of the overlying epidermis. A transmission and scanning electron-microscopic study of the surface and cortical cytoplasm of neural ectoderm cells involved in these processes is in progress. The results may provide information on 1) the mechanism of the closure of the neural tube itself, and 2) the relation between fine structure and properties of the external surfaces of these cells before and after closure of the neural tube, when the same surfaces are located internally.

2a. Transmission and scanning electron microscopy

The first step in the fixation procedure for neurulae (see previous report, sect. I.B.7b) has been modified by the addition of other aldehydes to the 1% acrolein solution. It was found that the neural ectoderm cells from stage 18 (N. & F.) onwards show a modification in the structure of their external triple-layered cell membrane. The outer osmiophilic lamella is 2–3 times thicker than the inner lamella. Enzymatic digestion

experiments on these thickened membranes, as described in 2b below, will be continued at the electron-microscopic level.

With scanning electron microscopy the surface of these neural ectoderm cells, which plays a major role in the closure of the neural tube, can be visualized. The shape of the neural groove shortly before closure makes it impossible to obtain an evenly distributed thin layer of conductive material over all irregularities of cell surface contour when using conventional evaporative metal-coating techniques. A procedure in which structurally bound osmium is used for specimen preparation has been worked out (see 3 below).

2b. The effects of concanavalin A, cytochalasin E, and enzymes on neurulation

The closure of the neural tube of neurulae inside their vitelline membrane can be inhibited with concanavalin A (10–100 $\mu\text{gr}/\text{ml}$). If the vitelline membrane is removed before treatment, however, little effect on the process of closure is observed, even after pretreatment with solutions containing trypsin or urea (cf. previous report, sect. I.B.7d).

Treatment of neurulae with cytochalasin E (1–10 $\mu\text{gr}/\text{ml}$) affects certain neural ectoderm cells. This effect, a disaggregation of small groups of cells which would normally adhere to each other during the closure of the neural tube, only occurs shortly before closure (st. 18½). The loosened cells prevent normal closure.

A membrane-associated material can be visualized with ruthenium red on the surface of neural ectoderm cells. If this material (acid glycosaminoglycans?) plays a part in the contacts between neural ectoderm cells that are necessary for the closure of the tube, its enzymatic digestion should influence the closing process. For these experiments solutions containing neuraminidase (1–50 U/ml) or hyaluronidase (0.01–0.1 mg/ml) were used. Generally treatment with these enzymes seems not to interfere with normal closure. The only macroscopically visible effects occur during later stages, after the closure of the neural tube. These experiments are being continued at the electron-microscopic level.

3. *The preparation of soft biological specimens for scanning electron microscopy* (R. O. Kelley, R. A. F. Dekker, J. G. Bluemink)

Soft biological specimens generally require a surface coat of some conductive material to prevent an uneven buildup of electrons when subjected to a scanning electron beam. Most commonly this charging phenomenon is prevented by depositing a thin layer of metal on the specimen surface. With certain biological objects, however, metal coating techniques cause insuperable difficulties. Not all surfaces can be evenly coated and fine structural details are easily obscured by an uneven coating.

We have used the osmium-bridging properties of thiocarbohydrazide to bind additional osmium to a variety of soft tissue surfaces before

critical-point drying. With this so-called OTO-procedure a structurally bound layer of osmium is obtained which is evenly distributed over all irregularities of cell surface contour (while in addition the osmium density in the cytoplasm is enhanced). This layer gives satisfactory protection to the specimens under a scanning electron beam at accelerating voltages between 5 and 25 kV. The procedure provides a simple and economical alternative to coating with heavy metals, which avoids either masking of fine structural detail or charging resulting from uneven coating. (See refs. 6, 18.)

4. *The effect of the geometry of transplanted blastemal mesenchyme on disto-proximal organization in the ensuing limb (Ambystoma mexicanum)* (J. Faber)

N. J. de Both, while at this Laboratory, found that the disto-proximal extent of limb differentiation of blastemal mesenchyme transplanted to the muscles of the flank could be enhanced by combining several epidermisless blastemas into one transplant (published in 1970). Since he used an experimental set-up resulting in a fused mass of mesenchyme that was elongated perpendicular to the wound epidermis (which is supposed to provide the information for polarity), the question arose whether the results could have been due to this particular geometry.

An attempt was made to produce a different geometry by grafting four naked regeneration blastemas in close linear apposition into a shallow groove made in the muscles of the flank, and allowing the epidermis to heal over the graft. Limbs grew out in many cases and a preliminary analysis indicates that they have enhanced transverse vs. reduced disto-proximal organization.

5. *Effects of axially rotated tissues in the limb stump upon the morphogenesis of the regenerate (Ambystoma mexicanum)* (B. M. Carlson)

It has been established that after rotation of the skin through 180° about the longitudinal axis of the axolotl forelimb and subsequent amputation of the limb through the level of the rotated skin, a high percentage of the regenerates develop into complex multiple structures. The mechanisms behind the phenomenon are poorly understood. The following experiments were designed to increase our understanding of this morphogenetically disturbed system.

Exchanging the flexor and extensor musculature before amputation, rotating the muscles through 180° as well, resulted in 80% multiple regenerates, whereas 180° rotation of bone (humerus) permitted normal regeneration. The combination of rotated dermis plus normally oriented epidermis resulted in 79% multiple regenerates, but rotated epidermis combined with normal dermis caused less than 20% of the regenerates to be multiple.

If a cuff of normal skin intervenes between the rotated skin and the

amputation surface, the regenerates are all normal. However, an experiment currently in progress indicates that if the skin is removed for 3 mm between rotated skin and the amputation surface, some multiple regenerates will form. This may be due to the distal migration of cells from the dermis of the rotated skin cuff. This experiment is being followed up by one in which a 3-mm space between the rotated skin cuff and the amputation surface is occupied by normally oriented dermis and rotated epidermis.

Another major question is whether the multiple limbs are due to the axial reversal of the rotated tissues and cells or to the physical displacement of tissues that accompanies rotation. A number of experiments which involve the displacement of tissues without concomitant axial rotation are in progress. The test tissues are skin, muscle, and bone.

6. *The signal relay system in slime mould aggregation (Dictyostelium discoideum)* (V. Nanjundiah)

To begin to understand multicellular development, one needs to focus on systems whose behaviour is 1) sufficiently simple in space and time, and 2) governed by a small number of critical parameters. The aggregative phase of the cellular slime moulds provides one such system. During this stage of their life cycle a large number of chemotactic amoebae gather together by means of a series of coordinated pulsatile movement steps in response to a signal in the form of cyclic AMP released originally by a small group of cells (the aggregation centre).

The present study started from two rival quantitative models for the speed of wave propagation: one model in which the centre signals periodically, the signal diffuses outwards through the (agar) substrate, and the other amoebae move in passively; and another model in which the signal of the centre is relayed outwards from one amoeba to the next, and each amoeba moves up its local signal gradient. A preliminary comparison of theory with measurements (on films made by Dr. K. Hara, amoebae supplied by Dr. Th. M. Konijn, Leiden) has already yielded some results: 1) the first model is ruled out by the simple observation that the speed of wave propagation seems to increase with distance from the centre; 2) the second model yields a quantitative fit to the data, and in addition indicates that a) extracellular phosphodiesterase is not important for the aggregation process, and b) the signal relay system involves a delay time of about 18 secs. within each amoeba, even before it has made contact with other amoebae. This delay time could correspond to the synthesis of a transmitter substance (as in nerve cell synapses) or to the time required to amplify the incoming signal before relaying it on. The study will be continued elsewhere.

VI. Miscellaneous

1. Dr. F. Müller and Dr. I. Müller (Fribourg, Switzerland) visited

the Central Embryological Collection for some days in April to study vascular patterns in the heads of lower Primates, in order to compare them with vascular patterns known from fossil Primates.

2. During a renewed visit of several months to the Central Embryological Collection, Dr. W. P. Luckett extended his studies on the early development of the foetal membranes and placenta in various species.

3. Dr. R. Chandebais spent the month of May in the library, studying literature in connection with the preparation of a developmental biology textbook.

4. Dr. B. M. Carlson made extensive use of the library facilities in writing several chapters for a book on human pregnancy.

P. D. NIEUWKOOP
J. FABER

VII. Papers published and accepted for publication in 1973

Published

1. BLUEMINK, J. G. and S. W. DE LAAT, New membrane formation during cytokinesis in normal and cytochalasin B-treated eggs of *Xenopus laevis*. I. Electron microscope observations. *J. Cell Biol.* **59**, 89-108 (1973).
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6. KELLEY, R. O., R. A. F. DEKKER and J. G. BLUEMINK, Ligand-mediated osmium binding: its application in coating biological specimens for scanning electron microscopy. *J. Ultrastruct. Res.* **45**, 254-258 (1973).
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12. NIEUWKOOP, P. D., The "organization center" of the amphibian embryo: its origin, spatial organization, and morphogenetic action. *Adv. Morphogen.* 10, 1-39 (1973).
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14. ——— and J. M. VAN DER MEER, Determination and regulation in the haltere disc. *Drosoph. Inf. Serv.* 49, 88 (1972).
15. ——— and ———, Differentiation capacities of the dorsal metathoracic (haltere) disc of *Drosophila melanogaster*. I. Normal organ map. *Wilhelm Roux' Arch. Entw. Mech. Org.* 172, 149-161 (1973).

Accepted for publication

16. BLUEMINK, J. G. and S. W. DE LAAT, Cell surface changes due to new membrane formation during cytokinesis. *Mikroskopie* (abstract).
17. KELLEY, R. O. and J. G. BLUEMINK, An ultrastructural analysis of cell and matrix differentiation during early limb development in *Xenopus laevis*. *Devl. Biol.*
18. ———, R. A. F. DEKKER and J. G. BLUEMINK, Surface coating of biological specimens with osmium for scanning electron microscopy (SEM). *Mikroskopie* (abstract).
19. LAAT, S. W. DE and J. G. BLUEMINK, New membrane formation during cytokinesis in normal and cytochalasin B-treated eggs of *Xenopus laevis*. II. Electrophysiological observations. *J. Cell Biol.*
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21. NANJUNDIAH, V., A differential chemotactic response of slime mould amoebae to regions of the early amphibian embryo. *Exp. Cell Res.*
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CENTRAALBUREAU VOOR SCHIMMELCULTURES BAARN AND DELFT

PROGRESS REPORT 1973

The Centraalbureau voor Schimmelcultures was founded in 1904 by the "Association Internationale des Botanistes". Dr. Johanna Westerdijk at Amsterdam was appointed as the first director in 1907. After the dissolution of the AIB, the Bureau was supported by various Netherlands scientific institutions and associations, esp. by the Royal Netherlands Academy of Arts and Sciences. In 1920 the institute moved to Baarn; the yeast collection has been kept since 1922 at the Laboratory of Microbiology, Technical University, Delft.

After Prof. Westerdijk retired in 1959, she was succeeded as director by Miss A. L. van Beverwijk (1959–1963). In 1964 the CBS moved into a new building in Baarn (Oosterstraat 1). Since 1968, the CBS is an institute of the Royal Netherlands Academy of Arts and Sciences.

The Centraalbureau voor Schimmelcultures maintains a collection of living fungi, yeasts and actinomycetes. In 1973 the total number of strains maintained was 21.700, including 3.600 yeasts. By supplying cultures, identifications and advice to workers in diverse fields of scientific and applied mycology, a service is rendered to all those interested in these micro-organisms. Scientific research is carried out mainly in taxonomy of fungi. In the division of human and animal mycology, problems pertaining to this field are studied. Investigations on the chemistry of fungal metabolites are carried out in the biochemical department.

Facilities are available to students and guest workers who want to study a mycological subject. Each year courses are given on general and on human and animal mycology.

Scientific Staff (as from December 1st 1973).

Baarn, Oosterstraat 1.

Dr. J. A. von Arx, director (general mycology, Ascomycetes, Melanconiales)

Dr. G. A. de Vries (human and animal mycology, Actinomycetes)

Miss drs. A. C. Stolk (Aspergillus, Penicillium and related genera)

Miss dr. M. A. A. Schipper (Mucorales)

Mrs. drs. E. J. Hermanides-Nijhof (Fusarium, Aureobasidium)

Mrs. drs. A. J. van der Plaats-Niterink (Oomycetes)

Dr. H. A. van der Aa (Sphaeropsidales)

Dr. G. W. van Eijk (biochemistry)

Dr. W. Gams (Verticillium, Acremonium and related genera, Mortierella)

Drs. R. A. Samson (Paecilomyces, Penicillium and related genera)

Drs. G. S. de Hoog (Dematiaceae)

Drs. J. A. J. M. Stalpers (Basidiomycetes)

Drs. A. C. M. Weijman (biochemistry, physiology)

Mrs. drs. G. de Bruin-Brink (documentation)

Yeast Division, Delft, Julianalaan 67A.

Prof. Dr. T. O. Wikén, Director Laboratory of Microbiology, Technical University

Drs. L. Rodrigues de Miranda (Basidiomycetous yeasts)

Miss drs. M. Th. Smith

D. Yarrow (Saccharomyces and related genera)

1. Division of Fungus Taxonomy

Dr. H. A. van der Aa

The manuscript of the first part of a revision of the genus *Phyllosticta* was finished and published in October as nr. 5 of the Studies in Mycology. In this paper the genus *Phyllosticta* Pers. sensu stricto, as typified by *Phyllosticta cruenta* (Kunze ex Fr.) Kickx, is monographed. *Phyllostictina* Sydow and the monotypical genus *Caudophoma* Patil & Thirumalachar are put in the synonymy. 46 species of *Phyllosticta* are described, based on herbarium material and sometimes also on fresh collections or pure cultures. A checklist is given of the 84 species published in *Phyllostictina*.

Special attention is paid to the *Guignardia*-ascigerous states, which have been observed in 12 species. The relation with other genera, particularly *Botryosphaeria*, is discussed. A spermatial state was encountered in 17 species; in 10 of them for the first time. It is classified in *Leptodothiorella* Höhnelt sensu Sydow ex van der Aa.

The study of the species to be excluded from *Phyllosticta* was continued. A check-list of all the species described in *Phyllosticta* is in preparation.

In *Coniothyrium* a restricted number of new strains was obtained and provisionally described. Two strains were studied of an unknown *Coniothyrium* species isolated by Mrs M. Muntañola-Cvetković from fresh water of Skadar Lake in Yugoslavia; they form a conspicuous greenish pigment. Considerable numbers of *Phoma*-isolates were received from M. Muntañola-Cvetković and from J. Bissett, Canada, some of them are unknown species, which are being studied in cooperation with G. H. Boerema, Wageningen.

F. Brunck, Nogent sur Marne, France, sent a fungus culture isolated from diseased *Terminalia ivorensis* in Côte d'Ivoire for identification, which proved to be an unknown species of *Coniella*, differing from other species by large conidia measuring 25–30 × 16–20 μm. A manuscript with a description of this fungus is in preparation.

A sample of a leaf spot disease on Bean (*Phaseolus vulgaris*) was sent to the CBS by ISAR, Rwanda, for identification of the pathogen. The causal organism could be isolated and was identified as *Ramularia deusta* (Fuckel) Baker & Snyder (syn.: *Hyalodendron album* (Dowson) Diddens).

An unknown fungus species isolated a number of times from leaves of *Panicum laevifolium* was obtained from Dr. W. F. O. Marasas, Pretoria, South Africa. It appears to be a *Stauronema* species; a study on this fungus will be published in cooperation with Dr. Marasas.

Ripe perithecia of *Acanthostigma parasiticum* (Hartig) Sacc. together with a *Pyrenochaeta* conidial state were observed on living needles of *Abies alba*, received from Forstbotanisches Institut in Munich, W. Germany. By means of mono-ascospore cultures it was proved that both states belong to the same life cycle.

Dead stems of *Dioscorea composita* systemically affected by *Glomerella*

cingulata (Stoneman) Spaulding & v. Schrenk were sent to the CBS by Steromex, Mexico; on this substrate also an undescribed species of *Nectria* was observed; by mono-ascospore isolations a conidial state was obtained which belongs to the genus *Kutilakesopsis* Agnihotrudu. *Dendroochium macrosporum* Sacc. & Ellis was isolated from the same substrate. From a fallen leaf of *Populus nigra*, collected on the banks of the river Dommel near Valkenswaard, the Netherlands, *Uncigera cordae* Sacc. was isolated. The latter two Hyphomycetes were not reported in pure culture up to now.

Dr. J. A. von Arx

From a continued comparative study of filamentous and yeast-like fungi associated with bark beetles (ambrosia fungi) it is concluded that most of them belong to a phylogenetically related group. The perithecial members of this group have been classified by Hunt (Lloydia 19: 1-58. 1956) in the genus *Ceratocystis*. This genus, however, is heterogeneous and should be restricted to species with a *Chalara* (*Chalaropsis*, *Thielaviopsis*) conidial state. For species forming blastoconidia in sympodulae (form genera *Sporothrix*, *Verticicladiella*, *Graphium*), the genus name *Ophiostoma* can be reintroduced. *Ophiostoma* species usually develop on bark or wood attacked by bark beetles. The genus *Europhium* is very close to *Ophiostoma* and can be distinguished only by its non-ostiolate ascomata. It also is connected with *Verticicladiella* conidial states. Other related genera without perithecia but with asci on free ascophores, hitherto classified in yeast-like ascomycetes, are *Cephaloascus*, *Botryoascus*, *Hormoascus* and *Ambrosiozyma* (von Arx, Antonie van Leeuwenhoek 38: 289-309. 1972). In the monotypic genera *Cephaloascus*, *Botryoascus* and *Hormoascus* the asci are formed in chains on ascophores, comparable with the conidiophores of the form genera *Verticicladiella* and *Raffaelea*. Such fungi may have lost the capacity to form perithecia during their evolution, the ascigerous state being transferred to structures of the vegetative mycelium or conidial state. In *Cephaloascus* and *Hormoascus* the vegetative propagation was replaced by yeast-like budding structures; in *Botryoascus*, on the other hand, a *Raffaelea*-like conidial state was retained. In a natural system of the fungi all the above mentioned genera would have to be classified in the family Ophiostomataceae. Some more species, described in the unnatural yeast genera *Pichia*, *Hansenula* and *Candida* (e.g. *C. diddensii*; cf. van der Walt & al., Antonie van Leeuwenhoek 37: 449-460. 1971) would have to be added.

The CBS-strains belonging to *Microascus*, *Petriellidium* and related genera have been compared. The new genus name *Pithoascus* has been proposed for *Microascus nidicola*. In *Petriellidium* and *Pithoascus* 6 species are accepted in both genera, 4 in the former and 2 in the latter genus are described as new. Most of the new species were isolated from soil (mainly desert soil) and sent to the CBS for identification.

Among a large number of cultures isolated by Ir J. H. van Emden (Wageningen) from soil, two cleistocarpous ascomycetes were encountered which could not be classified in any described genus. In cooperation with Drs. R. A. Samson, the new generic names *Leucothecium* and *Xanthothecium* were proposed from them.

The manuscript was prepared for a fully revised second edition of the handbook "The genera of fungi sporulating in pure culture", which will be published early in 1974. In cooperation with Prof. E. Müller (Zürich, Switzerland), a revision of the bitunicate ascomycetes was started with keys to families and genera, descriptions of the families and lists of the accepted genera.

Dr. K. W. Gams

While attending the Symposium on Fungal Taxonomy in Madras, India, in Jan. 1973, fungal specimens were collected, mainly in the Botanic Gardens of Bangalore—India, and Peradeniya and Hakgala—Ceylon. From this material numerous cultures were isolated, amongst which several *Cephalosporium*-like Hyphomycetes. Together with cultures sent to the CBS for identification, 23 tropical species will be described in a supplement to the *Cephalosporium*-monograph. Apart from this paper, a supplement on *Niesslia* and *Monocillium* is in preparation, encompassing 3 tropical *Niesslia* species found to be connected with *Monocillium* conidial states, several undescribed conidial states and a revision of the collections available at the Commonwealth Mycological Institute and the Kew Herbarium.

For the monographic work on *Verticillium* and *Gliocladium* further strains were collected.

For the revision of the genus *Chloridium* further collections were made; living material of the type species *Chloridium viride* Link ex Link was obtained from Dr. M. B. Ellis at the Commonwealth Mycological Institute, Kew; collections available at this institute, Kew Herbarium, Farlow Herbarium and the Biosystematic Research Institute at Ottawa are being revised, before this study can be finished. The work on *Phialophora* species with catenulate conidia was continued and can soon be finished with a description of about 8 species, 6 of which are new. The revision of the genus *Coniochaeta* takes on much larger dimensions than expected, because of the vast numbers of specimens available in various herbaria. The study of the collections in both institutes at Kew has been started. *C. xylariispora* (Cooke & Ellis) Cooke was commonly isolated from soils after steam treatment; first G. J. Bollen isolated several strains from green-house soils in this country and recently J. H. van Emden sent some 20 strains isolated from a soil sample from Surinam; variability of this species could be studied in detail.

An obligately parasitic Hyphomycete with annellated conidiogenous

cells and cubical conidia growing on *Tubercularia vulgaris* has been found for the second time in the Netherlands and grown in culture together with the host fungus. Without the host fungus, conidia germinate on agar media, but produce only very limited yeast-like growth by phialidic conidium formation. Hyphal growth could not be induced by several other species of the Nectriaceae. In slide cultures no connections between hyphae of the host and the parasite could be seen. The fungus will be described in a new genus as *Tympanosporium parasiticum*.

Several *Oidiodendron* strains isolated from Swedish forest soils were received from B. Söderström, Lund. Identification of these strains prompted a comparative study of numerous CBS strains. Optimal growth and sporulation was obtained on acid malt extract agar (pH c. 6). At least one new species which forms chlamydospores will have to be described. This study is supplemented with scanning electron-micrographs of conidial ornamentation.

Prof. K. H. Domsch, Braunschweig, plans a continuation of the compilation work started in the book by Domsch & Gams "Pilze aus Agrarböden" (G. Fischer, Stuttgart, 1970; Engl. translation Longman, London, 1972) in collaboration with W. Gams and Mrs. H. Anderson. The aim is an amplified edition which will contain almost 400 species of common soil fungi and much more documentation. W. Gams continues to compile the taxonomic part and to store all the information available in the "Abstracts of Mycology", which subsequently will be worked out by means of a computer in Braunschweig.

During a joint visit to the Commonwealth Mycological Institute, Kew, the herbarium folders of the less common species were scanned in order to compile the information on species distribution.

Drs. G. S. de Hoog

The work on the revision of the genus *Sporothrix* was continued. *Sporothrix* is characterized by hyaline, elongate conidiogenous cells with apical groups or dense series of distinct conidium-bearing denticles, and by an irregular pattern of branching without differentiated conidiophores. The type species is *S. schenckii*; 41 synonyms are listed, among which 16 obligate. 7 species will be described as new, and 4 new combinations will be proposed. Two of the new species were published separately in a joint article with Dr. G. A. de Vries. About 13 species of *Ophiostoma* have *Sporothrix* conidial states, which were studied in detail. The conidial state of *O. stenoceras* is almost indistinguishable from *Sporothrix schenckii*. The delimitation and distinction of these *Sporothrix* states is often difficult, due to the tremendous variability within the strains. For identification purposes large numbers of conidiogenous cells and conidia often have to be compared. Some species have identical *Sporothrix* states and can only be identified by means of the perfect states. In addition *Graphium*-like

conidial states may be present, which occasionally serve in distinguishing species. On the other hand, perfect states of several *Ophiostoma* species are very difficult to distinguish from each other, especially in pure culture, but their *Sporothrix* states may well be recognizable. Using both perfect and imperfect states for species delimitation a sound taxonomy of *Ophiostoma* can be achieved. The differences between *Ophiostoma* and *Ceratocystis* are mainly based upon the characteristics of their conidial states. The distinction of these genera is being worked out in cooperation with Dr. J. A. von Arx.

The genus *Calcarisporium* is restricted to only one species, characterized by erect, well differentiated conidiophores. A list of doubtful and excluded species is in preparation.

Apart from the above revision, some smaller projects were started. In the herbarium T. Petch (K), the type collection of *Sporotrichum isariae* was encountered. It contained a species of *Tritirachium* not mentioned in the latest study on this genus (G. S. de Hoog, Stud. Mycol. 1: 1-41. 1972). The study of this species made a better delimitation of *Tritirachium* and *Nodulisporium* possible: on the rachids of *Tritirachium* the conidial scars are very difficult to see with the light-microscope, whereas the rachids of *Nodulisporium* are conspicuously cicatrized or have blunt denticles. As a consequence, *N. cinnamomeum* was described again in *Tritirachium*, and *Tritirachium* sp. A (H. C. Evans, Trans. Br. mycol. Soc. 57: 241-254. 1971) was newly described as *Nodulisporium cylindroconium*.

Dr. Gertrud Franz (Bonn, Germany) visited the CBS in 1972 and left some strains isolated from soil in Nepal. Amongst these, a species of *Cordana* could not be identified with any of the known species in this genus. It was described as a new species, *C. ellipsoidea*. Dr. J. Grinbergs (Valdivia, Chile) sent a soil isolate for identification. Morphologically it resembled some strains of *Wardomyces*, which after prolonged cultivation had lost the ability to produce differentiated conidiophores, e.g. the strains of *W. ovalis* maintained in the CBS-collection. However, no germ slits were observed. Consequently the fungus could be classified in the genus *Trichocladium*. It is somewhat reminiscent of *T. pyriforme*, but differs by its small conidia. A manuscript was prepared to describe it as *Trichocladium minimum*.

In cooperation with Dr. J. A. von Arx the genera *Scolecobasidium* and *Pleurophragmium* were studied. The type species of *Scolecobasidium*, *S. terreum*, differs rather considerably from most other species described in the genus. Hence, the name *Scolecobasidium* was restricted to species with Y- or T-shaped conidia, and *S. constrictum* was made the type species of a new genus, *Ochroconis*. Six species, most of them formerly described in *Scolecobasidium*, were transferred to *Ochroconis*. Several *Scolecobasidium* species which did not fit the new genus were classified in *Dactylaria*. The generic limits between *Pleurophragmium* and *Dactylaria* are vague. In pure culture the type species of *Pleurophragmium* is very similar to

the type species of *Dactylaria*, *D. purpurella*. The best solution for this problem was to combine *Pleurophragmium* with *Dactylaria*. A list of species excluded from *Pleurophragmium* is provided; most of them have already been classified in *Spiropes* by Ellis (Mycol. Pap. 114: 1-44. 1968).

Drs. E. J. Hermanides-Nijhof

The study of the genus *Aureobasidium* was continued. Many strains were collected and compared, the majority of them being of the common type of *Aureobasidium pullulans* (de Bary) Arnaud (group A).

Another group of strains could be recognized as the imperfect states of *Dothiora*, *Pringsheimia*, *Guignardia* or *Potebniamyces* (group B). The characters that can be used for identification of the two groups are:

1. Type of conidium-production.

In group A the conidia are generally formed on the tip of the hyphae or on the tip of small side branches, in both cases simultaneously and close together.

In group B conidia arise one after another, scattered laterally over the hyphae, often on small denticles.

Endoconidia are commonly formed in group A; in group B, however, they were never seen.

2. Size and shape of the conidia.

The conidia in group A are generally larger than in group B, in the latter they are more elongated in shape. In both groups, however, there is a great variability.

3. Size and shape of detached dark cells.

In group A these cells are often very big; they are thick-walled and sometimes covered by a gelatinous substance. There has not been any evidence of budding, but it was observed that the cells germinate or burst open (giving rise) to a hyaline cell. The dark cells of group B are smaller than those of group A and they often form conidia.

4. Colour of the colony.

Generally cultures of group B are already black or dark green when they are still young, while young cultures of group A are often white or light brown, sometimes changing to black in older cultures.

5. Shape and structure of vegetative hyphae.

In group A the cells are proportionally longer than in group B. Longitudinal septation in cells is common in group B, but does not occur in group A.

Strains belonging to the recently described *Aureobasidium prunorum* Dennis were studied. This species resembles group B more closely than group A. The groups are still heterogeneous; great differences have been found between several strains of the same group, e.g. in growth-rate and in colour of the culture.

A number of strains deviated very strongly from either group, so that incorporation in one of the two groups did not seem justified. Investigation of possible further differentiation of the groups is going on.

Drs. A. C. Stolk

In cooperation with Dr. G. F. Orr an ascomycetous fungus belonging to the Eurotiaceae, but superficially resembling the Gymnoasaceae, was studied and described as a new genus, for which the name *Sagenoma* is proposed. The single species *S. viride* was isolated from soil from Australia. The ascomata are surrounded by loose networks of dark peridial hyphae, asci are formed in chains, ascospores are 1-celled and the conidial state is of the *Acremonium* type. The ascospores are finely striate longitudinally under the light microscope.

The study of the genus *Eupenicillium* was continued, with special emphasis on ascus development. In cooperation with Mrs J. W. Veenbaas-Rijks a new *Eupenicillium* was described: *E. osmophilum*, isolated from arable soil near Wageningen, the Netherlands. This osmophilic species is characterized by single asci, lenticular ascospores with two closely appressed equatorial ridges and warty convex surfaces (when viewed with S.E.M.). The conidial state is abundantly and most typically produced on hay infusion agar, malt agar with 40% sucrose and Czapek agar with 70% sucrose. The penicilli are divaricate, the phialides ending in long tubes. Dr. Udagawa sent several strains of *Eupenicillium* isolated from New Guinean soil for examination. Some of them could be identified with described species; two new species will be described by Dr. Udagawa. Among the other strains probably some more undescribed species are present.

Drs. R. A. Samson

The study on the entomogenous fungus flora in Ghana was continued in cooperation with Dr. H. C. Evans (Tafo). *Gibellula pulchra* (Sacc.) Cavara, *G. leiopus* (Vuill.) Mains and *G. alata* Petch were regularly collected on spiders. *Gibellula formicarum* Mains appeared to be common on a wide range of insects. This species differs from the other typical *Gibellula* species with phialides by the production of conidia borne singly on sympodial conidiogenous cells. Moreover, it grows and sporulates abundantly on agar media, whereas the other species do not sporulate in pure culture. For these reasons *G. formicarum* has been transferred to the new genus *Pseudogibellula* (Acta bot. neerl. 22: 524. 1973). Four species belonging to the stilbaceous genus *Akanthomyces* were collected. *Akanthomyces araneorum* (Petch) Mains was found to be rather common on spiders. *Isaria pistillariaeformis* Pat. and *Trichosterigma arachnophilum* Petch were transferred to *Akanthomyces*, because the conidiogenous cells arranged in a hymenium-like layer along the synnema are phialidic and produce catenate conidia. One species which occurs on a wide variety of

insect hosts and particularly on ants, differs sufficiently from the other *Akanthomyces* species, to be described as a new taxon, *A. gracilis* Samson & Evans. The studies on *Hymenostilbe*, *Hirsutella* and unidentified genera are in progress.

Amongst the numerous specimens collected by Dr. H. C. Evans several species belonging to the genus *Paecilomyces* were encountered. Most of them could be isolated in pure culture. Four species can be regarded as new. Pure culture studies of *Isaria amoene-rosea* P. Henn. and *Isaria tenuipes* Peck brought further evidence that these species belong to *Paecilomyces*. The descriptions of these species were added to the monograph of the genus *Paecilomyces*. During a visit at the Commonwealth Mycological Institute and the Royal Botanic Gardens in Kew (England) several type specimens of *Spicaria* and *Isaria* were examined. The study on *Paecilomyces* and its relatives was finished and much time was spent on the preparation of the manuscript.

A stilbaceous fungus isolated by Dr. V. Hintikka (Helsinki) from pellets of small rodents in Northern Finland was studied and compared with *Mycosylva clarkii* Tulloch. The Finnish isolate differed from the type culture of *M. clarkii* mainly by reticulate conidia, by the presence of ramoconidia and by the olive green colour of the synnematal heads. It is therefore described as a new species, *M. reticulata* Samson & Hintikka. Both species appeared to be psychrophilic showing optimal growth at 15° C.

In cooperation with Mr J. Mouchacca (Paris) the study on some osmophilic and thermophilic fungi from desert soil was continued. Two strains of *Aspergillus* differed from all known taxa. Furthermore an interesting cleistothecial ascomycete with optimal growth at 35° C was isolated. After comparison with other cleistothecial genera it is regarded as a new species of *Xylogone*.

Jointly with Mr L. P. Kish (Gainesville, USA) a manuscript on the genus *Nomuraea* was prepared. The genus *Nomuraea* is retained for a fungus originally described as *Botrytis rileyi* Farlow (syn. *Spicaria rileyi* (Farlow) Charles). The species is entomogenous and has conidiophores which form dense whorls of phialides and branches below each septum. The phialides have no neck and produce conidia in divergent chains. Another species, *Isaria atypicola* Yasuda, with similar conidiogenous structures belongs to this genus.

During a 3-months stay at the Università Cattolica del Sacro Cuore in Rome (Italy), the fungus flora on Italian cheese and salami sausages was studied. Furthermore fungi were isolated from soil with various techniques and screened on their antibiotic or antiviral properties.

Dr. M. A. A. Schipper

The methods used in the study on *Mucor hiemalis* and related species (Studies in Mycology 4, 1973) were also applied to strains close to the species *Mucor flavus* Bainier. This selection comprises strains described

or identified as: *M. flavus* Bainier, *M. sciurinus* Naumov, *M. attenuatus* Linnemann, *M. peacockensis* Mehrotra & Nand, *M. mephitis* Ellis & Hesselteine, *M. meridionalis* Milko & Kormilizina and *M. piriformis* Fischer sensu Pidoplichko & Milko.

Common characters of the strains studied are: Colonies yellowish-greyish; sporangiophores up to 35 μm in diam., with a constriction next to the sporangium, at first unbranched, then branched sympodially in the upper part of the main axis; sporangia whitish when young, changing to bluish hyaline or yellowish-brownish hyaline while moist in appearance, then turning to a yellowish sand colour (mat glittering, dry), up to 175 μm diam., with echinulate deliquescent walls; columellae ovoid-ellipsoidal to slightly pyriform (sporangiospores are often found sticking to the wall in water mounts); sporangiospores ellipsoidal, with granular contents; zygosporos borne in the upper portion of the aerial mycelium, globose or slightly compressed between the suspensors, roughened by short projections, glossy black.

Variation is found in odour of the colony, time required for the formation of side branches on the sporangiophores, occurrence of fully grown sporangia on short sporangiophores and, particularly, the maximum size of the sporangiospores.

The strain CBS 234.35, *M. flavus* (Linnemann, 1936) will be proposed as neotype. The type strains of *M. meridionalis* (CBS 197.71) and *M. piriformis* sensu Pidopl. & Milko (CBS 378.66) differ from the other strains in their ability to produce sporangiospores of two sizes: large ones in sporangia in the under portion of the colony, smaller ones in sporangia on tall sporangiophores.

Mating experiments revealed that:

- a. The mating reaction signs of CBS 234.35 (+) and CBS 235.35 (—) (Linnemann's strains received in 1935) had to be reversed. These mating-partners were received at the CBS with an annotation that the mating signs were arbitrary. After responsive contrast of CBS 234.35, *M. flavus*, and Blakeslee's tester strain IV (+) (and other combinations) the mating reaction types were determined:
CBS 234.35 is a (—) strain, CBS 235.35 is a (+) strain
- b. Abundant zygosporos were obtained in matings of
CBS 127.70, *M. mephitis* (+) \times CBS 234.35, *M. flavus* (—)
CBS 127.70, *M. mephitis* (+) \times CBS 210.71, *M. peacockensis* (—)
CBS 127.70, *M. mephitis* (+) \times CBS 197.71, *M. meridionalis* (—)
CBS 235.35, *M. flavus* (+) \times CBS 197.71, *M. meridionalis* (—)

A few zygosporos were yielded in the mating CBS 378.66, *M. piriformis* sensu Pidopl. & Milko \times CBS 127.70, *M. mephitis*.

No mating reactions were observed in combination with strains CBS 230.35, *M. attenuatus* (type) and CBS 893.73, *M. sciurinus* (neotype),

though other strains, quite similar in morphology to these, did produce zygospores in matings within the group.

As a result of the comparative morphological examination and the outcome of contrasts, synonymy of the afore-mentioned species was concluded.

Drs. A. J. van der Plaats-Niterink

The investigations on the occurrence of *Pythium* species in soils in the Netherlands were finished. A paper on the results is in preparation.

In the study of Oomycetes, water cultures play an important role. Tap water and distilled water are often not suitable for a good production of zoospores. Most useful is water of a pond or river, diluted with one or two parts of distilled water. Also polluted water cannot be used. Suitable water was found in the summer of 1972 in a pond water near the castle Broekhuizen near Leersum. However, in the spring of 1973, after many ducks had visited the pond during winter, sporulation could not be induced in Oomycetes cultivated in this water nor could Oomycetes be isolated from it. In June 1973 the results were much better. The water could again be used for water cultures. The pH was 5.9. Strains of *Saprolegnia diclina* Humphrey, *S. parasitica* Coker, *Pythium angustatum* Sparrow and at least 3 different types of *Pythium* species which produce only filamentous, non-swollen sporangia were isolated.

The water of the Hijker Meertje near Hijken (Dr.) was also rich in Oomycetes. The pH of the water on 30 September 1973 was 5.4. Strains of *Saprolegnia diclina* Humphrey, *S. ferax* (Gruithuizen) Thuret, *Aphanomyces irregularis* Scott and four types of *Pythium* species which produce only filamentous, non-swollen sporangia, were isolated.

Aphanomyces irregularis has not been recorded for the Netherlands before. The fungus forms filamentous zoosporangia, which are not differentiated from the vegetative hyphae. The zoospores are produced in a single row and discharged through a common mouth, they collect at the top of the sporangium. The oogonia are terminal on short side branches, spherical, mostly 16–23 μm in diam., their wall is irregularly roughened but does not form definite spines; oospores measure mostly 15–19 μm with a 1–2 μm thick wall, antheridia are usually absent or there is sometimes a single small clavate antheridium.

Species of *Pythium* with filamentous, non-swollen sporangia form a conspicuous part (83.5%) of the *Pythium* isolations from water. Most of the *Pythium* isolations from soil have spherical sporangia or form only hyphal swellings (85–90%). Soil which is in contact with water (river banks; soil in Zuidelijk Flevoland, shortly after this polder became dry) also have a high percentage of *Pythium* species with filamentous non-swollen sporangia. In the soil of Zuidelijk Flevoland the percentage of these *Pythium* species declined from 89 in 1968 to 9 in 1972.

A number of soil samples originating from Czechoslovakia, Corsica, Sardinia and Ibiza (Spain) were investigated for the occurrence of *Pythium* species. Interesting was the high percentage of *P. heterothallicum* Campbell & Hendrix in Corsica (56% of all isolates), Sardinia (40%) and Czechoslovakia (21%). In soils in the Netherlands *P. heterothallicum* is rather rare (1% of all isolates), but *P. sylvaticum* Campbell & Hendrix is very common (19%). In the Czechoslovakian soil samples *P. sylvaticum* was less common but still present at a percentage of 9.5. In Corsica this percentage was only 2.5, in Sardinia 1. None of these species was isolated from the soil samples from Ibiza. *P. intermedium* de Bary was not isolated from the three mediterranean islands, but made up 4% of the isolations from the Netherlands and 2% of the isolations from Czechoslovakia.

The influence of temperature on oogonium formation in dual cultures of these three heterothallic species was different. *P. intermedium* formed oogonia only at temperatures of about 20° C, *P. heterothallicum* produced more oogonia at 25° C than at 20° C, for *P. sylvaticum* no influence was noticed at temperatures between 20 and 25° C.

Drs. J. A. Stalpers

The study on wood-attacking Aphyllophorales in pure culture was continued. The spectrum of species taken in culture has been enlarged. Several groups of the Corticiaceae can not or hardly be cultured on artificial media, viz. Botryobasidioideae, Botryohypochnoideae, *Tubulicrinis*, *Cristella* and several species of *Phlebia* sensu lato. Athelioideae (*Athelia*, *Byssomerulius*, *Phanerochaete*), Hyphodermoideae (*Basidioradulum*, *Hyphoderma*, *Hyphodontia*, *Hypochnicium*, *Peniophora*) and to a lesser extent Phlebioideae (*Merulius*, *Mycoacia*, *Phlebia* sensu stricto), Gloeocystidielloideae, Ceratobasidioideae and Aleurodiscoideae usually grow in pure culture. Coniophoraceae (*Coniophora*, *Jaapia*, *Serpula*) and Stereaceae can as a rule easily be cultured, whereas Thelephoraceae (*Thelephora*, *Tomentella*) give constantly negative results.

Much time was spent on the preparation of keys to fruitbodies of the non-poroid resupinate Aphyllophorales, comprising the European species and also some well-known extra-European Corticiaceae. Up to now the keys cover 880 species, divided over 115 genera. In co-operation with Dr. W. Jülich (Rijksherbarium, Leiden) the project will be expanded to cover all published European and North-American species.

The study on the genus *Oedocephalum* is finished and a manuscript is in preparation. *Oedocephalum* is confined to species with ascomycetous affinity (8), while a new genus, *Spiniger*, is erected for those with a basidiomycetous nature. The differences can be summarized as follows: In *Oedocephalum* the conidiophore passes abruptly into the warty or minutely denticulate vesicle, which is usually separated by a septum; the conidia are white, pinkish or fawn coloured in mass, warty, sometimes

apparently smooth, but under the scanning electron microscope always roughened; growth on malt agar is very rapid to moderately rapid, on malt agar pH 4 no or very little growth. In *Spiniger* the conidiogenous part widens gradually towards the tip, is covered with tapering or cylindrical denticles and never separated by a septum from the non-conidiogenous part; the conidia are always white in mass and smooth, even under the S.E.M.; growth on malt agar (normal or pH 4) rapid to very slow.

The hitherto unidentified Basidiomycete QM 806 is of interest because of the abundant production of exo- β -D-1,3 glucanase (Reese, E. T. & Mandels, M. – Can. J. Microbiol. 5: 173–185. 1959), which causes almost complete solubilization of cell walls of yeasts (Bauer, H., Bush, D. A. & Horisberger, M. – Experientia 28: 11–13. 1972; Kanetsuna, F. & Carbonell, L. M. – J. Bact. 106: 946–948. 1971). It was identified as *Sporotrichum dimorphosporum* v. Arx. A comparative study was made of *Sporotrichum pulverulentum* Novobranova and *Chrysosporium lignorum* Nilsson (nomen nudum) and they turned out to be conspecific. The species is a potential source of food protein (von Hofsten, Uppsala, pers. comm.).

2. Division of Biochemistry and Physiology

Dr. G. W. van Eijk

The production of several acetates by acetylation of the red pigment of *Arthrinium phaeospermum* (CBS 142.55, type culture of *Botryconis sanguinea*) was reported in 1972 (CBS progress report). The main product obtained in a pure state was characterized as an anthraquinone triacetate by means of mass spectroscopy and spectrophotometric analysis. This substance with molecular formula $C_{22}H_{18}O_9$ showed physico-chemical properties similar to the so-called compound VII described by Noda et al. (Tetrahedron 26: 1346. 1970). The identity was confirmed by comparison of the IR spectrum of our product with the IR spectrum of compound VII, kindly provided by Dr. Noda. Compound VII is one of the products obtained by Noda et al. in their study on the structure of bostrycin, a tetrahydro-anthraquinone pigment produced by the fungus *Bostrychonema alpestre*. Therefore it was thought that the *Arthrinium* pigment might be identical with bostrycin. This was established by direct comparison with authentic bostrycin, also a gift of Dr. Noda. The *Arthrinium* pigment showed complete agreement in all respects. The strain labelled as *B. alpestre*, provided by Dr. Takada did not produce conidia. The original specimen, also sent by Dr. Takada appeared too scanty for recognition. The systematic position of the genus *Bostrychonema* is still problematic. Since no type material is available and the original description is too vague, the genus should be regarded as doubtful. Thus it was not possible to compare the relationship of *A. phaeospermum* with *B. alpestre*.

During the isolation procedures of catenarin and helminthosporin

needed for reference purposes from *Drechslera catenaria*, CBS 191.29, two minor pigments were also detected on thin layer plates. The compounds were isolated and identified as chrysophanol and emodin respectively. A manuscript on this investigation has been submitted for publication.

A particular strain of *Penicillium citrinum*, CBS 222.73, yielded large crystals in agar media. They were picked up and analyzed spectrophotometrically. This led to the identification of the compound as curvularin. Besides this substance, two other metabolites were isolated from liquid cultures. One compound was identified as dehydrocurvularin. The structure of the other substance, also a phenol derivative with molecular formula $C_{22}H_{26}O_9$, is still under investigation.

An unidentified fungus isolated from air in Munich by E. Stix cultivated on malt agar produced yellow crystals in the medium. The pigment was extracted from the medium and purified. The spectrophotometric characteristics were identical with those reported for mollisin, a chlorine containing naphthoquinone derivative isolated from *Mollisia caesia*.

Investigations are going on concerning the carotenoid pigments and terpenoids produced by an entomogenous hyphomycete. Several carotenoids are produced. The main pigment is β -carotene; one of the minor carotenoids was identified tentatively as torulene. The nature of the other pigments is under investigation. The presence of ergosterol in the mycelium was proven by ultraviolet spectroscopy and gas liquid chromatography. So far two terpenoids were isolated in a pure state. The molecular formulae $C_{30}H_{52}O_2$ and $C_{30}H_{52}O_3$ were shown by mass spectrometry. The infrared spectra contained strong hydroxyl absorptions and no carbonyl absorptions. By acetylation with acetic anhydride and pyridine the diol terpene gave a mono-acetate and the triol terpene a di-acetate. These results combined with data obtained from mass spectroscopy and nuclear magnetic resonance indicate that both triterpenoids belong to the class of hopane derivatives. These assignments will be studied further.

Drs. A. C. M. Weijman

A study was started on the chemistry of cell-wall constituents of yeast-like fungi, a group regarded as heterogeneous and of polyphyletic origin (von Arx, Antonie van Leeuwenhoek 38: 289. 1972).

Cell-wall chemical data are regarded as a useful aid for the understanding of natural relationships between taxonomic groups of fungi (Bartnicki-Garcia, A., Rev. Microbiol. 22: 87. 1968). According to the scheme given by this author, most filamentous Ascomycetes, Hyphomycetes and Basidiomycetes belong to the chitin-glucan group, while true yeasts (Saccharomycetaceae) constitute the mannan-glucan group. The Sporobolomycetaceae and Rhodotorulaceae contain chitin and mannan in their cell walls. A detailed analysis of cell-wall components may allow further subdivision.

The program was started with a group of 21 yeasts and yeast-like

Ascomycetes. Spore or hyphal suspensions were grown in a liquid medium of 2% glucose, 1% peptone and 0.5% yeast extract with continuous shaking at 25° C. Cultures were harvested 10 days after inoculation by centrifugation or filtration and washed twice with 0.9% NaCl, followed by 3 washings with deionized water.

Various techniques were applied for cell disintegration. Filamentous fungi can be easily disintegrated by the X-press method (Edebo, J. biochem. microbiol. Technol. Engng 2: 452. 1960), e.g. *Ascoidea rubescens*, whereas fungi with a more yeast-like appearance, e.g. *Pichia membranaefaciens*, need glass beads for disintegration (Braun MSK cell homogenizer). After disintegration, the residue was washed repeatedly with 10% sucrose, then with 0.9% NaCl and finally with deionized water. The washings were effected by ultrasonic treatment. After the washing procedure, the purified cell-wall fractions were lyophilized, ground and stored as a powder in an evacuated dessicator.

Infrared spectroscopy of purified cell-walls, intact cells and commercially available polysaccharides is in progress in order to determine the occurrence of characteristic peaks in the spectra. Gas-liquid chromatography, however, seems to be the most promising technique in the qualitative and quantitative analysis of cell-wall hydrolyzates, as well as for the determination of the products resulting from methylation analysis, a method used for the determination of the cell-wall polysaccharide linkage composition.

3. Division of Human and Animal Mycology

Dr. G. A. de Vries

A paper on "A case of Lobo's disease in the dolphin *Sotalia guianensis*" by G. A. de Vries & J. J. Laarman was published in Aquatic Mammals 1, 3: 1-8. 1973. In the dolphinarium at Harderwijk a biopsy was taken from the skin of a dolphin (*Tursiops truncatus*) suspected of a dermatomycosis. With the microscope no fungus elements were seen. Dr. S. Hechmann Andersen promised to send material from Odense in Denmark where mycotic skin disease in dolphins seems to be fairly common.

In another project, type material was examined of the pathogen *Monosporium apiospermum*, the conidial state of *Petriellidium boydii*. All efforts to obtain type material of the pathogens *Glenospora graphii* and *Graphium eumorphum* were unsuccessful. A pure culture of a fungus isolated from a case of otitis externa in a human patient at Prague and identified as *Graphium eumorphum* by P. Fragner & J. Hejzlar was received from the authors for comparative cultural study. This strain is compared with 4 marine strains of *Petriellidium boydii* received from Dr. P. W. Kirk Jr. at Blacksburg (Va., USA) and with the already available strains of *Monosporium apiospermum* and *Glenospora graphii*.

A blue pigment-producing, probably saprophytic fungus which was isolated from skin lesions in human patients was described as *Sporothrix cyanescens* nov. spec. in a paper entitled "Two new species of *Sporothrix* and their relation to *Blastobotrys nivea*" by G. S. de Hoog & G. A. de Vries (Antonie van Leeuwenhoek 39: 515-520. 1973).

A culture of the newly described *Trichophyton candelabreum* was received from the author H. Listemann. In cultural experiments this species did not appear to be different from *Trichophyton rubrum* var. *nigricans*.

In June, August and October, 88 soil samples were collected in S. Flevoland, E. Flevoland as well as in the N.E. Polder on and around the former island of Schokland. With the hair-bait technique three keratinophilic fungi were isolated: *Trichophyton terrestre* from an onion field in the N.E. Polder, *Arthroderma* species from E. Flevoland and *Arthroderma uncinatum* (st. con. *Trichophyton ajelloi*) from loose soil in a *Picea abies* plantation at the bottom of the dike of Schokland. The samples collected in S. Flevoland have not yet produced any keratinophilic fungus in culture.

Thermophilic and thermotolerant fungi and actinomycetes were common in all soil samples. *Aspergillus fumigatus* was the most abundant thermotolerant fungus just as in 1972. *Thermoactinomyces vulgaris* which is regarded as one of the principal etiological agents of farmer's lung disease was the commonest thermophilic actinomycete.

All the actinomycete strains tested for antimycotic activity against *Trichophyton rubrum* and *T. mentagrophytes* appeared to be inactive.

4. Division of Yeasts (Laboratory of Microbiology, Delft)

Head: Prof. Dr. T. O. Wikén

Drs. L. Rodrigues de Miranda

Some yeast strains of different origin isolated from the phyllosphere of cereals were studied. There appeared to be a number of strains of undescribed species among them. The description of two species isolated from the leaves of barley has been prepared in cooperation with Dr. Hoang G. Diem of the Laboratory of Botany and Microbiology, University of Nancy, France, and submitted for publication to the Canadian Journal of Botany. A third still undescribed species was isolated from the leaves of rye by Dr. N. J. Fokkema of the Phytopathological Laboratory Willie Commelin Scholten, Baarn. One of the two species isolated from barley and the species isolated from rye leaves belong to the genus *Candida* and are closely related. Both species assimilate nitrate and inositol, and do not ferment. A comparison of the nitrate positive and inositol assimilating species, together with the description of the new species isolated from rye, will be published in Antonie van Leeuwenhoek.

The species described by Goto et al. as *Sporobolomyces antarcticus*

appeared not to form ballistospores but fragile aerial pseudomycelium. Detached hyphal pieces form new colonies, by which means a mirror image of the colonies on the plate can be obtained in the inverted plate. *Sporobolomyces antarcticus* also assimilates nitrate and inositol. On the basis of the cell structure, the absence of true spores and the formation of pseudomycelium, this species has to be placed in the genus *Candida* and appears to be identical with *Candida edax*. Small differences in the assimilation pattern disappear on repeating the assimilation tests.

Two yeast strains isolated from a gazelle and described as *Candida nouvelii* Saëz were received from Dr. H. Saëz of the Paris Zoo. This species appears to be identical with *Candida albicans*. Also from Prof. Saëz, two strains were received of a red species isolated from deer (*Axis axis*) and described as *Cryptococcus hungaricus* var. *gallicus*. This species appears to be identical with *Cryptococcus macerans*. Mating types of *Cryptococcus laurentii* were received from Dr. Kurtzman of Peoria. His mating experiments were repeated and 11 of the strains of *Cryptococcus laurentii* present in the CBS collection were tested. Only two strains nr. 2208 and 4919 formed mycelium after mating with CBS 6476 mating type a, the other 9 strains mated with CBS 6473 mating type α . A large number of yeast strains were received from Denmark of a species which showed a close relationship with *Cryptococcus laurentii* but which differed so greatly morphologically that, according to the definition of the genus, this species would not belong to *Cryptococcus*. Most strains produce pseudomycelium abundantly and even true mycelium after a long time. The strains nr. 5451 and 5489, both in the collection as *Cryptococcus laurentii*, show the same morphology. It was intended to describe these 2 strains and the 23 from Denmark as a new *Candida* species. Mating experiments showed, however, that the strains 5451 and 5489 mate with the strain 6473 and the typical binucleate mycelium is formed which completely differs in structure from the mycelium formed by the two individual strains. The strains from Denmark still have to be tested. Attempts were made to find mating types among the strains of *Cryptococcus albidus* var. *albidus*, *Cryptococcus terreus*, *Cryptococcus uniguttulatus*, *Cryptococcus hungaricus* and *Cryptococcus macerans* in the collection. These experiments have not yet had any positive results.

Drs. M. Th. Smith

A taxonomic study of the genera *Kloeckera* and *Hanseniaspora* was started. All strains in the CBS culture collection were examined: 20 strains of *Kloeckera* and 40 strains of *Hanseniaspora*, including three not yet identified strains.

Two strains are identical and do not correspond with any of the three *Hanseniaspora* species recognized by Miller & Phaff (Mycopath. Mycol. appl. 10: 113. 1958). They will be described as a new species. The third

strain (CBS 2827), assimilating saccharose, could not be identified with any of the *Hanseniaspora* species either. As it has the same physiological properties as the asporogenous form *Kloeckera africana* and as it forms ascospores, CBS 2827 could be considered as the perfect state of *K. africana*, which has not yet been found. Phaff & Miller use the assimilation of saccharose as a characteristic to separate *K. africana* and *K. corticis*, whose perfect form is *Hanseniaspora osmophila*. Since Nakase & Komagata (J. gen. appl. Microbiol. 16: 241. 1970) found the GC content of *K. africana* and *K. corticis* to be the same and the assimilation of saccharose to be variable, *K. africana*, *K. corticis* and *Hanseniaspora osmophila* have to be studied further to see whether CBS 2827 can be identified with *Hanseniaspora osmophila* or whether it is a new *Hanseniaspora* species.

Checking the 14 *Hanseniaspora valbyensis* strains, it was found that they could be divided into two groups. One group not growing at 37° C forms 1–2 spores per ascus, the second group growing at 37° C forms 1–4 spores per ascus. This was suggested already by Kreger-van Rij & Ahearn (Mycologia 60: 104. 1968) and is supported by Nakase & Komagata (l.c.), who found a difference in GC content between both groups. A closer study of these groups will be made for revising *Hanseniaspora valbyensis*.

Strain CBS 5934 (=NRRL Y-915) listed as *H. valbyensis* was found to form 1–2 round spores per ascus. *Hanseniaspora valbyensis*, however, is characterized by forming hat- or helmet-shaped spores. Strain CBS 5934 has to be reidentified with *Hanseniaspora uvarum*, which forms 1–2 spherical spores.

D. Yarrow

In view of the many species of *Rhodosporeidium* that have been described recently it became necessary to examine the collection strains of *Rhodotorula* to see whether any of them are haploid heterothallic strains of that genus. All the strains of *Rhodotorula glutinis* were mixed with the mating types of *Rhodosporeidium diobovatum*, *R. sphaerocarpum*, and *R. toruloides*. CBS 315 (*Torula rubescens* Saito = *Mycotorula rubescens* (Saito) Ciferri & Redaelli = *Rhodotorula rubescens* (Saito) Harrison = *Rhodotorula glutinis* var. *rubescens* (Saito) Lodder), CBS 350 (*Rhodotorula gracilis* strain Äggfors), and CBS 2370 are *Rhodosporeidium toruloides* mating type α . CBS 994 (*Torulopsis terrestris* Verona nom. nud.) is mating type α and CBS 5540 is mating type α of *Rhodosporeidium diobovatum*.

Of the strains that failed to mate with the above-mentioned *Rhodosporeidium* species, 3 were found to be misidentified. One was reidentified as *Rhodotorula graminis* (CBS 4477) and two as *Rhodosporeidium infirmo-miniatum* (CBS 5014, mating type A2; CBS 5015, mating type A3).

None of the strains of *Rhodotorula pallida*, *R. marina* and the varieties of *R. minuta* mated with the types A1B1 and A2B2 of *Rhodosporeidium dacryoideum*.

All strains of *Cryptococcus infirmo-miniatum* were transferred to *Rhodosporeidium infirmo-miniatum*; one (CBS 2427) is mating type A1; three (CBS 2204, 2205, 6136) are mating type A2. Another strain (CBS 2207) is unusual in that it mated to produce hyphae and teliospores with the A1 mating type but not with either the A2 or A3 type. None of the strains of *Rhodotorula macerans* mated with *Rhodosporeidium infirmo-miniatum*.

All strains present in the collection as unidentified *Rhodotorula* species were also examined. One strain of unknown origin (CBS 6230) was identified as *Rhodosporeidium capitatum*. Another strain (CBS 5143) was identified as *Rhodotorula araucariae*. This strain differed from the type in the assimilation of the β -glucosides, L-arabinose and dulcitol. However, as the assimilation of these sugars is described as variable for many species of this group of yeasts, there seems to be no objection to considering them variable in this species also.

A culture of *Kazachstania viticola* Zubkova (Bot. Mater. Gerb. Inst. Bot. Akad. Nauk kazakh. SSR 7: 53-56. 1971) was obtained via the BKM collection in Moscow. This strain (BKM Y-1659 = CBS 6463) is identical with *Saccharomyces dairensis*.

J. A. VON ARX

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Plate I - Heteren: the youngest sprout of the Institute



Plate II - and its study area.

INSTITUTE OF ECOLOGICAL RESEARCH

PROGRESS REPORT 1973

1. History and function of the institute

The institute was founded in 1954 by the Division of Sciences of the Netherlands Academy of Arts and Sciences on the initiative of the Committee on Ecology. This proposal was based on the lag in some aspects of ecological research in The Netherlands noted by the Committee.

The function of the institute is to perform and to encourage terrestrial ecological research in a broad sense and to co-operate with other organizations engaged in such research.

At the start, several already existing institutes of scientific research were incorporated, viz. the Bird Migration Station, the Bird-Ringing Department of the National Museum of Natural History at Leiden, and the Weevers' Duin Biological Field Station of the Foundation for Scientific Dune Research. The population research on the Great Tit, which had been carried out until then by the Phytopathological Service at Wageningen, was also taken over. The distributional research on organisms in the reclaimed IJsselmeer polders was started after the foundation of the institute.

The headquarters of the institute are at Arnhem; the Department of Botanical Ecology has its seat at Oostvoorne. Recently a new seat for experimental research was founded at Heteren near Arnhem.

The institute is supervised by a committee appointed by the Division of Sciences of the Academy.

The address of the institute is: Kemperbergerweg 11, Arnhem, Holland.

2. Scientific Staff

J. W. Woldendorp (Director)

Population ecology

J. H. van Balen (Head, Ecology)

J. A. L. Mertens (Physiology)

H. N. Kluyver (Guest worker)

Christina W. Eshuis-Van der Voet (Guest worker)

Bird migration

A. C. Perdeck (Head, Bird Migration)

A. J. Cavé (Orientation)

Distributional ecology

J. H. Mook (Head, Zoology)

J. Haeck (Zoology)

R. Hengeveld (Zoology)

J. van der Toorn (Botany)

Ph. Stoutjesdijk (Micrometeorology)

Botanical ecology Weevers' Duin, Oostvoorne

P. J. M. van der Aart (Head, Zoology)

A. H. J. Freijsen (Experimental Ecology)

C. P. W. M. Blom (Experimental Ecology)
P. A. I. Oremus (Microbiological Ecology)
C. van Dijk (Microbiological Ecology)
D. van der Laan (Synecology)
J. H. Wessels (Soil analysis)
M. J. Adriani (Guest worker)

3. Population Ecology

3.1. POPULATION DYNAMICS OF THE GREAT TIT, *Parus major*

3.1.1. *Mortality, dispersal, and moult in 1972/1973* (J. H. van Balen)

The project was continued along the lines described in earlier Progress Reports.

In the spring of 1973, when due to severe storm damage the study area had to be modified, a five-year period was terminated in which the numerical fluctuations of different categories of Great Tits were investigated in detail. The results of this study will be analyzed in the near future.

After the 1973 breeding season the routine field work was resumed. The investigation of numerical fluctuations is to be combined with a study (starting in 1975) on behavioural interactions between Great Tits.

3.1.2. *Factors affecting clutch-size* (J. H. van Balen, L. A. Kajim)

The experiments on the relationship between the size of the nesting hole and the size of the clutch (discussed in previous Progress Reports) were continued. An attempt was made to determine the properties of the nesting hole with an influence on the clutch-size. This was done by offering the tits holes of normal diameter but with reduced depth. The results were very similar to those obtained in 1971-1972, when the experimental nestboxes were of normal depth and smaller diameter. The volumes of both types of experimental boxes were similar, *ca.* 800 cc.

The clutches in both types of experimental box were of equal size, both being considerably smaller than in normal boxes. This indicates that the volume of the nesting hole plays a role in determining the clutch-size. This mechanism may be ultimately related to the thermoregulation of the brood, in particular to the risk of hyperthermia. Experiments on the survival of large and small broods in small nesting holes are planned for next year.

The experiments (see previous Progress Reports) on the possibilities of modifying clutch-size by the removal and addition of eggs were continued. The results of these experiments indicate that egg production can be influenced to some extent provided the nest does not contain more than two eggs.

3.1.3. *Experiments on the regulation of numbers in the Vlieland population*
(J. H. van Balen, H. M. van Eck)

The experiments on the effect of removing part of the breeding population were continued. As in the three previous years, a number of parent tits were removed in June during the second breeding period. The effect of this interference on the annual survival percentage of the remaining adults and the juveniles was assessed.

Last year's findings confirm earlier results indicating that the removal of part of the breeding population increases the survival of both adults and juveniles. It seems that the survival of the adults is mostly increased (due to the experimental conditions) in the period between November and May, and in the juveniles the survival between fledging and November is also favourably affected.

3.1.4. *The behaviour of Great Tits toward the parasite Protocalliphora azurea* (Fall.) (H. N. Kluyver)

As a continuation of previous studies on the importance of parasitism by *Protocalliphora azurea*, the behaviour of the parent tits toward larvae and pupae of the parasite was studied.

Examination of nests for the presence of parasites often shows only 1-4 pupae per nest, which points to a considerable mortality among the larvae, since the fly lays its eggs in packets of 5-15 at a time. Part of this mortality is probably due to the parent tits, which search for larvae in the nest material. After feeding the nestlings, the parents often dig in the nest material, and the female also does this during the night. This digging or shaking, which causes all sorts of small objects to sink to the bottom of the nestbox, possibly helps to remove *Protocalliphora* larvae, but this has never been observed. When larvae were offered to the parents in the nestbox, the parents reacted in different ways: some of the larvae were eaten by the parents and some were fed to the nestlings, but the great majority were actively removed from the nestbox. Pupae of *Protocalliphora* as well as larvae and pupae of *Calliphora* and other insects were all eaten by the parents or fed to the young.

When *Protocalliphora* larvae were fed to the nestlings by the observer, they were accepted but most of them reappeared in the nestling's bill within a short time. The impression was gained that the larvae were not vomited, but left the oesophagus actively. A small number of larvae remained in the nestling's stomach, however. Further observations on this behaviour are planned.

3.1.5. *The effect of parasitism by Protocalliphora* (C. W. Eshuis-van der Voet, R. H. de Reede)

The research on the influence of the larvae of *Protocalliphora* on the haemoglobin level in the blood of nestling Great Tits was continued (see 1972 Progress Report).

The data collected so far show a regular decrease of the Hb level at increasing degrees of parasitism (Table 1).

Table 1. Mean Hb level (in g/100 ml) in the blood of nestling Great Tits at different ages and different levels of parasitism by *Protocalliphora*.

Total weight of larvae per nestling (in mg)	Age of nestling		
	9 days	12 days	15 days
0	9.28 (20/64) ¹	10.32 (29/129)	11.70 (34/139)
0- 200	7.42 (5/16)	10.55 (6/16)	11.47 (13/53)
200- 400	6.84 (4/13)	8.81 (6/20)	10.17 (1/7)
400- 600	6.32 (6/2)	6.50 (1/4)	9.47 (1/4)
600- 800	5.92 (1/2)	8.23 (1/4)	—
800-1,000	—	—	8.02 (2/6)

¹ Values between parentheses: number of nests sampled/number of birds in these nests from which blood samples were taken.

The variability of the decrease at 12 days is due to the fact that the Hb level is related not only to the degree of parasitism and the age of the nestlings but also to body weight. In this age class we had an irregular distribution of body weight in some classes of parasitism. A more detailed analysis of the data is being carried out.

In 1973 the breeding season of the Great Tit started rather late, viz. in the beginning of May. Although *Protocalliphora* infections were not proportionally delayed, we had a good opportunity to study the influence of parasitism on relatively young broods.

Table 2 shows the laying date of the first egg, the first hatching date, and the date of the first *Protocalliphora* infection for the four years during which the data were collected.

Table 2. Dates of laying of the first egg, the first hatching, and the first *Protocalliphora* infection, for the Great Tit in four years. (Dates counted from: 1st April = 1).

1	2	3	4	5	6	7	8
Laying date of first egg	First hatching date	Period of laying + incubation in days (= 2-1)	Date of first <i>Protocalliphora</i> infection	Difference between 4 and 1	Difference between 4 and 2	Year	Locality
6	31	25	39	33	8	1972	Bischofszell
13	35	22	44	31	9	1969	Renkum
18	40	22	45	27	5	1970	Renkum
32	54	22	56	24	2	1973	Bischofszell

Our previous finding that in years when the breeding season starts later the nestlings are infected at a younger age (1971 Progress Report) was confirmed, but the dependence of the oviposition time of *Protocalliphora* on the photoperiod seems to be less direct than was assumed.

In 1973 the *Protocalliphora* infections occurred so early that larvae had completed their development when the tits were 9–12 days old. Consequently, it could be seen, as in previous years, that *Protocalliphora* causes anaemia in the Great Tit, but also that the nestlings can recover quite rapidly even after heavy infections. In most cases the nestlings showed a normal Hb level as early as three days after the larvae had stopped sucking blood. Parasitism by *Protocalliphora* seems to have no effect on nestling weight; possible effects on nesting success and on the duration of the nestling stage remain to be worked out. This quick recovery raises doubts concerning our earlier hypothesis, i.e. that anaemia directly or indirectly causes mortality of Great Tits after fledging, at least for late breeding seasons. This point will be investigated in more detail.

3.2. ECOLOGY OF THE COLLARED DOVE, *Streptopelia decaocto* (J. H. van Balen, D. Westra)

The study on the productivity of the Collared Dove population near Ermelo was continued, and experiments on the rearing of broods of more than two young were added.

The number of nests in the study area agreed closely with that of 1972. The seasonal distribution of egg-laying was similar to that found in 1971, but differed from the pattern seen in 1972, when there were more clutches in March. Generally, fresh clutches are found from early March until early September, the highest numbers occurring during May and June. Calculation showed that per pair, between 2.4 and 3.4 clutches per year are produced.

As in previous years, mean clutch-size amounted to 1.96 eggs and hardly varied throughout the laying season. Egg losses were very high, especially in the clutches produced from March to May. Nestling losses were smaller than in 1972, and were smallest in the middle of the breeding season. The productivity (i.e. number of young fledged) per clutch also varied strongly throughout the season and showed a peak in June and July.

Many Columbidae species invariably produce two eggs, and several authors have speculated on the mechanisms responsible for the evolution of such a small and constant clutch-size. Observations on the incubation of 3-egg clutches and on the rearing of broods of three (or more) nestlings may indicate the factors playing in this mechanism.

During 1973, an attempt was made to study this aspect by transferring fresh eggs or newborn nestlings, and by regular inspection of the resulting extra-large clutches and broods. The formation of 3-egg clutches gave

many difficulties and will be repeated next year with a modified experimental design.

The formation of broods of three in June resulted in the fledging of one or two nestlings, but in experiments performed later (July-August) all three nestlings fledged. In broods of four (August-September), three nestlings usually fledged. It was concluded that in this region, at least during the second half of the breeding season, the doves are capable of rearing more than the usual two young.

3.3. ECOLOGY OF THE COOT, *Fulica atra* (J. H. van Balen, J. Visser)

The field work during the breeding season was continued in three study areas, i.e. the Westeinderplassen (W), Ringvaart Haarlemmermeer (R), and Molenpolder (M). R differs from W by the scarcity of suitable nesting sites, M differs from the other two areas by the absence of an important food species, *Dreissena polymorpha*. The main object of this study is to determine the relationships between breeding density, clutch-size, and laying date. These relationships are being studied both between and (over a series of years) within the areas. Some of the results are summarized below.

The overall clutch-size is largest in R, slightly smaller in the neighbouring area W, and much lower in M. Annual differences in clutch-size exist, and in recent years there has been a tendency for clutch-size to decrease, especially those in W.

The average laying date of the first clutches is earliest in W, and is 1-2 weeks later in R and M. In both W and R the laying season has tended to become earlier in recent years.

In general, clutch-size tends to decrease during the breeding season, but comparison of the annual means of clutch-size and laying date shows the reverse of this relationship, i.e. the later the average laying date the larger the mean clutch-size. Both clutch-size and laying date are negatively correlated with the breeding density.

The scarcity of suitable nest sites in R is mainly reflected in the much lower density of breeding pairs, and the feeding conditions in M, which differ from those in W and R, may be responsible for the late laying dates and small clutches in this area.

4. Bird migration

4.1. DISPLACEMENT OF JUVENILE STARLINGS DURING SPRING MIGRATION (A. C. Perdeck, B. J. Speek)

In previous studies it was established that during autumn migration adult starlings are able to compensate for an experimental sideways displacement by means of goal orientation toward their winter quarters (PERDECK 1958, 1967). Juveniles did not compensate, but moved in a

direction normal for autumn migration, apparently using one-direction orientation. It was concluded that starlings are able to use goal orientation only when aiming at a known area.

Since it would be of interest to know whether a juvenile starling can find its hatching area by means of goal orientation, a displacement experiment was carried out during the spring. From 1964 to 1971 nearly 3,000 juvenile starlings (2nd calendar year) were caught in The Netherlands in February and March, transported to Zürich (Switzerland), and released there.

During the breeding period 13 recoveries were obtained, 9 of these within the normal breeding area and 4 near the point of release. This suggests that a number of the displaced birds returned to their area of hatching and that their method of navigation was goal orientation. Since the 4 recoveries made near the point of release in the breeding period concern the four breeding seasons following the displacement, it is possible that these birds had simply stayed at the release point, but this can be rejected, because no recoveries have been made in this area outside the breeding season. It is more likely that such birds stayed there only during the breeding season, bred here, and then migrated. This is consistent with the occurrence of a considerable number of recoveries of displaced birds in the southern half of France. It is difficult to attribute these recoveries to birds that returned to their area of hatching. A detailed report on these experiments is in press.

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4.2. SPONTANEOUS MIGRATION ACTIVITY OF CHAFFINCHES IN THE KRAMER CAGE (A. C. Perdeck, C. Clason)

In the previous report evidence was given that chaffinches in a Kramer cage show a directional preference turning 360° clockwise over the year such that in the migration months these preferences are in the migration directions. The birds were held in a naturally changing day-night rhythm and were tested under the natural sky in sunny conditions.

The factors responsible for this annual shift in direction choice can be internal (circannual clock) or external, such as for instance the day length. If the day length is an important factor, it must be possible to influence the direction choice by experimentally changing the day length. Technical difficulties connected with the test situation under the sunny sky led to failure of these experiments (see previous report). To overcome these

difficulties, a primitive solarium was built with a fixed sun arc of 21 March/21 September. It was established that the birds accepted the artificial sun as a directional clue.

Moreover, the more or less constant direction throughout the day proved not to be related to fixed clues of the experimental setup.

A number of birds were held under constant conditions with 12 hours of light and 12 hours of darkness and tested from June to December in the solarium. As Table 3 shows, there is no indication of a shift in direction, as was found with changing day-night rhythm. This points to the absence of an internal circannual clock related to direction choice.

Table 3. Mean direction choice by four chaffinches held under constant conditions from June to December.

	ind. A	ind. B	ind. C	ind. D
June, July	SE by E (123°)	W by S (258°)	S by E (258°)	E by N (74°)
Sept., Oct.	ENE (68°)	W by S (256°)	SE (133°)	ESE (110°)
Nov., Dec.	E by N (75°)	SW (229°)	SE by S (146°)	N (358°)

4.3. EXPERIMENTS ON DISCRIMINATION BY THE STARLING BETWEEN GEOGRAPHICAL LOCATIONS (A. J. Cavé, C. Bol, G. Speek)

The purpose and design of these experiments are discussed in detail in the 1970 Progress Report (pp. 50–60). In 1972, the conclusion that the birds can distinguish between two localities 200 kilometre apart east-west was confirmed.

Tests performed in a different landscape at a short distance from the training places showed that the birds were still capable of distinguishing the correct location provided that the sun was visible. When the landscape was screened off, the birds failed. These two results, taken together, suggest that both landscape and sun are essential, but that the landscape does not necessarily have to be a familiar one. We are inclined to think that the horizon is necessary to measure the altitude or change of altitude of the sun. The latter is not possible with a screen at short distance. Suggestive in this respect is the finding that birds trained and tested before the culmination time of the sun generally perform better than birds trained and tested during culmination. At the former time of day the differences in the height of the sun between the two places and the changes in altitude are larger than around culmination time.

5. Department of Distributional Ecology

5.1. COLONIZATION OF THE IJSSELMEER POLDERS BY CARABID BEETLES AND PLANTS (J. Haeck, R. Hengeveld)

In our study on the dispersal and establishment of organisms in relation to environmental conditions we concentrated mainly on two problems:

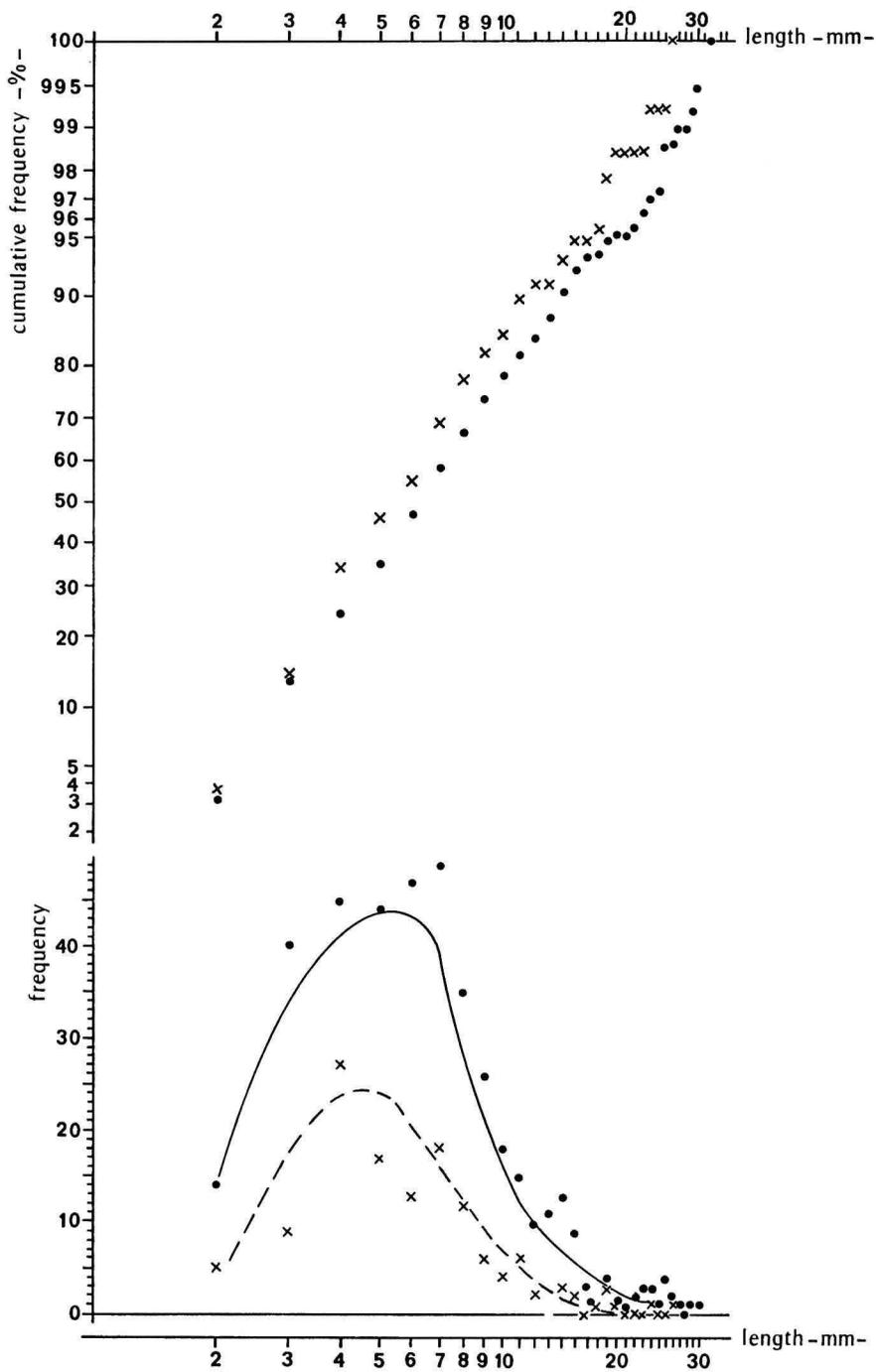


Fig. 1. Distribution of body length of carabid species from The Netherlands (●) and from the IJsselmeer polders (×).

1. the factors determining which carabid species invade a new area, and
2. the effect of the age of an environment on the species composition.

5.1.1. *Comparison of the species composition of carabids in the polders with the total Dutch beetle fauna* (Together with H. Turin)

In earlier Progress Reports (1968, 1969, and 1970) HAECK described the effect of the ability to fly on the capacity to invade a new area. There may, however, be other factors which also determine whether or not a certain carabid species will be found in a new terrain of this kind. For example, species from a dry habitat will probably not be found in marshy land such as a newly drained polder.

To find out which factors are operative, we compared, as a first approach, the mean sizes of the species occurring in the polders with those occurring on the mainland of The Netherlands. It proved to be the relatively small species which were present in the polder area (Fig. 1). This fact can be explained in two ways: either, there is a correlation between the dispersal capabilities and size, or there is a correlation between the ability to settle in this area and size. A combination between these factors is of course also possible.

In effect, we found arguments in favour of both of the first two explanations. As mentioned above, the macropterous species or morphs invade the new area sooner than the brachypterous species, which, with one exception, are still absent. Since the brachypterous species are the relatively larger ones among the Dutch carabids, this finding favours the first explanation.

However, if we compare the sizes of beetles from some randomly chosen areas (grids on a map) in the western part of The Netherlands with those of some eastern areas, we find a difference between the two geometric means, the value of the western part being smaller than that of the eastern part. Distinct differences between the habitats present in the western and eastern areas are to be expected. For instance, the western part of the country is the lowest and may be the wetter of the two, especially since the soils in the grid locations contain a high proportion of clay, whereas those in the east have more sand. This may be an indication that the second hypothesis is valid, although we still do not know the factors concerned.

Another indication in this direction is offered by the trend in the monthly geometric means of the beetle sizes shown in Fig. 2. The larger species among the beetle fauna are relatively more abundant at the end of the summer than in the winter, the other months being intermediate. This fact too suggests a relationship between beetle size and environmental factors, as postulated in the second explanation. These two facts favouring this explanation are in accordance with the findings of MOSSAKOWSKI (1970), who reported that the relatively larger species occur more frequently in drier habitats and the smaller ones in both dry and wet habitats.

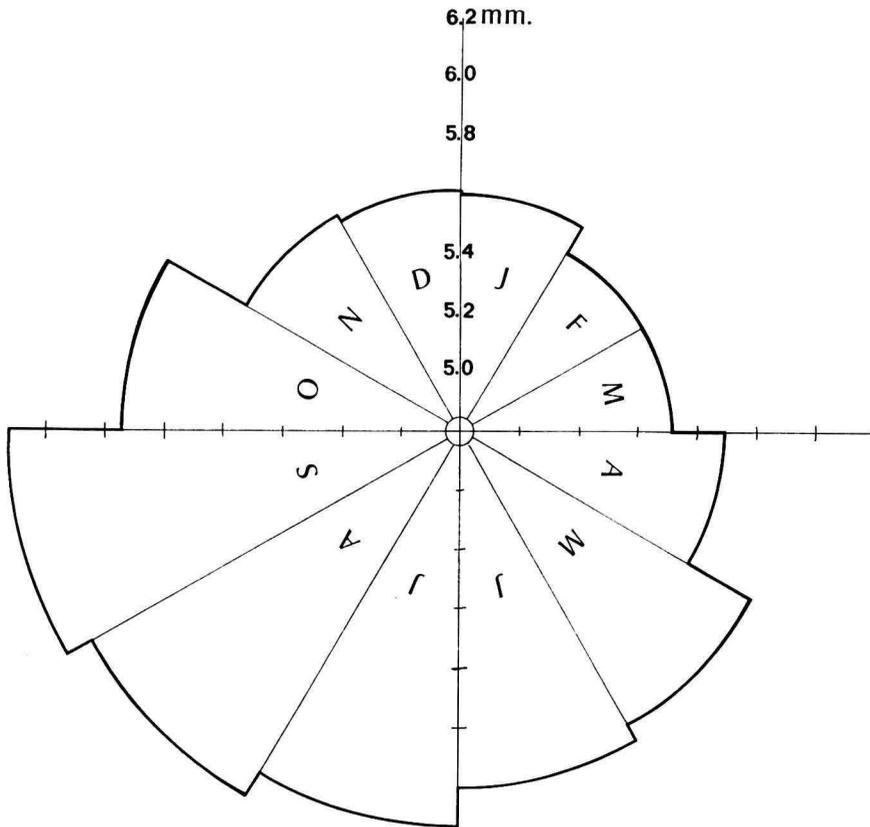


Fig. 2. Monthly geometric mean of the length of carabid species from the IJsselmeer polders.

5.1.2. *Effect of the age of an environment on the species composition of plants* (Together with H. Nip-van der Voort)

As described in the 1972 Progress Report, we sampled animals and plants along road verges in the IJsselmeer polders and compared the sampling sites according to the species composition to find out whether there is a correlation between the ranking as to the age of the sites and the dispersal mechanisms of the plants.

We started by assigning the plants to two classes, namely the early and the late immigrants (see VAN DER TOORN c.s., 1969). Fig. 3 gives the results expressed as percentage of the late immigrants. These percentages are the highest for the oldest areas (mainland and Noordoost Polder) and the lowest in the youngest (Zuidelijk Flevoland).

Next, we divided the plant species into seven categories according to the dispersal mechanisms. Fig. 4 gives the distribution of the mean number of species per road for each category. In this Figure the roads are ranked according to age and the dispersal characteristics according to dispersal

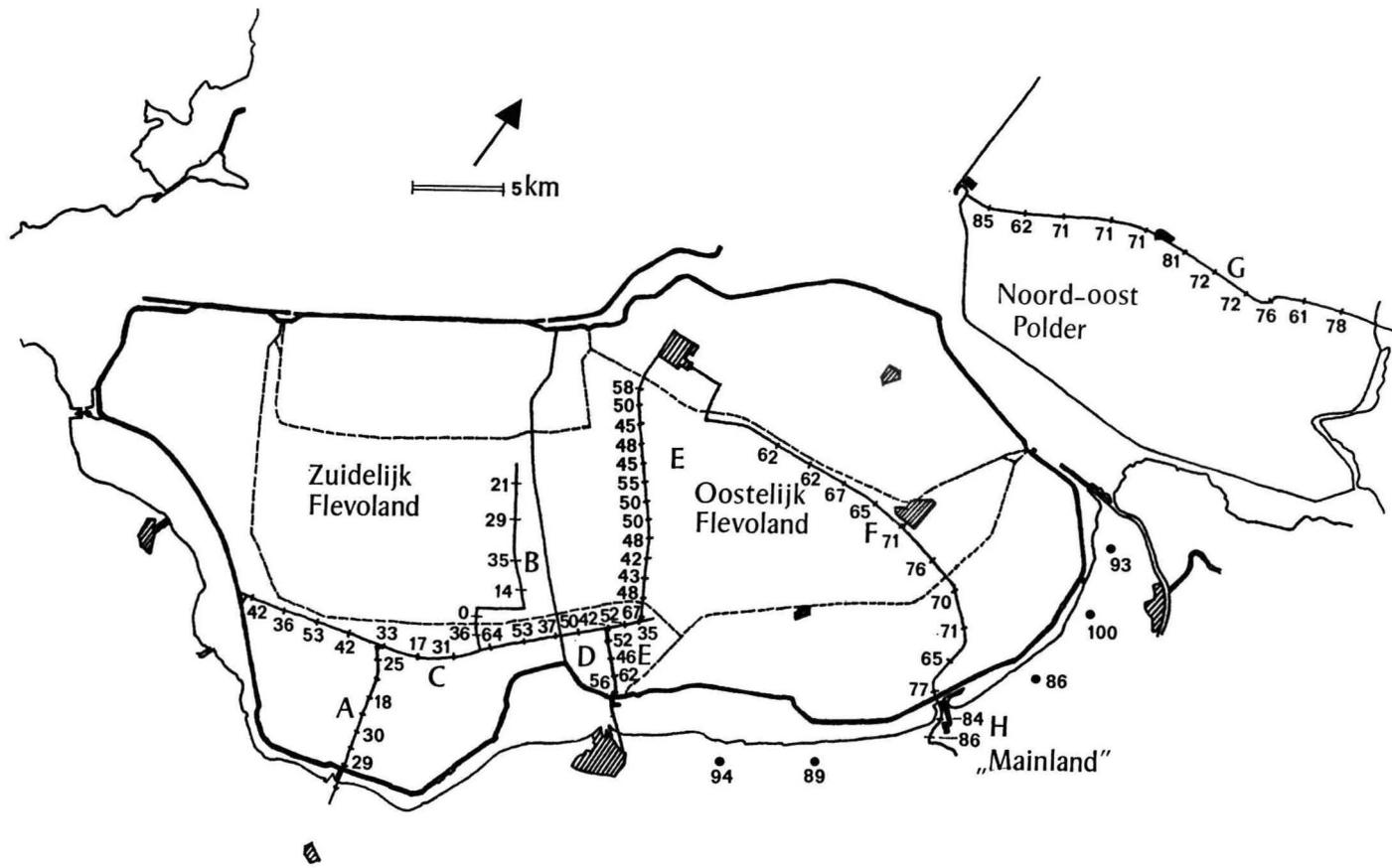


Fig. 3. Percentage of late immigrants among plant species found in various road verges in the IJsselmeer polders.

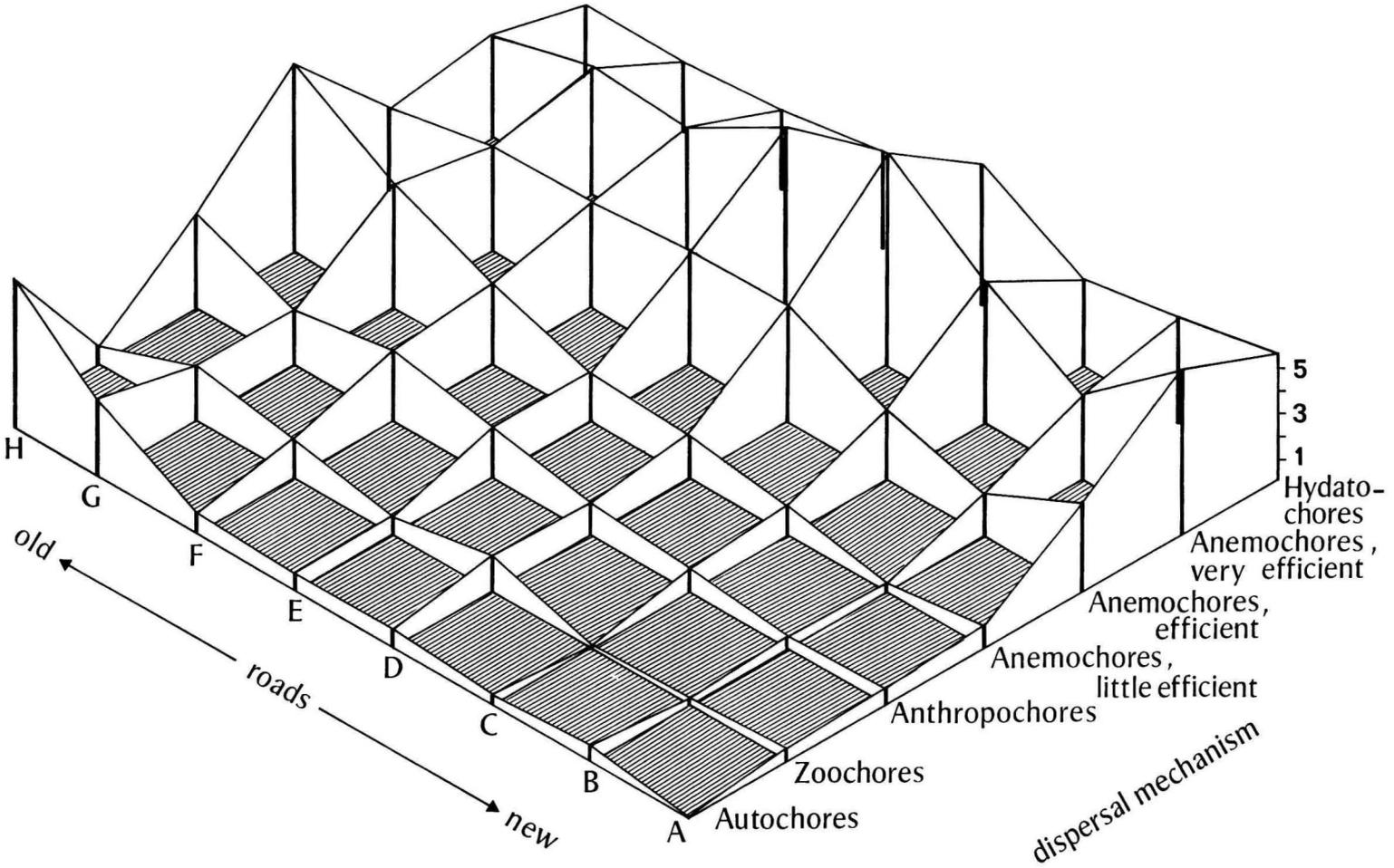


Fig. 4. Frequency of plant species with various dispersal characteristics in road verges of different ages.

efficiency. For this latter characteristic we used data from FEEKES (1936). The Figure shows that in the youngest polder the most efficient invaders prevail, these categories also occurring in the oldest polder and on the mainland, but that the least efficient migrants are prevalent in the latter two areas. The Figure also shows that the change in vegetational composition does not consist simply of a substitution of species but rather that the process is an additive one. This explains why the oldest areas or road verges have become the richest in species.

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5.2. A FIELD EXPERIMENT ON THE DEVELOPMENT OF REED VEGETATION (J. H. Mook, J. van der Toorn)

A vegetation starts to develop rapidly soon after the drainage of an IJsselmeer polder, resulting in a more or less closed reed stand (FEEKES and BAKKER, 1954). This development is accelerated by artificial sowing of the reed, leading to an almost completely closed and well-developed stand after three years. Later on, reed growth diminishes, and this permits the establishment and further development of other plant species (HEMINGA and VAN DER TOORN, 1970). A number of simultaneous changes in the environment of reed can induce this development. The most important of these are probably the following: the drying-up of the soil; a decrease in the mineral nitrogen content of the soil (VAN SCHREVEN, 1965), and a heavy infestation of the reed by larvae of noctuid moths, especially *Archanara geminipuncta* (MOOK, 1972) and *Rhizedra lutosa*.

To investigate the influence of each of these factors, we gratefully accepted the opportunity to work in an experimental reed field set up in Zuid-Flevoland by the IJsselmeer Polders Development Authority. The experiment had been started in 1971 (3 years after drainage of the polders), when reed growth was still very good. The soil of the experimental field consists of heavy clay. Parts of the field were given different treatments, as shown in Table 4.

Some preliminary results will be given here. During the growth season of 1972 and 1973, shoots were harvested every 2-3 weeks on 8 plots (1/4 m²) in each of the four treatments. The rhizomes were sampled only once a year giving 40 bore samples per treatment in October (bore diameter 13.5 cm).

Burning of the reed in the spring eliminates the overwintering stages

Table 4. Treatments in the experimental reed field in Zuid Flevoland. (Burning applied in the winter.)

Variation in water-level (1 March-1 Nov.)	
+10/+20 cm	-40/-90 cm
Wet, burned	Dry, burned
Wet, not disturbed	Dry, not disturbed

of most insects. *Archanara* and *Rhizedra* both overwinter in the egg stage and the larvae do not migrate, so that burned stands are free of damage by these insects during the following summer (see Table 5). In such stands many young shoots are killed by the burning procedure and by night frosts in the spring. These shoots are replaced by one or more new ones, leading to a density of more than 300 shoots per m². As a result of this high density, many shoots (mainly the smaller ones) die off during the summer, probably due to intraspecific competition.

Table 5. Damage occurring under various treatments, due to four habitat factors. Experimental reed field, Zuid Flevoland. (Damage graded as +++=heavy, ++=moderate, +=slight).

Treatment	Caterpillars (April-July)		Burning (March-April)	Night frost (April-May)	Intraspecific competition between shoots (August- September)
	Rhizedra	Archanara			
Wet, burned			++	+	++
Dry, burned			+	++	++
Wet, not disturbed		+++			
Dry, not disturbed	+++	+++			

With respect to the damage caused by moth larvae there is a difference between the two species. *Archanara* is a shoot borer occurring in both wet and dry places, whereas *Rhizedra* is a rhizome borer occurring only in dry stands. *Archanara* lives in the upper part of the shoot, which dies off after some time. Later on, regrowth starts by the formation of secondary shoots on the lower nodes; new shoots also arise from the rhizome. Since each caterpillar needs about 3 shoots for total development, a high percentage of the shoots may be damaged. This results in a strong reduction of the total leaf area during June and July.

Rhizedra attacks the young shoots first, and then bores downward to the rhizomes. All damaged shoots die off (this is in contrast to the situation in *Archanara*). The extent to which this also holds for the rhizomes remains to be investigated. Only restricted regrowth in the form of new shoots occurs, resulting in a low shoot density.

The effect of the moth larvae on the reed growth can be demonstrated

by comparing the differences in the annual increase in biomass in the different stands (Table 3). This increase closely approaches the annual total production of dry matter, the mortality of the rhizomes—at least in the burned objects—probably being low. According to FIALA (1973), rhizomes live for at least 3 and probably for 5 years; the stand in the present experiment is about 4 years old. From the data in Table 6 it can be concluded that in the wet undisturbed stand *Archanara* gave rise to a production decrease amounting to about 50 per cent as compared with the burned stands. In the dry undisturbed stand there was a combined effect of *Archanara* and *Rhizedra* giving a decrease of 70 per cent in 1972 and of 86 per cent in 1973.

Table 6. Yearly increase in shoot and rhizome biomass (in gram dry weight per m²). Values between parentheses indicate the percentage of decrease as compared with the respective burned stands.

	Production of shoot biomass		Increase in rhizome biomass		Production of shoot and rhizome biomass	
	1972	1973	1972	1973	1972	1973
Wet, burned	1,549	1,402	182	313	1,731	1,715
Dry, burned	1,201	1,286	245	509	1,446	1,795
Wet, not disturbed	917	871	-59	-84	858 (-50)	787 (-54)
Dry, not disturbed	721	540	-285	-291	436 (-70)	249 (-86)

The burned stands cannot be regarded as controls, because of the introduction of various other effects with a mainly negative influence on production. Nevertheless, the production of the burned stands closely approaches that of the optimal value for agricultural plants, viz. 2000 grams per m² (ALBERDA, 1970). This indicates that growth conditions were favourable and the effects mentioned above had little influence. With respect to the nitrogen and phosphorus content of the soil these conclusions were confirmed by fertilizer experiments in which no increase in production was obtained with high dressings of these elements.

From these results it may be concluded that the decrease in reed growth in the IJsselmeer polders occurs mainly under the influence of moth larvae. The influence of the water-level on this decrease is an indirect one, since it does not affect reed growth directly but rather via the damage inflicted by the insects.

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5.3. MICROMETEOROLOGY: THE MICROCLIMATE OF A REED VEGETATION (Ph. Stoutjesdijk)

As a part of the formation of a general picture of meteorological differentiation, measurements were made in a stand of reed. This vegetation was chosen because we expected it to be an interesting case in the range of possible microclimates and in relation to the work done here by the Department of Distributional Ecology.

The measurements were made in a high stand of reed on soil covered with a few centimetres of water. The main level of the vegetation tops is at 2.40 m, but some of the stems project above the highest level at which measurements were taken (2.80 m). The first curve (R) in fig. 5 shows that about 88% of the solar radiation absorbed between the tops of the vegetation and 1 m level. Below this level, where there are hardly any leaves, only a small additional percentage of the radiation is absorbed. The transmitted fraction (about 10%) is taken over the full solar spectrum. We estimate, on the basis of the results of the measurements in other vegetations, that of the assimilable light only 2–3% is transmitted.

From 2.80 to 2.40 m there is no significant temperature gradient (fig. 5, curve t). From here on downward there is a continuous decrease of temperature, the water surface being 16.1° C or almost 8° C cooler than the air at the vegetation tops. Apparently there is a continuous transport of heat from the highest measured level downward. Although the upper limit of the measurements is not quite high enough to permit a definite conclusion, it seems probable that the vegetation uses all of the net absorbed energy for evaporation and even takes up some heat from the air.

The vapour pressure curve (e) is more complicated than the temperature curve. There is a clear maximum at 1.80 m, apparently due to the strong transpiration by the leaves. From this level there must be both an upward and a downward transport of water vapour. The weak secondary maximum at 50 cm might be explained by errors or, less probably, by lateral transport. In any case it is clear that there must be a downward transport of water vapour to the water surface. This could be demonstrated directly by condensation on a piece of aluminium foil placed on the water surface. It is hard to estimate the magnitude of the downward transport rates of heat and water vapour, partly because the exchange coefficient is unknown and partly because of the complicating effects of lateral transport. An acceptable approach seems to be base to the calculation on the gradients in the lower decimetre above the water surface, using transfer numbers for still air. We then arrive at a condensation equivalent to about 0.004

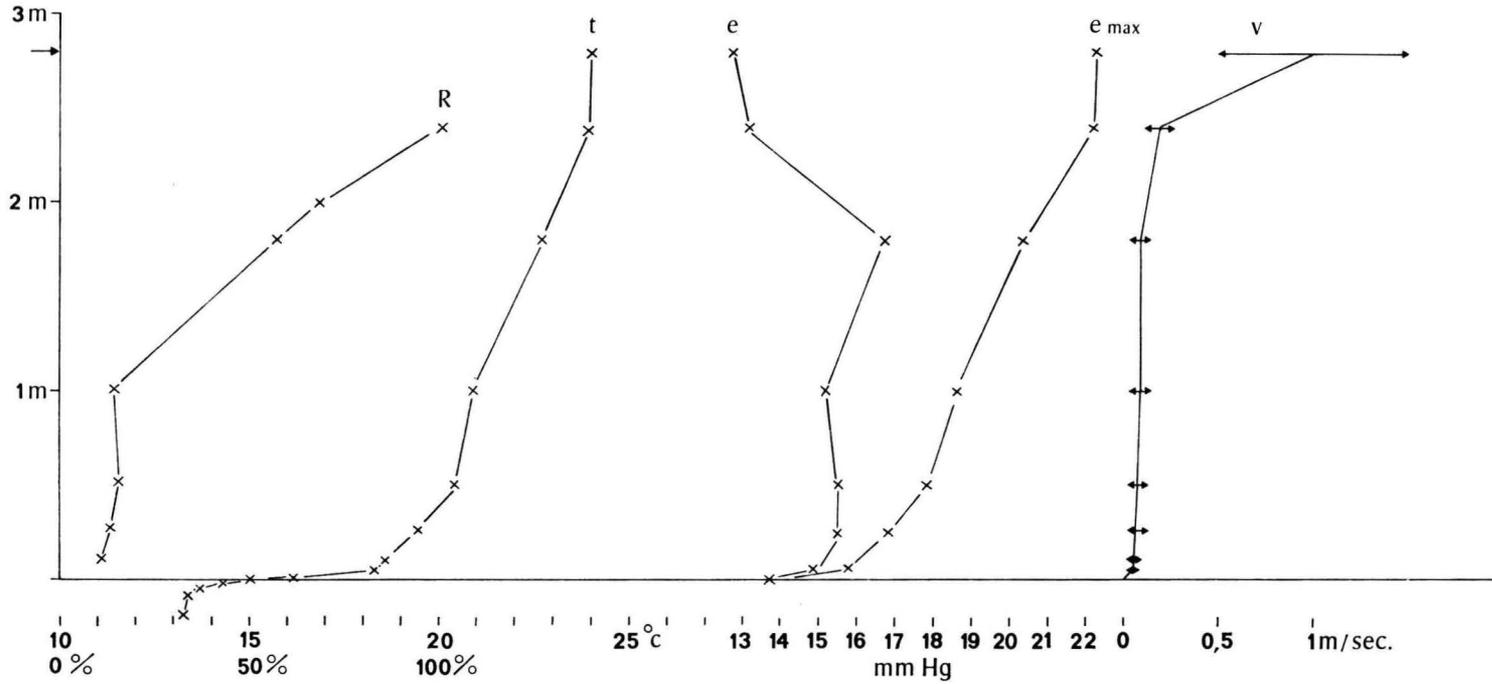


Fig. 5. Profiles of solar radiation (R), temperature (t), vapour pressure (e), max. vapour pressure at the relevant temperature (e_{max}) and wind velocity (V) in a high reed vegetation.

cal/cm² min., which is a very small value, certainly less than 1% of the energy dissipated in transpiration by the reed vegetation. The curve marked 'e_{max}' gives the maximum vapour pressure possible at the relevant temperature.

The difference between vapour pressure and maximum vapour pressure is the saturation deficit of the air. This deficit decreases continuously going downward and becomes zero at the water surface.

The wind velocity (v) decreases first rapidly on entering the vegetation and then slowly, also downward. The term wind velocity has no strict meaning inside the vegetation; in fact, heat-transfer numbers are measured by means of a hot-wire anemometer and converted into wind velocities.

Other measurements showed that the lowest air temperature is always found near the surface, but temperature differences are smaller when there is more wind and weaker solar radiation. The vapour pressure, however, is sometimes highest near the surface with only a weak secondary maximum at 1.80 m where the leaves are most densely developed. This situation is well developed when there is little radiation and hence transpiration.

6. Botanical Ecology (Biological Station "Weevers' Duin")

6.1. SYNCOLOGICAL RESEARCH OF THE DUNE SLACKS ON VOORNE; ANALYSIS OF VEGETATIONAL AND ENVIRONMENTAL DATA (D. van der Laan)

6.1.1. *Introduction*

Within the scope of the synecological study of slack vegetation on the dunes of Voorne, a large number of vegetational and environmental data have been collected. Most of the records were made in permanent plots. The present communication summarizes attempts to analyze these data by various numerical methods.

For the analysis of the data, various floristic and vegetational characteristics of the vegetation can be used, such as the presence or absence of the relevant species, their abundance, coverage, sociability, and phenological stage. It appeared that, depending on the method of analysis, not all of these characteristics have to be considered. In such cases no surplus information was obtained as compared with the use of only one characteristic, for instance the presence or absence of species. Two examples of methods are given below.

6.1.2. *Ordination of the stands*

An arrangement of the vegetation records of dune slacks was carried out according to Bray and Curtis (BRAY, J. R. and CURTIS, J. T. 1957) using the following characteristics:

- a. presence or absence of the species,

- b. presence or absence and phenological stage of the species,
- c. presence or absence, abundance, and phenological stage of the species.

Subsequently, the correlation between various environmental factors and the differentiation in vegetation, as obtained with the above method, was investigated. The analysis of soil factors was performed on the basis of samples collected at the same time as the vegetation records were made. The ground-water table proved to be a highly variable factor whereas only very small differences occurred in the other factors analyzed. Therefore, only the correlation between the differentiation in the vegetation and the ground-water table is given. Depending on the characteristics used to arrange the vegetation records the following correlation coefficients were obtained:

- a. presence or absence $r=0.855$,
- b. presence or absence and phenological stage $r=0.845$,
- c. presence or absence, abundance and phenological stage $r=0.856$.

Each of the three correlation coefficients appeared to be significant ($n=13$, $p<0.005$).

Since the same levels of significance are obtained with the three operations, preference is given to the least laborious operation, i.e. the arrangement of the vegetation records based on the basis of presence or absence only.

6.1.3. *The ecological amplitude of the species*

In a different approach the species were arranged between the limits found for the various environmental factors. In this case, likewise, the question is which of the characteristics must be used in the analysis to obtain an optimum amount of information. To answer this question the occurrence of a number of species over a wide range of ground-water tables was established using the same characteristics (a, b, c) as described above.

Fig. 6 shows the amplitude of two of the species. It is clear that in this particular analysis more information is obtained by using more characteristics. From part a of the Figure it can be concluded that the two species react differently to the ground-water table. Unlike *Samolus valerandi*, *Parnassia palustris* does not occur at the highest ground-water tables of the range.

When in addition the phenological stage is used as a characteristic (part b of the Figure), it can be shown that at the highest values of the range of ground-water tables at which *P. palustris* is observed, the species was found only in the vegetational stage.

Part c of the Figure gives the data for which besides the presence or absence and phenological stage, the abundance and the frequency distribution were also been taken into account. In this way the most detailed

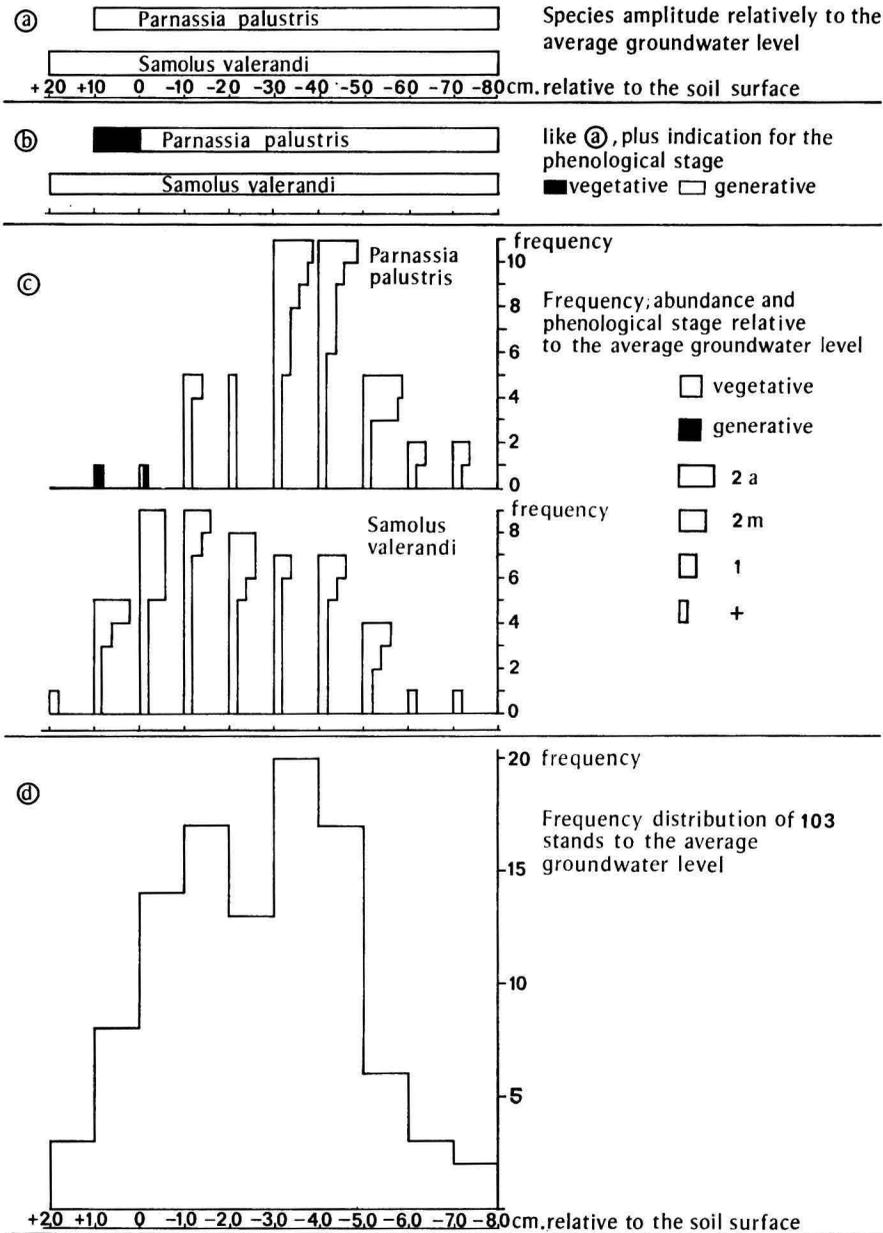


Fig. 6. Characterization of two species of the dune slacks.

picture of the relationship between the occurrence of both the species and the ground-water table is obtained. It is evident that *P. palustris* occurs more frequently and with a higher abundance in the dry part of the wet-dry gradient. The reverse holds for *S. valerandi*, i.e., the species occurs more optimally in a wet environment and its contribution to the vegetation is relatively higher there.

6.1.4. *Concluding remarks*

From the foregoing it appears that for the various comparative operations different numbers of characteristics of the vegetation have to be used to obtain an optimum output of each method. In dealing with a vegetation rich in species such as those found in the dune slacks of Voorne, large amounts of data have to be handled. Formerly, this was done by means of hand sorting, but now, to avoid this very laborious work, the vegetational and environmental data will be transferred to punch cards for computer processing. It will be possible to carry out more operations with more extensive material and to award different weights to the characteristics involved. This development is now in progress.

Reference

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6.2. CULTURE EXPERIMENTS WITH SOME COASTAL PLANTS, MAINLY IN RELATION TO THE PROBLEMS OF SALT TOLERANCE (A. H. J. Freijsen)

6.2.1. *Differences in growth physiology and salt tolerance between varieties of Centaurium littorale (Turner) Gilmour (Gentianaceae)*

Since 1968, various culture experiments with the halophyte *Centaurium littorale* have been carried out. In the first series of experiments specific adaptations of this species to the environment were studied. *C. littorale* proved to have a low growth rate and a medium salt tolerance. These properties enable the plant to grow on oligotrophic brackish sandy soils along the coast of NW Europe (FREIJSEN 1971). In the 1970 Progress Report preliminary water culture experiments with two varieties of *C. littorale* were described.

In 1971, culture experiments with five varieties of *C. littorale* were carried out to compare growth rates and salt tolerances. In 1973, a paper dealing with the results of these comparative culture experiments was prepared. The main results of this study can be summarized as follows. There are differences in growth rate between the varieties investigated. These can be explained in terms of different values of the shoot/root ratio and of the dry-matter content of the leaves. Differences in leaf morphology are also of importance with respect to the growth rate. There is a correspondence between the level of the growth rate and the maximal plant size in the field. There are also varietal differences in salt tolerance. *C. littorale* var. *latifolium*, occurring on soils of lower salinity than the others, has a rather low salt tolerance caused mainly by an excessive uptake of Na^+ and Cl^- . Although two other varieties differ in physiological and morphological respects they occur together in many places in The Netherlands. The question of how these two varieties differ in ecological requirements is still unanswered.

6.2.2. Differences in salt tolerance between three subspecies of *Festuca rubra* L. (Gramineae) (In cooperation with J. J. L. Huber)

In 1973, the experiments on salt tolerance were continued with another plant species, namely *Festuca rubra*. Culture experiments with the three Netherlands subspecies of this grass were started. *F. rubra* ssp. *litoralis* (Mey.) Auquier grows near the spring-tide level on salt marshes. *F. rubra* ssp. *arenaria* (Osb.) Richter is a common plant in sandy outer dunes. *F. rubra* ssp. *rubra* is an inland plant.

These three environments form a gradient from mesohaline to non-saline conditions. The aim of the experiments is to determine the various salt tolerances of these subspecies and to relate them to properties of the ionic balance and the growth physiology. In the first series of experiments a close agreement was found between the salt tolerances of the subspecies investigated, on the one hand, and the salinity levels of their natural environments, on the other.

6.2.3. Methodical culture experiments

For these culture experiments, containers with a circulating culture solution were used in which a number of experimental plants can be grown at an equal and constant solution level. The water circulation favours the aeration of the rooting medium (STEINER 1965). The primary purpose of these small-scale experiments was to become familiar with a new culture technique, but during their execution it was decided to take advantage of this opportunity to determine the variation in yield and mineral composition of plants grown under such homogeneous and constant conditions. The experiments were carried out with three species: *Ranunculus repens* L., *R. acris* L. (Ranunculaceae), and *Cynoglossum officinale* L. (Boraginaceae). The results concerning the last-mentioned species are briefly described below.

Sixteen juvenile rosette-plants of *C. officinale* having the same size at the start of the experiment were used. After they were harvested, dry weights (70° C) and certain mineral contents were determined, the minerals being analyzed in the leaves of half of the plants.

The variation of the dry-weight values is rather high (Table 7). The roots show the most variation. However, the shoot/root ratio varies less. This pattern of variation probably results from small differences in age

Table 7. Variation in dry weight (mg) and shoot/root ratio within a set of 16 plants of *Cynoglossum officinale* cultured in the greenhouse between 21 March and 25 April.

	rosette	root	total plant	ratio
mean (\bar{x})	1594	589	2183	2.75
coefficient of variation (\bar{x}/s)	0.35	0.38	0.35	0.13
95% confidence interval	1296-1891	469-709	1771-2594	2.57-2.94

and initial weight of the experimental plants, which could lead to great differences in absolute plant weight during the stage of exponential growth. Although the shoot/root ratio is known to change during early growth, the effect of small differences in age on this property of the plant is much smaller. The variation of the mineral contents is much lower than that of the dry weights (Table 8). The Ca^{++} content varies more than the others. This difference is caused by the rather great variation in plant size. Old leaves accumulate Ca-ions and large plants having more old leaves than small plants will have a higher average Ca^{++} content.

Table 8. Variation in mineral composition within a set of 8 plants of *Cynoglossum officinale* (contents in meq of mK/kg dry leaf matter).

	K ⁺	Ca ⁺⁺	N-org.
mean (\bar{x})	1540	1366	3297
coefficient of variation (\bar{x}/s)	0.03	0.12	0.04
95% confidence interval	1502-1579	1230-1501	3177-3416

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6.3. THE INFLUENCE OF SOIL MOISTURE AND TRAMPLING ON GERMINATION AND DEVELOPMENT OF THE SEEDLINGS OF FOUR *Plantago* SPECIES AT VARIOUS DEGREES OF SOIL COMPACTNESS (C. W. P. M. Blom)

6.3.1. Introduction

Within the scope of the research on the influence of recreation and grazing on the dune vegetation of Voorne and Goeree, the experimental study on the influence of soil compactness on germination of some *Plantago* species has been continued (see 1972 Progress Report, pp. 106-112), with the addition of experiments on the influence of trampling on germination and the growth of seedlings.

Preceding experiments concerned the influence of soil compactness under optimal soil moisture levels on the germination of *Plantago lanceolata*, *P. coronopus*, *P. major*, and *P. media*. The present report gives results of experiments on the influence of soil compactness on germination under conditions of a limited water supply and also on the influence of trampling on germination and growth of seedlings. In addition a distinction was made between the germination of seeds lying on the soil surface (uncovered) and those covered with 3 mm sand.

In general *Plantago* species are found on more or less compacted or trodden soils. For purposes of comparison, *Potentilla tabernaemontani*, which grows mainly on non-compacted or non-trodden soils in the dune area, was included in the present investigations.

6.3.2. Germination of seeds lying on the surface of soils differing in compactness

6.3.2.1. Material and methods

The experiments were carried out with seeds of *Plantago lanceolata*, *P. coronopus*, *P. major*, *Potentilla tabernaemontani*, all collected in the dune area of Voorne and Goeree in 1971 and 1972, and with seeds of *Plantago media*, which were collected in Zuid-Limburg in 1972. The seeds were stored in envelopes at room temperature. Germination and growth took place in a phytotron (18 hours light, 6 hours darkness, temperature 24° C, relative air humidity 70%) in wooden boxes (30 × 30 × 10 cm) filled with sand originating from the older dunes (humus content about 0.5%, pH ± 9). This substrate was chosen because except for *P. media* the *Plantago* species mentioned and *Potentilla tabernaemontani*, are normally present in the older dunes. *Plantago media* was included to permit comparison of the *Plantago* species occurring in the dunes with one of different origin.

The following treatments were applied. Three series were prepared: in series A the soil was not compacted, in series C there was a maximal compaction, and in series B the compaction was intermediate between A and C. Compaction of the soil was achieved by ramming down the substrate. Soil compaction was determined by measurement of the soil resistance with a penetrometer. The soil resistance in the upper 2 cm was 0 kg/cm² in series A, 5 kg/cm² in series B, and 10 kg/cm² in series C. These soil-resistance values correspond with the following values of pore volume: series A: 45%, series B: 42%, and series C: 39%. Each box had the same quantity of water; the percentage of soil water (expressed as percentage of dry soil) were 10% in series A, 16% in series B and 14% in series C. During the experiments soil water was replenished every 24 hours, at which time the water contents were about 4% lower than the initial values. Since this water content was adequate for maximal germination, the sole limiting factor in this experiment was considered to be the soil resistance.

Unlike the experiments described in the 1972 Progress Report, the seeds were not covered with sand. In each box two plant species were sown separately (200 seeds of each species). In the control series the seeds were placed in the phytotron on wet filterpaper in petri dishes. During the tests the germination rate and the maximal germination were determined. At the end of the experiments the soil resistance was determined again.

6.3.2.2. Results

The results of these experiments are shown in figs. 7a-d, and 10 (mean values of three experiments). One month after the beginning of the experiments the soil resistance had not changed.

6.3.2.3. Conclusions

It can be seen that the highest germination percentages occurred on soils without compaction. The difference between the maximum germination on non-compacted and compacted soils was significant (X^2 -test). In all series *Plantago major* showed the highest percentage of germination. Therefore, it may be concluded that uncovered seeds of *Plantago major*

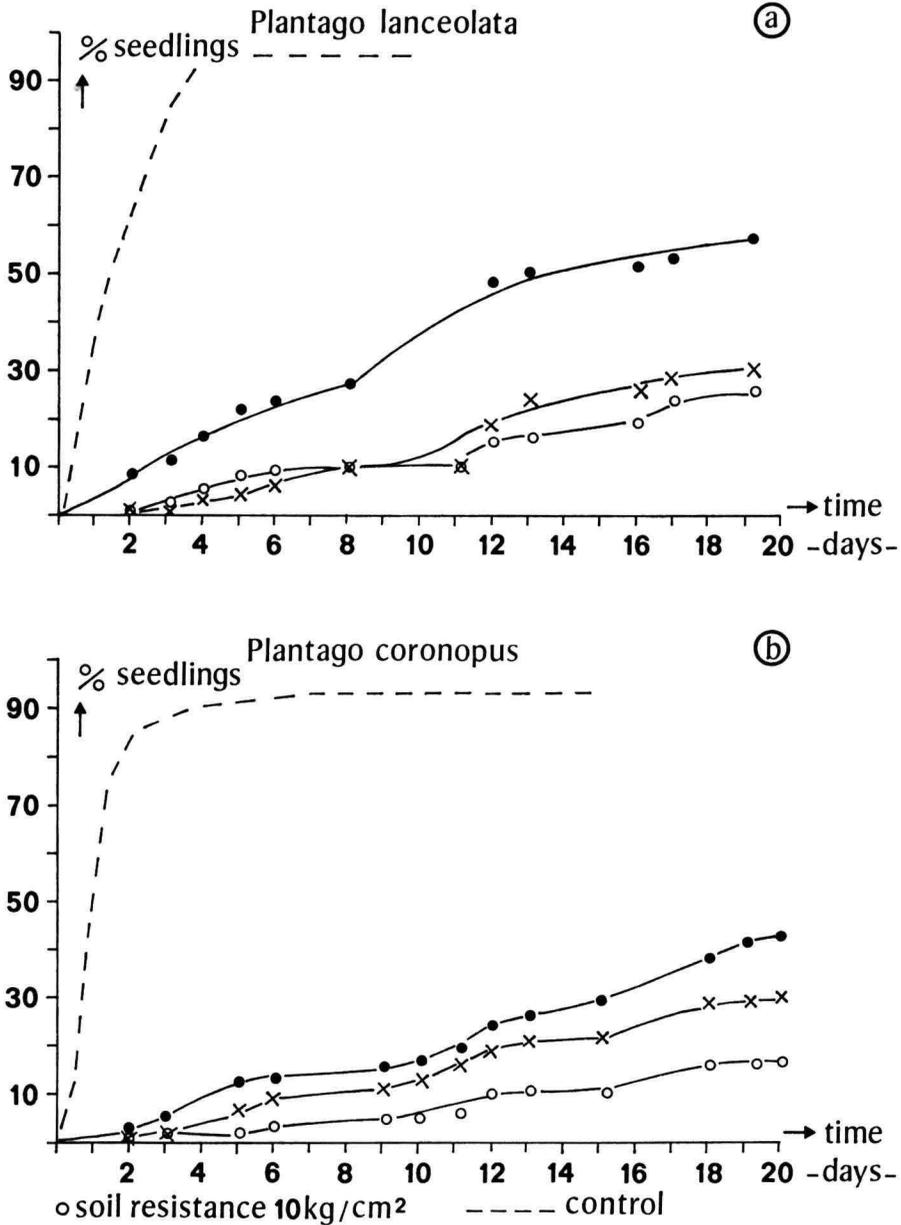


Fig. 7. The influence of soil compactness on germination of four *Plantago* species (uncovered).

lying on a compacted soil are more capable of germination than those of the other species investigated.

6.3.3. *The influence of soil compactness and trampling on germination*

6.3.3.1. Material and methods

To study the influence of trampling on the germination and development

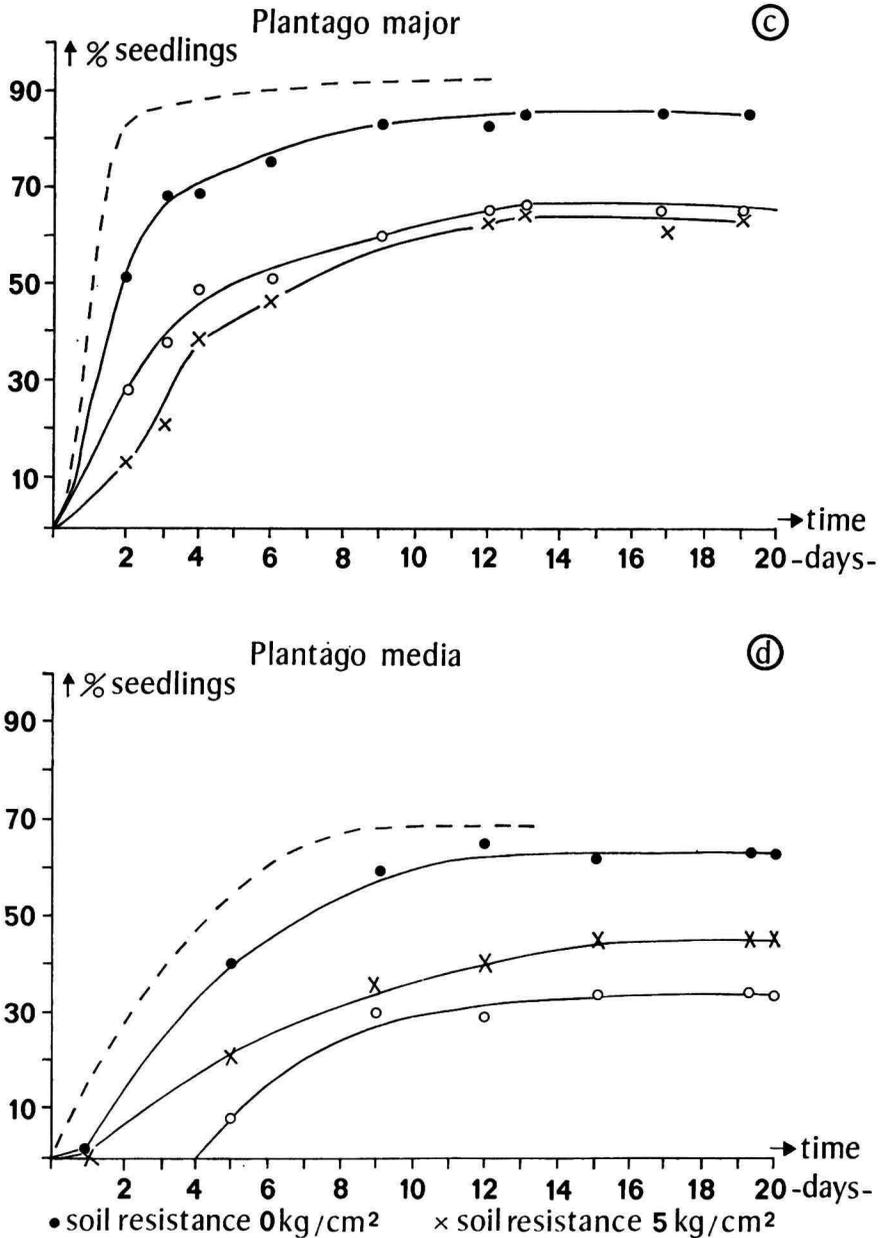


Fig. 7.

of the seedlings of the four *Plantago* species, the following treatments were applied. Three series (strong compaction, moderate compaction, and no compaction) were prepared in the same way and with the same substrate as described in section 2. The tests were carried out in pots (content 5 litres) and were placed in the greenhouse in July of 1973. The day

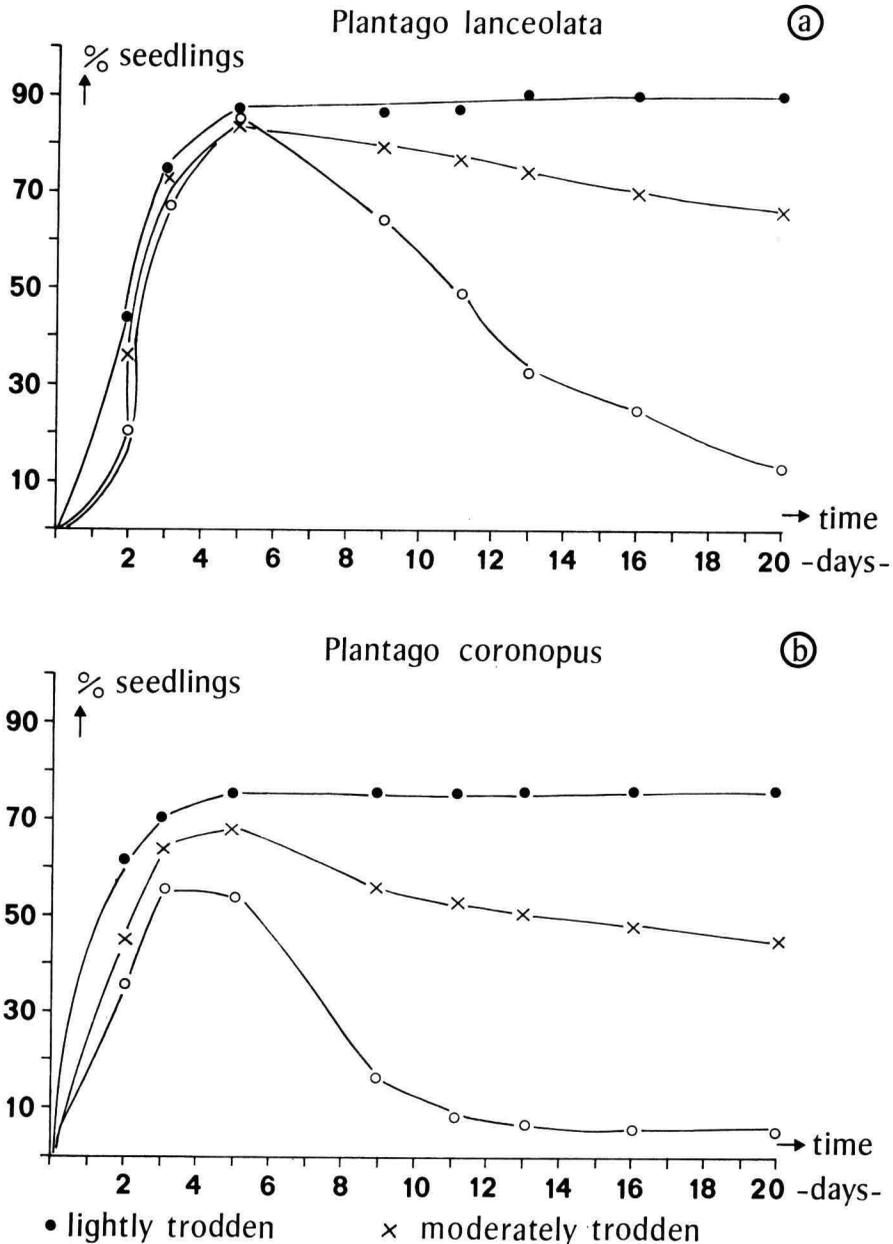


Fig. 8. The influence of trampling on germination of four *Plantago* species.

temperature in the greenhouse was about 27° C, the night temperature about 20° C, and the relative air humidity 60%. Per pot, 100 seeds of one species were sown; the seeds were covered with a 3 mm layer of sand, and the percentages of soil water were as described in section 2. Immediately upon sowing, the following trampling treatments were started.

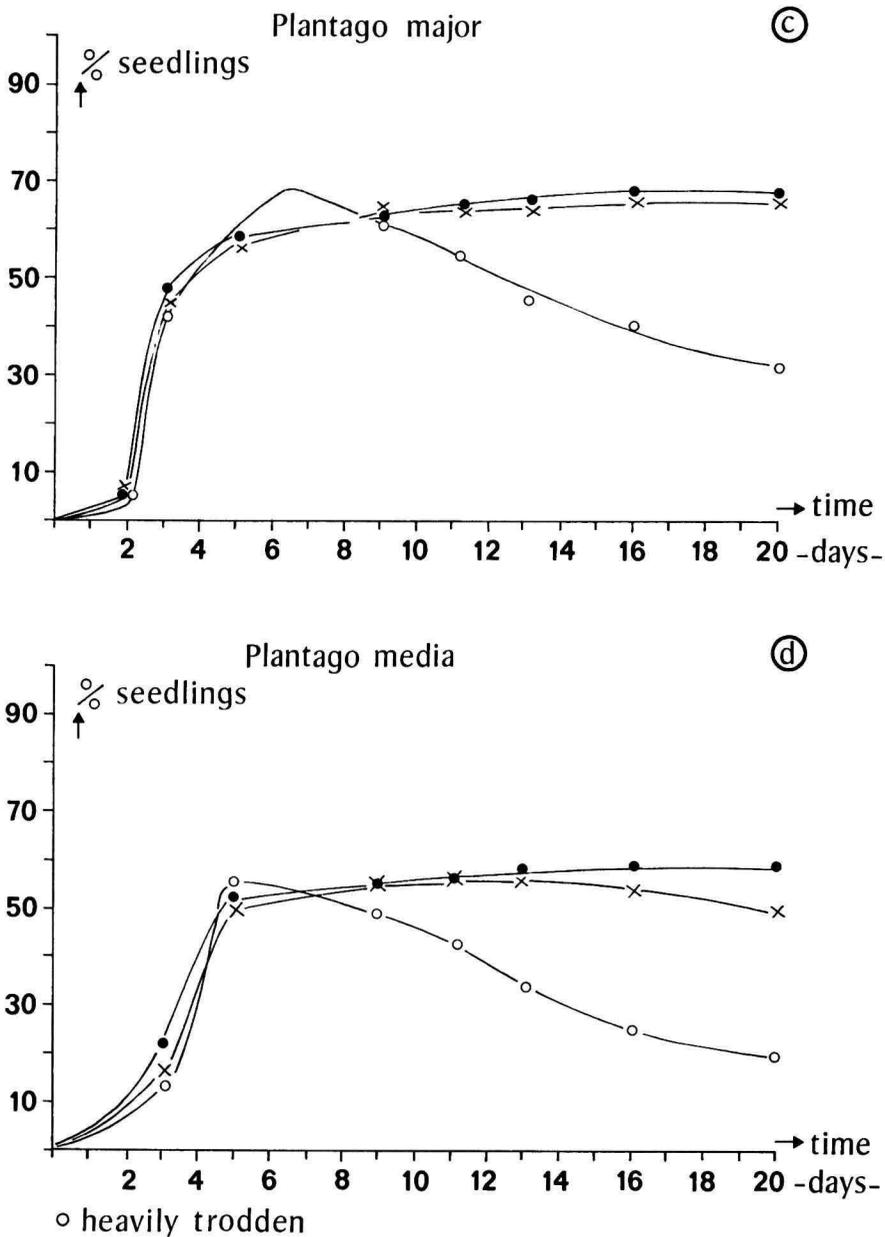


Fig. 8.

Series A (no compaction) was trodden once a day with a strength of 0.05 kg/cm²; series B (moderate compaction) was trodden twice daily at 0.15 kg/cm², and series C (strong compaction) was trodden four times a day at 0.25 kg/cm². In each series a untrodden control was included. During the tests the soil resistance was determined.

6.3.3.2. Results

The results of the trampling experiments are shown in fig. 8a-d. The results of the control tests were compared with those of series A.

In series A and C there was no important difference in soil resistance before and after the trampling treatment; in series B the soil resistance increased by 2 kg/cm².

6.3.3.3. Conclusions

The following conclusions may be drawn from this experiment. Moderate trampling on a moderately compacted dune soil with optimal humidity resulted in a recession of 21% for the seedlings of *Plantago lanceolata*, 34% for *P. coronopus*, and 15% for *P. media*. All of the seedlings of *P. major* survived this treatment. Intense trampling of seedlings growing in a strongly compacted dune soil caused a mortality of 90% for *P. coronopus*, 85% for *P. lanceolata*, 68% for *P. media*, and 50% for *P. major*.

It may therefore be concluded that the seedlings of *P. major* are less sensitive to trampling, whereas those of *P. coronopus* and *P. lanceolata* are highly sensitive to trampling.

In the future, experiments will be undertaken on the influence of trampling on full-grown plants of *Plantago* and some other plant species.

6.3.4. *Soil compactness and soil humidity as variable factors in germination experiments*

6.3.4.1. Material and methods

In this part of the study two factors, i.e. soil compactness and moisture content of the soil, were considered simultaneously, since in natural situations at least two important limiting factors for germination are conceivably operative: i.e., mechanical impedance (penetration resistance) and restricted availability of water.

The experiments were carried out with seeds of the four *Plantago* species and *Potentilla tabernaemontani*. Except for the level of soil humidity, the series were prepared in the same way as described in section 2. The seeds were placed 3 mm below the surface. In the experiments with *Plantago lanceolata* and *P. coronopus* the initial soil water level was 5%. After 20 and 36 days the quantity of soil water was increased by 1%. Because *Plantago major*, *P. media*, and *Potentilla tabernaemontani* did not germinate with less than 7% soil water, these experiments were started

at 7% of soil moisture; the percentages being raised once a week by 1%.

During the tests the germination rate and the maximum germination were determined.

6.3.4.2. Results

The results are shown in fig. 9a-d (mean values of three experiments). At the end of the tests it was found that the water percentages in the upper layer of the soil were 2% lower than given in the graphs, whereas in the lower layer the water percentages had increased by 2%. In the graphs the mean values of the water percentages are given.

6.3.4.3. Conclusions

From these experiments it may be concluded that seeds of *Plantago lanceolata* and *P. coronopus* were able to germinate at a lower level of soil water than the other species studied.

From the experiments on the influence of soil compactness on germination of *Plantago* seeds it can be concluded that at an optimum availability of soil moisture, the soil compactness is the limiting factor for germination (Fig. 7), whereas at a low level of soil water, the soil humidity is the limiting factor. The water availability is better for seeds in a compacted soil, since the capillary rise is better. In the experiments with a low level of soil water this resulted in a higher percentage of germination in compacted soils.

In addition, it may be concluded that under all of the conditions studied the mechanical impedance is the limiting factor for the germination of seeds of *Potentilla tabernaemontani*. When, at the end of the tests with a low level of soil water, the water content was raised to the optimal level the results of these treatments showed that all of the ungerminated seeds of *Plantago lanceolata* and *P. coronopus* were still capable of germination, whereas those of the other species showed no additional germination. It is possible that during the experiment (see section 4) a number of just-germinated seeds of *Plantago major*, *P. media*, and *Potentilla tabernaemontani* died due to water restriction. The low germination of *Plantago major* could also have been the result of a seasonal factor, since these experiments were carried out in the winter. These tests are to be repeated in other seasons.

6.4. THE STUDY OF THE ROOT-NODULE SYMBIOSIS OF *Hippophaë rhamnoides*; A METHOD TO ESTIMATE THE NUMBER OF INFECTIVE PARTICLES PER UNIT WEIGHT OF NODULE MATERIAL (P. A. I. Oremus)

6.4.1. Introduction

To study the population dynamics of the microorganism that lives in symbiosis with *Hippophaë rhamnoides*, resulting in the formation of root-nodules, it is necessary to develop a method to estimate the number of

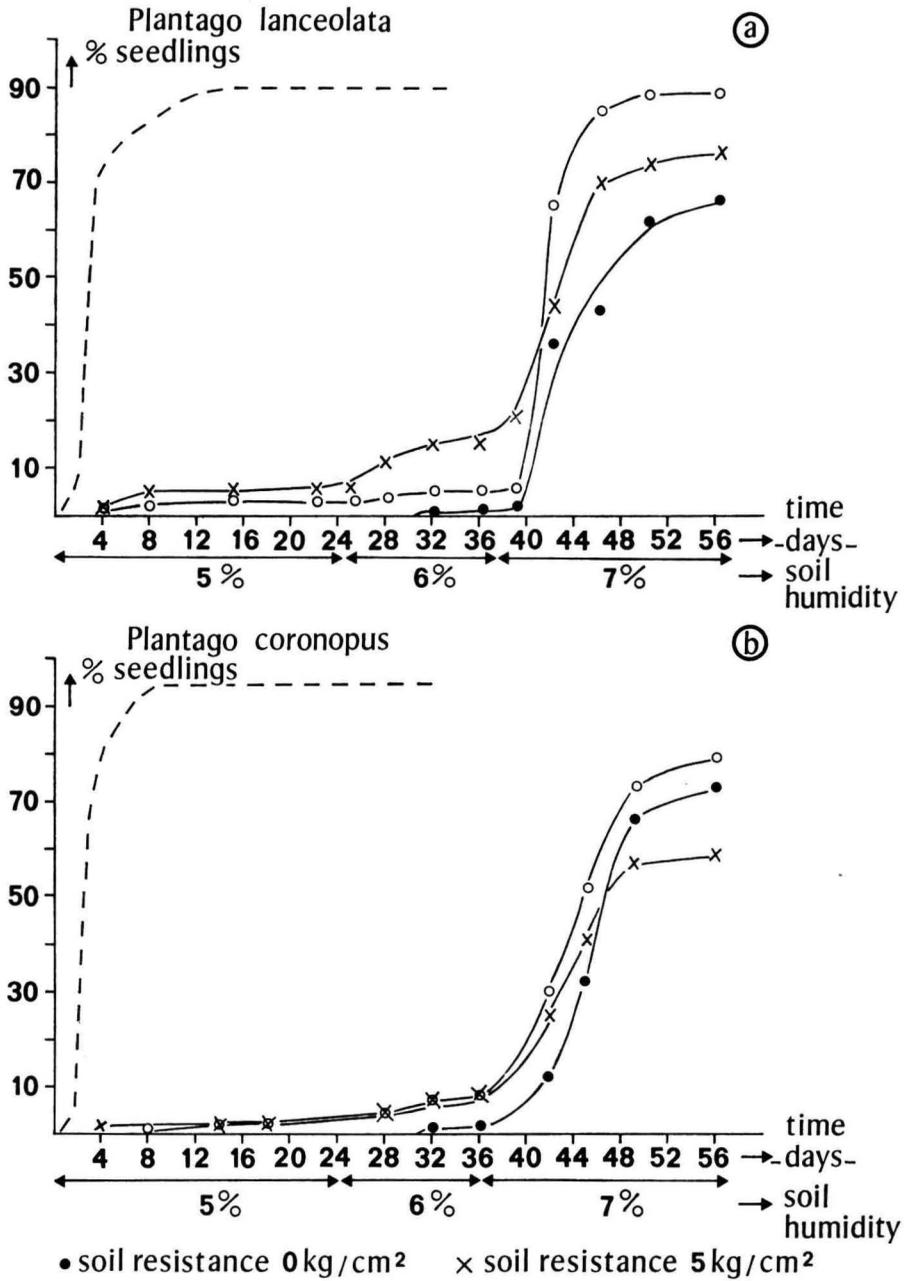


Fig. 9. The influence of soil compactness and soil humidity on germination.

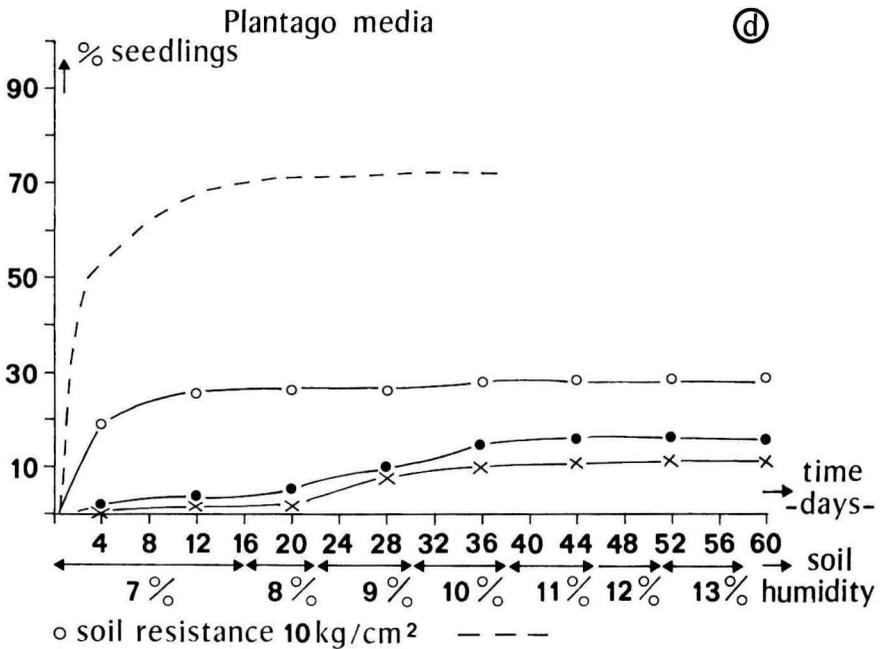
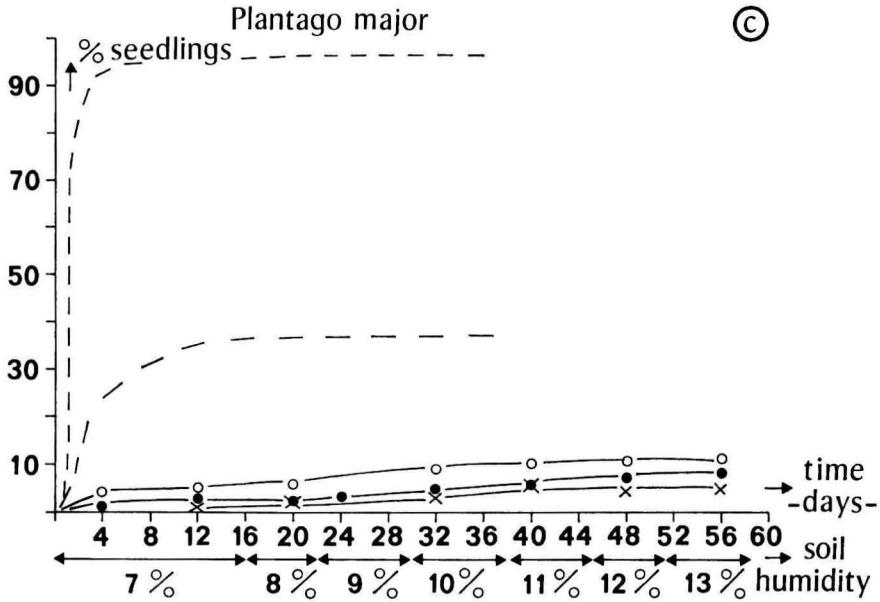


Fig. 9.

infective particles (the infectious state of the root-nodule endophyte) per unit weight of soil and nodule material. Because the microorganism concerned is an obligatory symbiont, the only way to estimate this number is by inoculation of endophyte-free plants with a suspension of soil or root-nodule material and counting the total number of newly formed

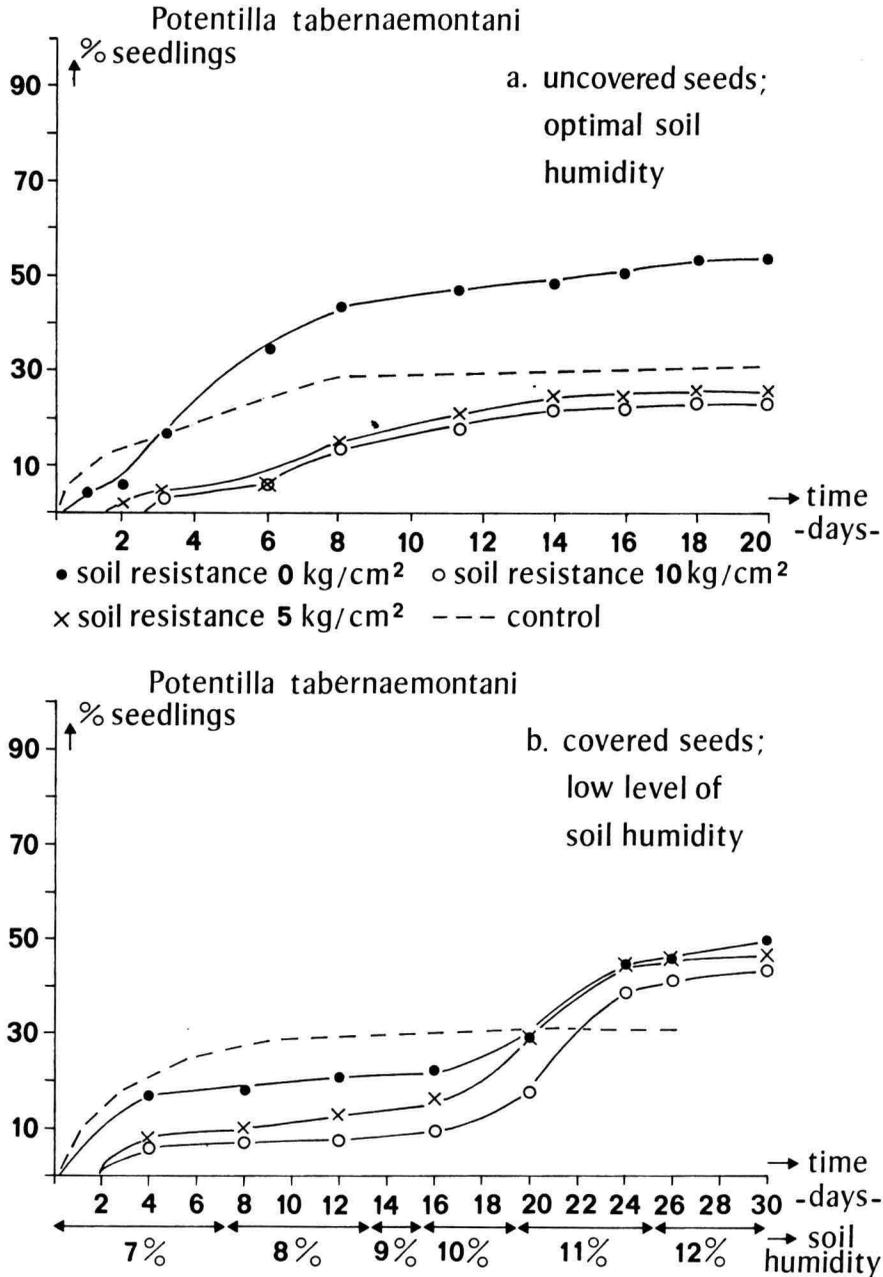


Fig. 10. The influence of soil compactness on germination of *Potentilla tabernaemontani*.

nodules. This number gives an indication of the total amount of infective particles in the examined soil or nodule material.

The experimental part of the study was therefore started with experiments on the germination, cultivation, and inoculation of endophyte-free *Hippophaë* plants (1971 and 1972 Progress Reports). The first experiments showed that there is no simple linear relationship between the amount of nodule material and the number of newformed nodules (1971 Progress Report). Therefore, there must be other factors besides the number of infectious particles affecting the formation of nodules. The first factor examined was the age of the test plants (1972 Progress Report). With plants older than 50–70 days, nodulation decreased sharply. This in contrast to the expectation of an increase in the total number of newly formed nodules with increasing age of the plants because of the enlargement of the available root-hair surface.

Repetition of these experiments indicated that aging of the test-plants did not depress nodulation: the acidity of the culture medium was responsible. In experiments with three plants (36 days old) placed together in 100 cc Crone solution the pH dropped within 5 days from 6.7 to less than 5.4, the minimum value for nodule formation (BOND *et al.*, 1954). With a higher number of test plants per culture unit this critical value is reached sooner. On the basis of these results we changed the inoculation method after optimization of the components involved, i.e., the culture medium, the age of the test plants, and the number of test plants per unit volume of culture medium.

6.4.2. *Inoculation method*

Six pre-cultivated *Hippophaë* plants (1971 Progress Report, pp. 94–96; culture medium Hoagland II instead of Crone) were placed 45 days after germination in 250 cc glass jars provided with 200 cc N-free Hoagland II solution (pH=6.8) and a known quantity of ground nodule material. This nodule suspension was prepared from a living nodule gathered in the field. The plants were placed in a phytotron with the following climatic conditions: temperature 23° C, air humidity 70%, light intensity at culture level 30,000 lux, 16 hours of illumination alternating with 8 hours of darkness.

After 7 days the plants were placed in sterile Hoagland II (-N) solution, which was renewed once a week. The pH was measured regularly and adjusted if necessary. About 14 days after inoculation the first nodules were visible. The nodules were counted once a week. The experiments were stopped if the number of nodules did not increase over a period of one week.

6.4.3. *Number of infective particles per mg nodule material*

Plants were inoculated with 10^{-3} , 10^{-2} , 10^{-1} , 10^0 , or 10^1 mg of nodule

material per jar. The use of low amounts of nodule material ($< 10^6$ mg per jar) leads to a linear relationship between the logarithm of the amount of nodule material and the logarithm of the number of nodules formed (fig. 11). With greater amounts of nodule material, root nodule formation is limited by some other factor, possibly having something to do with, for instance, the total amount of available root-hair surface.

The equation for the regression line calculated within the linear part of the curve is: $y = 0.40 X + 2.38$. The intersection of this line with the X-axis gives the amount of nodule material forming just one nodule. This intersection point is $(-6.0, 0)$, which means that 10^{-6} mg nodule material gives just one nodule; in other words: in 1 mg nodule material there are at least 10^6 infective particles. Since it is not yet certain that the conditions for inoculation as regards all factors involved are optimal by now, the real number of particles might still be higher.

Reference

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6.5. THE DISTRIBUTION OF THE ROOT-NODULE ENDOPHYTE OF *Alnus glutinosa* (L.) GAERTN. IN THE FIELD (C. van Dijk)

6.5.1. Introduction

The root nodules found on *Alnus glutinosa* are induced by actinomycete infection of young roots. The perennial root nodules thus formed are completely symbiotic. Growth of the root-nodule appears to be closely related to growth of the actinomycete within the nodule. Free growth of the root-nodule actinomycete in the soil has never been observed, and growth experiments under artificial conditions were also unsuccessful. This means that the root-nodule actinomycete behaves as an obligatory symbiotic microorganism that must be present in soil in a non-growing, latent form. The population of free soil endophyte is maintained by the input of vital actinomycete fragments from decaying root nodules, as confirmed by earlier experiments.

One of the complications in the study of the population dynamics of the root-nodule endophyte in soil is formed by the existence of two different strains which can only be distinguished by the presence or absence of granules within the root nodules. Granules are most probably spores. The granule-containing strain will be called Gr+, the granule-free strain is called Gr-. The number of infective particles per weight of living nodule is about 1,000 times higher for the Gr+ type as compared to the Gr- type nodule. This difference does not seem to be reflected in the ratio of Gr+ and Gr- nodules found in alder vegetations. In many cases Gr+ nodules and Gr- nodules are homogeneously distributed

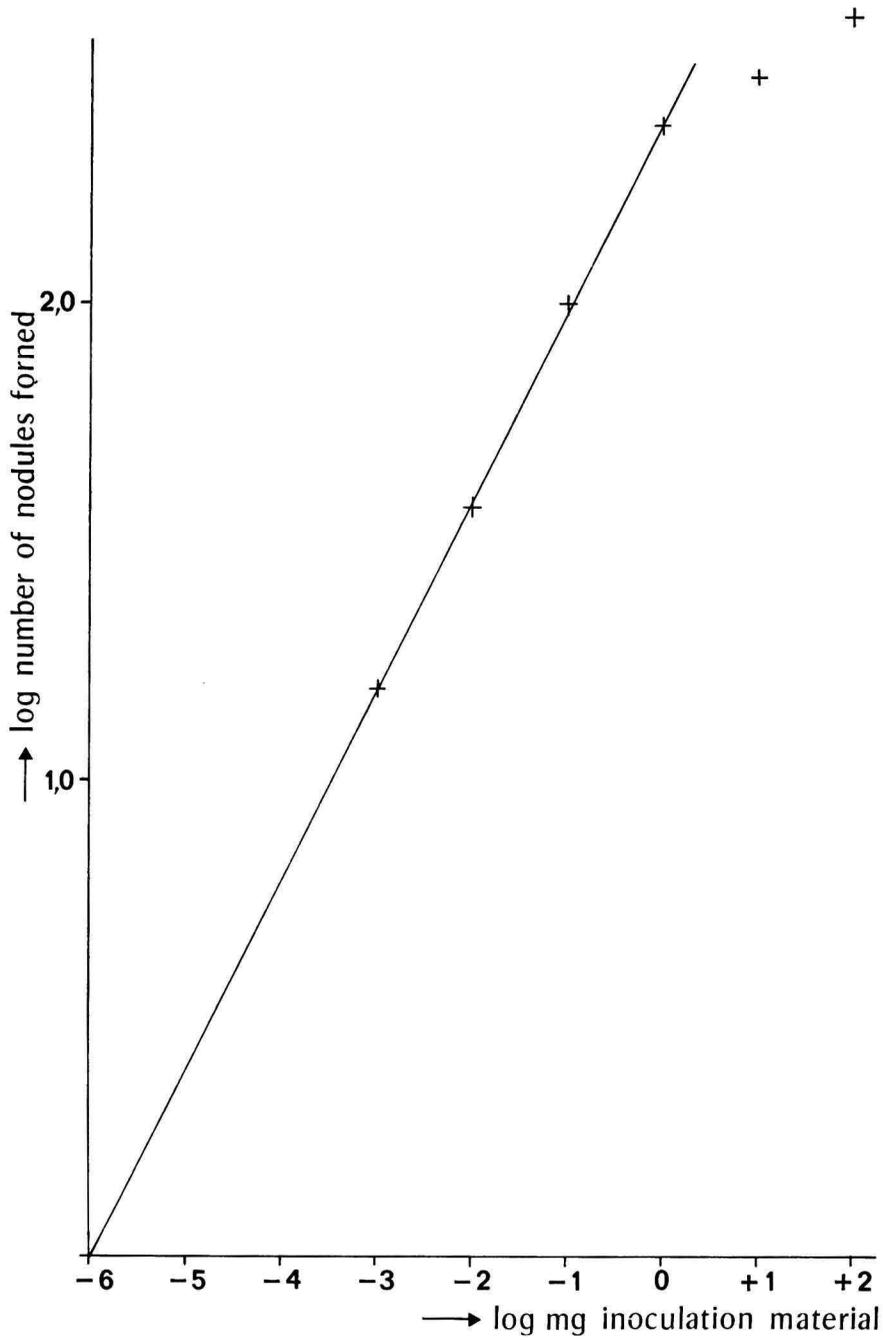


Fig. 11. Number of newly formed nodules in relation to the amount of nodule material used. Each point is the mean of 3 replicates.

or arranged in small clusters and over-all numbers are often about equal. In one case large-scale spatial separation was clearly observed within an alder vegetation in De Wieden, but this finding did not show any correlation with environmental differences. These observations suggest that differences in the population-dynamic behaviour of both strains are caused not only by differences in numbers of infective particles per nodule weight but also by environmental factors. To get some idea of these environmental factors, the occurrence of both strains in alder populations of different habitat will be investigated.

6.5.2. Description of alder populations in the dune region of Voorne

In the dune area of Voorne various more or less isolated alder vege-

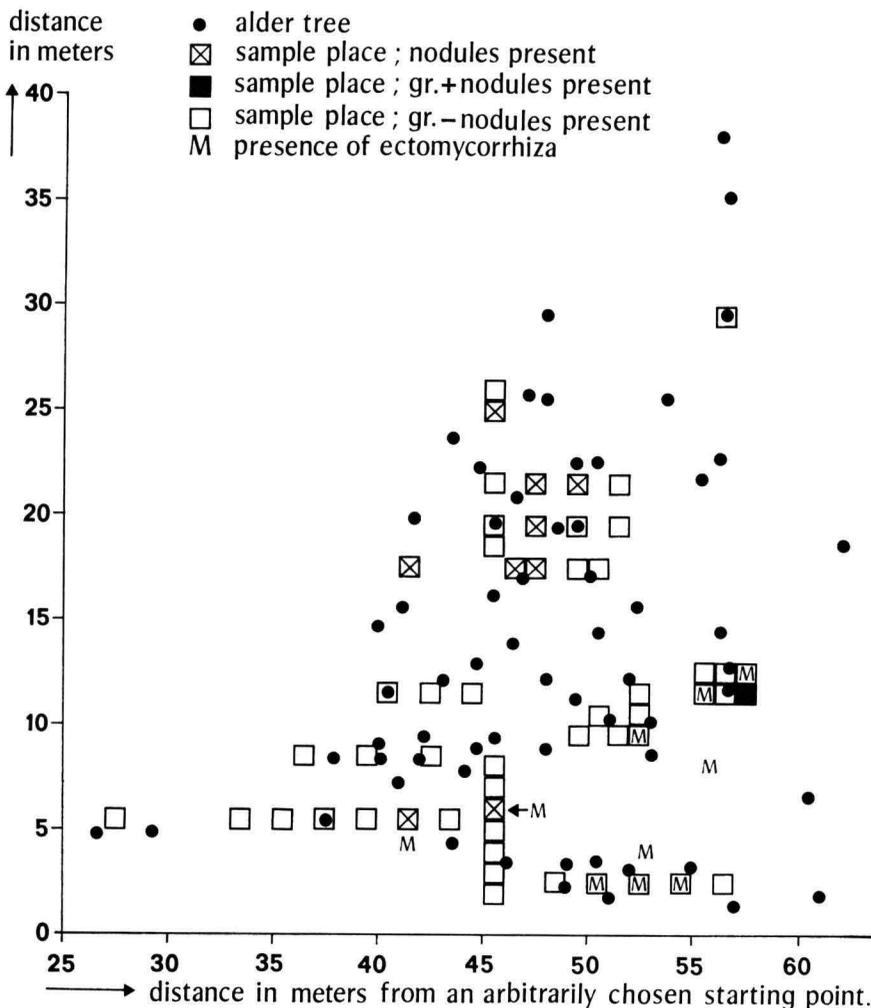


Fig. 12. Map of an alder vegetation in the dune region of Voorne. Trees and sample places are mapped according to an arbitrarily chosen co-ordinate system.

tations are present in humid valleys. In some cases it is not certain whether these populations settled spontaneously or were planted, but in general their development is rather undisturbed. This is favoured by the fact that almost the entire dune area is under the management of the nature conservancy program.

The oldest dune area consists of a weakly undulating landscape characterized by mosaic patterns of small cultivated areas and waste land. In this part alder is often used as windscreen around fields. The alder vegetation under investigation is situated in a dune valley which is about 50 years old and managed as a nature reserve. A high stand of about 20-year-old alder trees covers an area of 1,000 m². Within this area height differences are negligible. The thickness of the humus layer varies from 15 to 30 cm on a Ca-rich sandy subsoil. About 80% of the tree layer consists of alders with a total crown cover of about 70%. The herb layer is dominated by *Cannabium eupatorium* and *Urtica dioica*.

6.5.3. Methods

Root-nodule sampling was performed in squares with an area of 1 m². Sampling sites were chosen as shown in fig. 12. To prevent damage of the vegetation, sampling could not be carried out entirely systematically. If present, 10 nodules per square were collected for microscopical strain type analysis (presence or absence of granules).

6.5.4. Results

According to tree distribution, alder roots could be expected under a 750 m² surface. Root nodules were collected from 53 m² soil surface. Sample size was 7%.

Table 9 gives an impression of the distribution of root nodules in the area searched. The strain type is not taken into account in this Table.

Table 9.

Number of nodules per m ² (per sample place)	Number of sampling- sites/nodule classes	Total number of nodules/nodule classes
0	9 m ²	0
1-9	34	193
10-30	10	107-300
	53 m ²	300-500

It was not possible to calculate the exact number of nodules per 53 m². In most cases maximally 10 nodules per m² were collected and the exact surplus was not counted. Only a few of the sampling sites had up to about 30 nodules per m². The exact number of nodules in the area searched (53 m²) must lie between 300 and 500, the mean number of nodules per m² rooted soil being 6.9.

Samples taken in a comparable alder vegetation at a distance of about 1 km from the alder vegetation under study appeared to be exclusively of the Gr— type. Those taken from various alder hedges, on the contrary, appeared to be predominantly of the Gr+ type. Table 10 shows the results of the microscopical strain type analysis of the nodules collected.

Table 10.

	Gr+	Gr—	% Gr+ of total nr of sampling sites
Number of sampling sites	1	52	1.9
Number of nodules	3	297	1.0

6.5.5. Discussion

Except for 3 clustered Gr+ nodules, only Gr— nodules were found in the whole area. The sampling site where the Gr+ nodules were present apparently did not differ from the Gr— sites. The soil pH in the neighbourhood of the Gr+ nodules is consistent with the mean soil pH of the searched area (pH = 6.7).

The mean number of nodules per m² is about 10 times lower than the nodule density in an investigated Gr— area in De Wieden (peat bog). The results support the hypothesis the two strains have different ecological amplitudes which under certain environmental conditions leads to a spatial separation of the two strains.

Before conclusions can be drawn concerning the factors affecting nodule type in the field many more alder vegetations have to be investigated, and laboratory experiments are also necessary. The present results suggest that large-scale spatial separation of endophyte strain is a rather common feature.

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LIMNOLOGICAL INSTITUTE, NIEUWERSLUIS

PROGRESS REPORT 1973

History and function of the institute

The Limnological Institute in Nieuwersluis, a small village between Amsterdam and Utrecht, dates from 1957. Research is done on the biological and chemical hydrography of the fens and lakes near the institute and also in the large fen region in the north-eastern part of the Netherlands (Tjeukemeer). Special study is made of the ecological relations between the organisms and their milieu, with respect to its chemical composition which changes both in depth and in time; also the relations between the various organisms are investigated. The institute organizes courses in limnology for students of the neighbouring universities in Amsterdam, Leyden and Utrecht. It takes an active part in the International Biological Programme (IBP); it organized an IBP panel meeting in 1966 and offers facilities to an IBP-Unesco fellow from abroad wishing to train or do research in limnology.

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INTRODUCTION

The studies on the lakes Vechten and Tjeukemeer were continued. A working group on the morphogenesis of water plants was started with research on developmental and competitive phenomena in macrophytes. This group will expand its activities in the coming years and will include investigations on morphological aspects of algal development.

The other projects are a limnological survey of five lakes near the river Vecht and fourteen lakes in the Frisian polder system.

The perusal of the food budget of *Daphnia magna* was continued.

Project-group Tjeukemeer

ABIOTIC ENVIRONMENT (Dr. H. L. Golterman)

Routine analysis of the abiotic environmental factors provided basic data to explain the primary production. These abiotic factors change drastically under the influence of water movement. During summer there is an eastward water movement from the IJsselmeer, while in winter there is a westward water movement due to the inlet of polder water. The chloride concentration fluctuated around the value of 1.6 meq.l⁻¹ in March, increased almost linearly from April onward, reaching a maxi-

imum value of 6.6 meq.l^{-1} in September. From October to December it decreased linearly again to a value of 2 meq.l^{-1} . So from April to September, water from the IJsselmeer is continuously entering the lake, whereas from the beginning of October, polderwater is let into the lake. As a consequence, the values for calcium and bicarbonate increased during spring and summer from 2.4 to $3.2 \text{ meq.l}^{-1} \text{ Ca}^{2+}$ and from 1.5 to 2.0 meq.l^{-1} bicarbonate respectively. In June and July the bicarbonate value dropped to its original value again.

The (artificially) raised calcium content of the Rhine water is therefore noticeable even in the Tjeukemeer, as without pollution, the values for both calcium and bicarbonate should be below 1.8 meq.l^{-1} . The pH value of 8 in winter increased to 9.5 in early summer. During May and June the product of calcium and bicarbonate concentrations reached a value of thirty times the saturation value, corresponding with earlier published values. It is possible that the decrease of the bicarbonate concentration is due to this fact. During the second half of summer the pH value decreased to 9.

The o-phosphate, silicate, nitrate and ammonium concentrations showed the normal picture, i.e. high in spring and low in summer.

The concentration of o-phosphate decreased from 200 mg.l^{-1} to 10 mg.l^{-1} of $\text{PO}_4\text{-P}$, the particulate phosphate fluctuated between 100 and 150 mg.l^{-1} in summer. The low values for o-phosphate in summer might be explained by saturation of hydroxyapatite, as its solubility product is exceeded.

During March the silicate concentration decreased from 4.0 mg.l^{-1} to 0.1 mg.l^{-1} of $\text{SiO}_2\text{-Si}$. There was an abnormal increase during June and July to a value of 1.4 mg.l^{-1} , which decreased again afterwards. As there occurred a similar variation in the silicate concentrations in the lakes Grote Brekken and IJsselmeer, it seems probable that the silicate came from the IJsselmeer. In February the nitrate concentration increased from 1.4 to 2.4 mg.l^{-1} of $\text{NO}_3\text{-N}$, while the same amount of $\text{NH}_3\text{-N}$ disappeared. As the water temperature was still below 5°C , this could be an indication of possible bacterial nitrification at this low temperature. Between the end of March and the beginning of May approximately 2.5 mg.l^{-1} of $\text{NO}_3\text{-N}$ disappeared, of which only 1 mg.l^{-1} is bound by algae. This denitrification which is observed every year, was shown in preliminary experiments to be related to light and algae. Therefore, the earlier suggested importance of bacterial denitrification does not seem likely.

BACTERIOLOGICAL AND ECOLOGICAL IMPORTANCE OF HUMIC SUBSTANCES (Drs. H. de Haan)

Investigations on the humic substances in Tjeukemeer have been continued with emphasis on the dynamics of amino-nitrogen and hexoses in relation to humic fractions of different molecular size. The problem is to try and distinguish between amino-nitrogen and hexoses which are

free and those which are bound to humic substances. From the elution patterns at different pH values after Sephadex G-25 separation, it has been calculated that 35-45% of the hexoses are linked to humus fraction (F I); the value for the amino-nitrogen being 30-60%.

In the further study of possible co-metabolism of humic compounds, benzoate was chosen as a biodegradable "analogue" of humus. The F I fraction appeared to have two different effects on a Gram-positive unidentified bacterium growing on benzoate. In the first place, this humic substance fraction did not allow the occurrence of the diauxic growth pattern by enzyme induction. In the second place the addition of 0.1% of this F I fraction to a medium containing 0.1% of benzoate increased the cell yield by 100%. Since this bacterium is also capable to grow slowly in a medium with the F I fraction as the only carbon source, the observed cell yield increase is more likely to be due to a priming effect than to co-metabolism. Chemical analysis of the media before and after growth of this bacterium gave indirect evidence of a chemical change in the F I fraction by the action of this bacterium. Carbon analysis of the media before and after growth gave direct evidence of the use of the F I fraction by the bacterium, as an additional carbon source when growing in a F I containing benzoate medium.

Addition of fraction F II, having smaller molecular sizes, increased the cell yield of this Gram-positive bacterium three times more than the F I fraction does.

PRIMARY PRODUCTION (Dr. H. L. Golterman, Mr. R. F. Liqui Lung)

Primary production showed low values in March, but increased afterwards, reaching high values in June, when the maximum for 1973 was recorded. After this maximum there was a gradual decrease again, until during September and October the value fluctuated around 2-3 $\text{g.m}^{-2}.\text{d}^{-1}$ of O_2 . The mean value for the period between March and November was 4.68 $\text{g.m}^{-2}.\text{d}^{-1}$ of O_2 . The chlorophyll concentration was between 60 and 70 mg.m^{-3} , with incidental high values in May (150 mg.m^{-3}). There was no correlation between the maximal value of primary production in June with a corresponding high value of the chlorophyll concentration. The O_2 -uptake fluctuated around the value of 2.4 $\text{mg.l}^{-1}.\text{day}^{-1}$, corresponding to a value of approximately 4.8 $\text{g.m}^{-2}.\text{d}^{-1}$.

The uptake was measured, with DCMU being present to stop photosynthesis in the light, for two hours. If the O_2 -uptake is measured for a longer period, a lower value is found (75%). Apparently, calculations of net respiration remain uncertain. The uptake of oxygen remained at a value above that of the equivalent decrease in cellular carbon and nitrogen. The large variation between the fortnightly measurements, however, did not allow the calculation of a mean value.

Still it is clear, that part of the O_2 -uptake is due to bacterial decomposition of soluble organic substances. The Q_{10} value of the O_2 -uptake

was between 2 and 3, with incidental deviations. Therefore, artificial raising of the temperature could cause a strong increase of the O₂-uptake. Mineralization of cellular carbon and nitrogen showed again a parallel course. They both increased gradually from March till June, after which they slowly decreased again. The number of proteolytic bacteria fluctuated strongly around a value of 1×10^6 per litre. There is no apparent relation between the number of proteolytic bacteria and the mineralization of cellular carbon and nitrogen.

PHYTOPLANKTON-ZOOPLANKTON ENERGY TRANSFORMATIONS (Dr. R. D. Gulati)

The productivity studies on the food, feeding and metabolism of the consumer zooplankton in Tjeukemeer were continued in the year 1973. The investigation was further extended to include the estimates of carbon and nitrogen in different seston fractions as well as the ¹⁴C-uptake capacity of these fractions.

The maximum value for the total organic carbon (TOC) during 1973, as well as in 1972, was recorded in spring. The total organic nitrogen value reached its maximum in summer for both years. The dissolved organic carbon contributed about 62% to the total organic carbon (average 24 mg.l⁻¹ of C during 1972 and 1973).

Particulate organic carbon as phytoplankton and zooplankton formed the rest of the total organic carbon, in a percentage of 35 and 3 respectively.

The figures for total organic nitrogen (average 2.8 mg.l⁻¹) were: dissolved organic nitrogen 46%, particulate organic nitrogen from phytoplankton 48% and particulate organic nitrogen from zooplankton 6%. Nitrogen as percentage of carbon in the three forms was 8.4, 16 and 21% respectively, in dissolved organic matter, phytoplankton and zooplankton.

A study of phytoplankton in the lake indicated that both the average carbon (5.4 mg.l⁻¹) and nitrogen (0.9 mg.l⁻¹) in the 4–15 μ size fraction formed two-third of both total carbon and nitrogen in phytoplankton and detritus. This fraction, however, contributed to only 50% of the total primary production in in vivo experiments at one light intensity. This relatively low production efficiency is due most probably to the high detrital content of this fraction. The fractions of phytoplankton above 15 μ contained together 36% of the total carbon and nitrogen, but contributed to 48% of the primary production. The fraction of phytoplankton above 85 μ is the most efficient, constituting 5% of the total standing crop but contributing to 12% of the production.

The biomass of consumer zooplankton was about 6% of the total phytoplankton standing crop in spring and summer. *Daphnia* spp., *Bosmina coregoni*, and *Chydorus sphaericus* were the most important filter feeders during summer and rotatorians the main consumers in spring.

The average consumption rate (1.75 g.m⁻².d⁻¹ of carbon), of herbivore zooplankton based on the phytoplankton fraction smaller than 15 μ in

summer 1973 was nearly twice the value of the year before. Nearly one-fourth of the ingested energy ($0.44 \text{ g.m}^{-2}.\text{d}^{-1}$ of carbon) was assimilated and of this amount about three-fourth was used as the energy of maintenance and metabolism. The remaining energy ($0.11 \text{ g.m}^{-2}.\text{d}^{-1}$ of carbon), went into the tissue growth or yield to predators, i.e. net productivity of the consumers (K_2 tissue growth efficiency 24%). In other words the net production of the herbivore zooplankton community during the production period May-September was $168 \text{ kg carbon.ha}^{-1}$ or 7×10^6 joules. ha^{-1} . The production rates in the other periods were: 0 in winter, 0.07 in spring and $0.03 \text{ g.m}^{-2}.\text{d}^{-1}$ of carbon in autumn.

POPULATION DYNAMICS AND PRODUCTION OF ZOOPLANKTON (Drs. J. Vijverberg)

The production of *Copepoda* and *Cladocera* is studied on a species basis. The chemical composition and energy content of four cladoceran species, one calanoid copepod and a mixed sample of four species of cyclopoid copepods were determined. The relative composition, expressed in percentages of the total organic matter, was very much the same for each of the species analyzed. The mean relative composition for all these species was: 71.2% protein, 9.5% carbohydrate and 19.3% lipids (16.5% total nitrogen and 2.1% total phosphate). A substantial amount of the total nitrogen was found to be of non-protein nature (with a mean value of 23.3%).

The chemical oxygen demand (COD) and the calorific values of the different species were determined.

The COD value calculated from the protein, carbohydrate and lipid content agreed well with the measured values. The relation between total organic matter and the calorific values were constant. They were almost the same for the different species. The calorific values ranged from 5847 to 6355 cal.mg^{-1} of total organic matter (excluding chitin). The organic matter over COD—ratio had a mean value of 0.51. (see table 1).

POPULATION DYNAMICS AND PRODUCTION OF THE *Chironomids* (D. M. Beattie, M.Sc.)

A detailed description of the production and distribution of the majority of chironomid species in the Tjeukemeer, using a population dynamics method, is expected to be completed next year. Special consideration is given to factors having an important influence on the magnitude of production. Most of the year has been spent in processing the collected data over the period 1968–1973, which will be submitted as a thesis.

In cooperation with Vijverberg, the variation in the chemical composition of the larvae of the overwintering generation of *Pentapedillum uncinatum* was studied over the period from October 1972 until July 1973. From October until mid-March the 2nd instar larvae were in diapause; the total biomass per larva decreased slightly, due to a decrease

Table 1. Constants of the equation: $\log W = a \log L + b$, relating body length (L; in mm) to content (W) of chemical constituents (in μg) or energy (in $\text{cal} \times 10^{-3}$) of individual Cladocera and Copepoda.

	Cladocera								Copepoda			
	Eucladocera						Haplopoda		Calanoida		Cyclopoida	
	Daphnia hyalina		Bosmina coregoni		Chydorus sphaericus		Leptodora kindtii		Eurytemora affinis			
	a	b	a	b	a	b	a	b	a	b	a	b
Carbohydrates	2.06	0.22	3.29	1.41	3.66	4.68	2.62	0.05	2.11	0.12	1.47	0.18
Protein	2.63	2.34	3.05	9.77	4.29	91.20	4.79	0.02	3.97	0.81	1.67	1.07
Lipids	2.23	0.69	2.51	1.66	6.67	416.90	2.97	0.04	2.10	0.35	1.81	0.26
Total organic matter	2.18	3.09	2.96	12.89	4.64	195.00	2.93	0.34	3.21	1.41	1.68	1.74
N total	2.27	0.47	4.36	7.94	5.97	123.10	2.86	0.05	2.76	0.29	1.95	0.32
P total	2.49	0.06	2.17	0.15	3.76	1.29	2.80	0.00	1.99	0.04	1.64	0.04
C.O.D.	2.83	7.24	3.60	51.29	4.75	467.80	3.08	0.48	2.39	3.39	1.68	3.31
Caloric content	2.19	19.50	2.90	75.86	4.87	1585.00	3.02	1.78	3.06	9.12	1.72	10.47

in sugar content, giving a relative rise of the protein content. After the diapause was broken and growth resumed in April, the percentage of protein, slightly increased, whereas the absolute amount of protein increased strongly until the end of May. The percentage of fat also remained fairly constant, but again an absolute increase occurred, as the reserves are formed especially in early June. Sugar percentage continued to decrease until the end of May when pupation caused the mobilization of fat and protein, at which time both absolute and relative sugar values rose sharply.

The biomass of the female insect is almost twice as large as that of the male (136.8 against 71.6 μg). The sugar and protein percentages however are much alike in males and females.

From the results accumulated over the last few years, it has become apparent that the diapause mechanisms in chironomids is an important factor in determining the magnitude of their ultimate yearly production. Experimental research on the diapause mechanism will be initiated.

POPULATION DYNAMICS AND PRODUCTION OF YOUNG FISH (Drs. J. Vijverberg)

Work in this project is directed to the estimation of the production of fish in Tjeukemeer and the study of the regulating factors. In collaboration with Dr. C. Goldspink, research has been concentrated on the problems of the fecundity of roach (*Rutilus rutilus*) and bream (*Abramis brama*). Further work will be restricted to young Ciprinids (O⁺, I⁺ and II⁺) and fish eggs.

SEASONAL PERIODICITY OF DIATOMS (Dr. J. R. Moed)

To study the controlling factors of periodicity of diatoms in Tjeukemeer, the numbers of these diatoms were counted, while concentration of silicate, phosphate (ortho- and total dissolved), nitrate, ammonia and iron were measured (after filtration through 0.2 μ filters).

The population of diatoms in early 1973 consisted mainly of *Diatoma elongatum* and *Melosira* spp., whereas numbers of unicellular centric diatoms remained relatively low. Decline of the diatom populations could be correlated with dilution of the Tjeukemeer water by polder water (20 February) and depletion of the silicate (9 March). Resuspension of sedimented cells by a spring storm introduced new maxima of *Diatoma elongatum* and *Melosira* at 2 and 3 April. The disappearance of the *Diatoma* population in May can not be attributed to a particular factor, as the concentrations of various nutrients have reached a low level.

It seems likely that depletion of silicate in March caused the diatoms to disappear as the diatoms showed good growth in samples with silicate.

After Si-deficiency numbers of cells dropped quickly, whereas at least for the upper layer (1 m.) a considerable increase of the percentage of bi-protoplasmic *Diatoma* cells was observed. Heavy winds occurring two

weeks later stirred up both diatoms and high densities were reached again. The percentage of bi-protoplasmic *Diatoma* cells was 40–50%. In the following few weeks numbers of *Diatoma* almost doubled while silicate concentration was low, with again a relatively high percentage of bi-protoplasmic cells. The numbers of *Melosira* decreased during the same period.

GROWTH AND STOMACH CONTENTS ANALYSIS OF YOUNG FISH (D. M. Beattie, M.Sc., Mr. W. van Densen, Drs. J. Vijverberg)

Growth and stomach contents of young fish have been recorded throughout the year. Data on the growth of roach, bream, pike perch (*Lucioperca lucioperca*), ruffe (*Acerina cernua*), perch (*Perca fluviatilis*) and smelt (*Osmerus eperlanus*) have been collected each month. Zooplankton was the staple diet of all fish larvae examined. Some species (reaching a length of 5–6 cm) changed to a microbenthos diet, especially benthic *Cladocera*. In the autumn of the first year the pike perch larvae changed their diet to the much larger crustacean species, *Neomysis integer* (*Mysidacea*, *Decapoda*). After their first year the bream feed mainly on microbenthos, the roach on zooplankton and the perch and ruffe on macrobenthos (chironomids, *Gammarus* and *Neomysis*).

During the coming year, research will be directed to observations on the diurnal periodicity in fish feeding behaviour.

Project-group Vechten

ABIOTIC ENVIRONMENT (Dr. H. L. Golterman, Drs. H. Verdouw)

The routine analysis of the abiotic factors in lake Vechten was continued with special emphasis on the increase of certain nutrients above the mud. Strong gradients were found for ammonia, phosphate, iron and manganese (for the latter two see Mud-water relations).

The phosphate concentration increased up to 1 mg per liter just above the sediments; at 2 m. from the bottom no increased concentration could be detected. For ammonia maximum values of 7 respectively 1 mg per liter were observed. The concentrations of silicate, HCO_3^- , Mg^{2+} and Ca^{2+} also showed gradients, but these were much less pronounced.

MUD-WATER RELATIONS (Dr. H. L. Golterman, Drs. H. Verdouw)

Experiments were done on the nutrient supply from the bottom sediments for the growth of phytoplankton. *Scenedesmus* showed good growth on media to which sediments were added as the only source of phosphate. The amount of phosphate taken up by *Scenedesmus* is approximately the same as the amount that can be extracted using 0.01 N NTA. Phosphate bound to unpolluted clay (from Uganda, the Provence and the river Jordan) could neither be taken up by *Scenedesmus*, nor could it be ex-

tracted using NTA. Recently adsorbed phosphate on the other hand, was easily taken up and extractable.

Further work was done on the chemistry of bottom sediments. Fe- and Ca-phosphates were extracted using 0.01 N NTA and Fe-phosphate using 0.01 N Ca-NTA.

The amount of phosphate taken up by *Scenedesmus* from Ca-phosphate (hydroxyapatite) was dependent upon the size of the crystals.

Verdouw followed the complete sequence of changes in the concentration of Fe^{2+} , Mn^{2+} , Ca^{2+} , Mg^{2+} in the eastern part of lake Vechten, during the whole year, in the water as well as in the mud. The samples were obtained using a Jenkins mud sampler.

The aim of this investigation is to elucidate the factors controlling the regulation of the concentrations of the different ions in the lake. The concentrations of all the ions mentioned (which also holds for HCO_3^-) increase with depth in the sediment. This is not only the case with the interstitial water but is also true for the total of the exchangeable fraction, as far as it has been determined. These gradients are present during the whole year, but most strongly so during the circulation period. Between 5 and 10% of the exchangeable fraction is in solution in the interstitial water. Loss of ions by diffusion can therefore be compensated from a large stored supply. The same is true for NH_4^+ , of which an average of 15% of the exchangeable fraction is in solution. Only in the case of Mn^{2+} , a decrease in the exchangeable fraction by diffusion to the hypolimnion is measurable. The concentration of Fe^{2+} increases during stratification, both in the hypolimnion and in the interstitial water in the upper 3 cm, from 30 to 55 mg.l^{-1} .

During stratification, a gradient of Mn^{2+} is present in the beginning, but disappears later. The same phenomenon has been observed for Fe^{2+} , but the gradient is present during the whole time of stratification. The Mn^{2+} concentration in the interstitial water is approximately constant at 5 mg.l^{-1} , showing a decrease during the overturn to 4 mg.l^{-1} .

PRIMARY PRODUCTION (Dr. H. L. Golterman, Mr. R. F. Liqui Lung)

In 1973 the primary production in lake Vechten was higher than in the years before, the mean value of 0.49 g.m^{-2} of carbon being 39% higher than the mean value of earlier years. Net production was low, as was the case with the primary production in Tjeukemeer. Oxygen uptake in dinitrophenol stabilized samples was very near to the production value. Therefore, only a small part of the production can reach the hypolimnion, where oxygen is consumed during its mineralization.

The number of proteolytic bacteria in the epilimnion, was rather low compared with the situation in Tjeukemeer, reaching values of 10^5 per litre with incidentally even higher values.

As the primary production is controlled by the phosphate concentration, which is high at the bottom of the lake (0.5–1.0 mg.l^{-1} of $\text{PO}_4\text{-P}$) and

low in the epilimnion (about $5 \mu\text{g.l}^{-1}$), the transport of phosphate in vertical direction is an important factor. This transport of phosphate from the hypolimnion to the epilimnion by diffusion, will be promoted by water movements, but decreases as a result of precipitation of Fe-hydroxide. The exact value of this transport, therefore, can not be calculated. However, photosynthesis experiments showed clearly the impact of this upward transport. Comparing two samples taken with 1 m difference in depth and then incubated with $\text{NaH}^{14}\text{CO}_3$ at the same depth, always showed the greater photosynthesis value in the sample from the deeper sampling site. These experiments will be repeated in 1974, together with phosphate analyses.

PHYTOPLANKTON-ZOOPLANKTON ENERGY TRANSFORMATIONS (Dr. R. D. Gulati)

The productivity studies on lake Vechten were carried out simultaneously with and in a similar manner to that for Tjeukemeer.

The break-up figures for the organic carbon (5.6 mg.l^{-1}) and organic nitrogen (0.62 mg.l^{-1}) in the total organic matter were as follows: 4%, 17% and 79% of the carbon in respectively the zooplankton, the phytoplankton and the dissolved organic matter and 7.7%, 20.6% and 72% of the nitrogen in the same sources. Whereas the carbon and nitrogen correlation both in zooplankton and phytoplankton was positive and significant at 5% significance level, particularly in summer and spring, such a correlation was both negative and insignificant in the dissolved organic matter.

The phytoplankton fraction between 4 and 15μ contributed on the average 85% (range 69–97%) to the total primary production as measured in the laboratory and to 81% of the phytoplankton biomass, both expressed as carbon and nitrogen. The other two fractions, $15\text{--}44 \mu$ and $44\text{--}85 \mu$ together constituted 15% of the primary production as against 20% of the phytoplankton standing crop in these two fractions.

The primary consumer zooplankton in lake Vechten had a summer as well as an autumn mean of 0.46 g.m^{-2} of carbon. During both these periods they constituted three-fourth of the total zooplankton and formed respectively 16 and 24% of the phytoplankton. The phytoplankton which had its average seasonal maximum of 5.7 g.m^{-2} of carbon in spring decreased to the summer average biomass of 2.8 g.m^{-2} of carbon. This could be attributed mainly to the grazing pressure of herbivores discernible first on 3 April 1973 and continuing till 29 May by which date the phytoplankton standing crop fell sharply from 6.9 a fortnight before, to 1.6 g.m^{-2} of carbon. This sharp fall occurred despite the average primary production in this period of $0.350 \text{ g.m}^{-2}.\text{d}^{-1}$ of carbon. The consumer zooplankton community in the 8 week interval increased significantly from 0.10 to 1.25 g.m^{-2} of carbon and the total zooplankton from 0.4 to 1.4 g.m^{-2} of carbon. A simultaneous study of the phytoplankton fractions

revealed that on 29 May, the 4–15 μ fraction contributed 63% to the total phytoplankton standing crop as against its average contribution of 85% during the investigation period. This is most likely due to the maximum grazing pressure on this fraction. Further more, phytoplankton peak in late summer and sharp fall in spring were inversely correlated to the herbivore standing crop. Assuming that all phytoplankton size fractions were equally and efficiently utilized, an average assimilation rate of 137 $\text{mg.m}^{-2}.\text{d}^{-1}$ of carbon in summer by the primary consumers was 40% of their daily ingestion rates. In other words 80% of daily primary production was grazed upon by the herbivores. More than two-third of the assimilated food was used in metabolism, resulting in a net summer production rate of 39 $\text{mg.m}^{-2}.\text{d}^{-1}$ of carbon, giving a mean gross growth efficiency (K_1) of 11.6% and a tissue growth efficiency (K_2) of 28.5%. The production rates in spring and autumn were 12.5 and 85 $\text{mg.m}^{-2}.\text{d}^{-1}$ of carbon, respectively. K_1 and K_2 efficiencies were, respectively, 12 and 49% in spring and 16 and 77% in autumn. The respiratory losses during spring formed 50% and in autumn 23% of the assimilated food.

ECOLOGY OF ANAEROBIC BACTERIA (Drs. Th. E. Cappenberg)

The work on the sulphate-reducing (acetate-producing) and methane-producing bacteria in the mud-water interface in lake Vechten has been continued. The object is mainly to elucidate ecological and physiological relationships between these two types of bacteria in the anaerobic part of the carbon cycle in the lake. The sulphate-reducing bacteria, by the formation of sulfide from sulphate and acetate from lactate have been shown to influence the distribution of acetate-fermenting, methane-producing bacteria. On the other hand, CH_4 and CO_2 produced by the latter type, may function as additional carbon source for the former type.

Experiments were continued using ^{14}C -labelled acetate and lactate, to determine their turnover rate constant (k) and the average substrate pool size of isolated mud samples. For lactate the values were respectively 2.37 hr^{-1} and 12.2 μg per gram wet mud, giving a turnover rate of 28.9 μg lactate per gram mud per hour. The turnover rate of acetate was 1.99 μg per gram mud per hour, (from disappearance measurements) or 0.45–1.74 μg acetate per gram mud per hour, estimated from the formation of $^{14}\text{CO}_2$. These and other results suggest that only part of the lactate dissimilated is metabolized through the acetate pool.

Parallel to the direct determination of turnover rates, mixed cultures of *Desulfovibrio desulfuricans* and *Methanobacterium sp.* were cultivated in a chemostat. From these experiments it seemed likely, that the two species show interlinkage, the *Methanobacterium* depending on the production of acetate by the other. The situation was, however, complicated by the release of hydrogen sulfide by *D. desulfuricans*, inhibiting the growth of *Methanobacterium*.

MORPHOGENESIS OF WATER PLANTS (Drs. P. H. Best, Dr. R. Soekarjo)

Work on the aquatic macrophytes in lake Vechten was started with a comparative study of the development of *Elodea canadensis* and *Ceratophyllum demersum*, to elucidate their competitive behaviour. The maximal occurrence of *Elodea* in lake Vechten is at a depth of 0.5 to 3 m, whereas *Ceratophyllum* is most abundant between 3 to 5 m. A clear boundary is observable, separating the areas of the two species. Monthly, both species are described morphologically and sampled in situ by scuba diving. Caloric values, nitrogen, total protein, mineral and starch contents are determined.

Laboratory experiments on the morphogenesis of *Ceratophyllum* under various conditions of light intensity, day length, temperature and phytohormones are in progress.

Other Projects

ZOOPLANKTON (Dr. R. D. Gulati)

Gulati continued his work on the food budget of *Daphnia magna* using ^{14}C tracer techniques. The relative importance of different size fractions in the lake food—both Vechten and Tjeukemeer—was determined. Despite the relatively low food concentrations ($2.5 \mu\text{g}\cdot\text{ml}^{-1}$) in Vechten water, the daily food ration of *Daphnia* equalled its body weight (mean of 22 experiments). In contrast, the Tjeukemeer had much higher food concentrations ($8\text{--}20 \mu\text{g}\cdot\text{ml}^{-1}$) but the daily food rations were much lower and varied between 24 and 60%. Only in one out of 32 experiments performed did the daily ration equal the body weight.

Further, keeping the food concentration constant but using different labelled fractions, in Tjeukemeer the smallest size fraction, $<15 \mu$, when used as a tracer yielded the maximal filtration rate, $0.7 \text{ ml}\cdot\text{hr}^{-1}\cdot\text{animal}^{-1}$. In contrast, using the $44\text{--}85 \mu$ tracer resulted in the lowest filtration rate $0.07 \text{ ml}\cdot\text{hr}^{-1}\cdot\text{animal}^{-1}$. The filtration data based on the $15\text{--}44 \mu$ and $>85 \mu$ fractions as tracers lay between the two extreme rates. The assimilation efficiencies of the ingested food was also the highest (50%) for the 15μ food and the lowest (28%) for the $44\text{--}85 \mu$ food type. To examine if low filtration rates in Tjeukemeer were due to poor food or water quality, two experiments using same concentration of *Chlorella* ($12 \text{ mg}\cdot\text{l}^{-1}$, D.W.) in the waters of Tjeukemeer and Vechten were performed. The ingestion rates of *Daphnia magna* were 1.5 and $1.8 \mu\text{g Chlorella D.W}\cdot\text{hr}^{-1}\cdot\text{animal}^{-1}$ in the filtered (0.45μ) Tjeukemeer water and 4.2 and $6.0 \mu\text{g Chlorella D.W}\cdot\text{hr}^{-1}\cdot\text{animal}^{-1}$ in the Vechten water. The assimilation efficiency of *Daphnia* was, respectively: in Tjeukemeer water 36 and 42%, and in Vechten water 89 and 61%.

This, besides the feeding experiments using natural lake food, hints at physiological factors, related to water chemistry, which probably inhibit

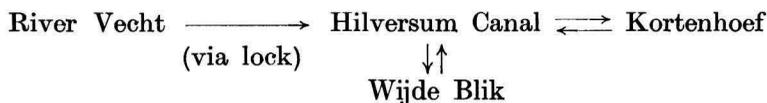
optimal feeding in Tjeukemeer water. This aspect of a study extended to in situ feeding of Tjeukemeer zooplankton is in progress.

LAKES IN THE EASTERN VECHT RIVER AREA (Dr. R. D. Gulati, Mr. H. C. Greven, Mr. H. van Leeuwen, Mr. D. A. Rootert)

Five lakes in the eastern Vecht river area have been investigated as to their water chemistry, phytoplankton and zooplankton. Based on these observations and productivity data, the lakes are arranged according to relative eutrophication level: Wijde Gat > Vuntus > Wijde Blik > Loenderveense Plas > Het Hol.

The causes of eutrophication and pollution of Wijde Gat (Kortenhoeft Plassen) were investigated. Compared to a study carried out by the "Rijksinstituut voor Natuurbeheer" in 1968, it appears that the chloride concentration in the lake has increased by nearly 100% (from 118 to 227 mg.l⁻¹). The source of pollution appeared to be water from the Hilversum canal, which is in direct connection with Kortenhoeft waters and the river Vecht.

Another aspect of the study was to investigate the extent of the influence of both the eutrophic water from the Kortenhoeft area and the polluted water from the river Vecht, both via the Hilversum canal, on the water quality of Wijde Blik.



The water quality in Wijde Blik is threatened by the fact that annually 26.10⁵ m³ of sand is removed from the lake, which is replaced to a great extent by water from the river Vecht via Hilversum canal. The chloride concentration in Wijde Blik (160 mg.l⁻¹) has increased by about 60% in the last 4 years, without a simultaneous increase in nutrients and primary production.

Primary production of *Chlorella* measured in the laboratory using filtered Wijde Blik water mixed with Hilversum canal water in different proportions resulted in higher production (up to 40%) than in the lake water, showing the effect of nutrient enrichment.

These studies have shown that pollution threat to the lakes in this area needs further investigation, though it is clear already that the present situation desires much to be corrected.

THE FRISIAN LAKES (D. M. Beattie M.Sc., Drs. J. Vijverberg, Dr. H. L. Golterman, Mr. W. van Densen)

To obtain a better insight into the factors regulating the abundance of animals occurring in the Tjeukemeer, thirteen other lakes belonging to the Frisian polder system were periodically sampled during 1971 and

1972. Data on *Copepoda* and *Cladocera* and on young smelt (*Osmerus eperlanus eperlanus* L.) were processed in 1973.

The species composition and the seasonal periodicity of *Copepoda* and *Cladocera* were very similar in the different lakes. Absolute population densities of *Copepoda* were relatively low and were not very different, but absolute densities of *Cladocera* were highly variable; the highest densities were found in the Leyen, the Slootermeer and the Brandemeer; the lowest densities in the Bergumermeer, the Princenhof, the Pikmeer, the Sneekermeer and the Morra. When the results of the Fluessen complex (Heegermeer, Fluessen and Morra) were omitted, there existed strong positive correlations ($r=0.85$ for 1971, and $r=0.88$ for 1972) between the mean yearly chlorophyll-a concentration of the lakes and the mean yearly population densities of the *Cladocera*.

The population densities of the O⁺ smelt was many times higher in 1972 than in 1971. Growth was poor during 1972 compared with 1971. At the end of the year the highest mean individual weights were registered in the Leyen (5.9 g in 1971 and 3.9 g in 1972). The mean individual weight of the O⁺ smelt showed a definite linear relationship with the chlorophyll-a concentration in each lake with the exception of Slootermeer and Fluessen complex that had unusually high O⁺ smelt densities in 1972.

The chlorophyll-a concentration was strongly correlated with the concentration of particulate phosphate in summer. In winter the ratio of particulate phosphate to chlorophyll concentration reached high values (ca. 20), mainly due to the presence of particulate humus phosphate. In summer this ratio decreased to about 2 in most lakes; small quantities of particulate humic phosphate will keep this ratio at a higher value than if only cellular phosphate is concentrated on the filter. The quantity of total phytoplankton seems therefore in the whole Frisian lake district to be controlled by the phosphate concentration.

In 1974 the sampling programme will be restarted on seventeen lakes. The south-west part of the lake district will be sampled more intensively. Emphasis will again be on the zoological side, i.e. benthos, zooplankton and fish, but the relationship between the amount of chlorophyll-a and the total phosphate concentration will also be studied.

R. SOEKARJO
H. L. GOLTERMAN

DELTA INSTITUTE FOR HYDROBIOLOGICAL RESEARCH YERSEKE

PROGRESS REPORT 1973 *

In 1957 the Division of Natural Sciences, reacting on an initiative of the Commission for Ecology, created an institute, to be established in the deltaic area of the south-west Netherlands, with the aim of studying the biological changes to be expected as results of the closing of the various river-mouths and sea-arms in this area.

When the Zuiderzee was closed by a dam and converted into the fresh Ysselmeer between 1920 and 1950, extensive biological research was carried out by a group of fishery biologists, members of botanical and zoological societies and academic staff, under the direction of Dr. H. C. Redeke. The important results obtained during this study, warranted the expectation that in the more diversified deltaic area of the rivers Rhine, Meuse and Scheldt, even more results could be achieved, especially so when one agency, located in the area, was given the task to make a co-ordinated effort to study the problems from various angles. After an exploratory phase, in which qualitative distribution of biota is to be studied from an ecological point of view, experimental work will be initiated, in order to elucidate the causal background of the changes observed.

The institute was erected under the name "Division Delta-Research of the Hydrobiological Institute", with the object to stress its affiliation to the Hydrobiological Institute at Nieuwersluis. As both institutes grew and matured the difference between the studies carried out in both of them gradually became apparent and in 1968 it was decided to change both names into their present form.

The institute is located at Yerseke on the Oosterschelde, the sea-arm to be closed in the last stage of the s.c. "Delta-plan".

The exploitation of the institute is financed by means of funds allotted to the Academy.

Scientific Staff

Dr. K. F. Vaas - Director

F. Vegter - Chemist

Dr. W. G. Beeftink - Botanist (Phanerogams)

P. H. Nienhuis - Algologist

R. Peelen - Planktonologist (Northern Delta area)

C. Bakker - Planktonologist (Southern Delta area)

Dr. S. Parma - Experimental Ecologist

Mrs. C. H. Borghouts - Zoologist

Dr. W. J. Wolff - Zoologist

Dr. A. G. Vlasblom - Experimental Zoologist

A. B. J. Sepers - Microbiologist

Miss A. W. Stienstra - Experimental Botanist

* Communication nr. 118 of the Delta Institute voor Hydrobiologisch Onderzoek, Yerseke, The Netherlands.

I. Introduction

Some progress was made this year in the effort to integrate and coordinate current research,—e.g. the work on the Grevelingen and on inland waters,—as well as to extend experimental work.

When the Deltaplan was initiated immediately after the flood-disaster of 1953, aspects of environmental hygiene and nature conservancy were not entertained to such an extent as is the case at present, nor had the pollution of the river Rhine reached such an alarming stage. For these and other reasons a strong movement in the Netherlands is opposed to the law passed in 1958, demanding the closure of various sea-arms including the—still open—Oosterschelde. The government was asked to consider alternative plans, in which safety against flooding is achieved but the Oosterschelde remains in connection with the sea. At the moment the whole problem is being studied by a special commission of experts installed by the Government. This year our institute has been deeply involved in this problem. Staff members were repeatedly called upon to write reports, state our views or give advice, among others to the above commission and to the Minister of Roads and Waterways, who visited the institute with members of his staff.

This year's progress in the technical works carried out by the Department of Roads and Waterways, and, owing to the changes involved, having consequences for our work, comprised dredging of the main gully through the Oosterschelde in connection with the construction of the Scheldt-Rhine canal and additional work on the reinforcement of the bottom of the deep gullies in the mouth of the Oosterschelde.

Miss A. W. Stienstra, an experimental plantecologist, will join the staff in Januari 1974.

II. Descriptive Research

The year 1973 was characterized by a hot and dry summer with low river discharges. That of the river Meuse dropped in August to 1/10 of the average value. Consequently the sluices in the Haringvliet were usually closed, and oxygen concentrations in the main rivers were very low. In the Rhine a minimum value of 2.6 mg/l (= 27% saturation) was reached.

1. *Brielse Meer, Brielse Gat*

As in previous years, R. Peelen found a spring bloom of green algae and diatoms in the Brielse Meer. Already in May *Microcystis aeruginosa*, again with *Phormidium muscicola* in its gelatinous envelopes, could be observed. *Aphanizomenon flos-aquae* and *Oscillatoria agardhii* occurred in smaller numbers than in 1972, probably because the salinity was lower this year (0.22‰ Cl') because less water was introduced.

On the other hand more water was introduced in the Brielse Gat and

here salinity and nutrients both increased. Stratification was again established and oxygen concentrations dropped to 3% saturation in the bottom layers. No H₂S was formed. Plans are being worked out to make the lake less deep. A fairly rich plankton again developed, although somewhat less diversified than in previous year. Among the zooplankton *Podon polyphemoides* can be mentioned as well as the ciliate *Cyclotrichium meunieri*. The μ -algae were again well represented.

Dr. W. G. Beeftink continued his observations on changes in the vegetation of higher plants of the Groene Strand. The vegetation on parts grazed by cows since 1969 is fairly well stabilized by now, on places grazed by goats since 1972 the vegetation of reed, tall herbs and brushwood – *Hippophae rhamnoides* – decreased. *Urtica dioica* developed in spite of grazing by goats.

2. *Haringvliet, Spui, Hollands Diep, Biesbosch*

The hydrographical situation this year was comparable to that of the previous year and thus no spectacular changes in the plankton can be mentioned. However, in the Biesbosch area a stronger development was observed, as witnessed by a doubling of the amount of chlorophyll to 300. 10⁻³ Extinction Units. Blue-green algae – *Microcystis aeruginosa*, *Aphanizomenon flos-aquae* and *Oscillatoria agardhii* were encountered in greater numbers in the entire Biesbosch.

The littoral algal vegetation of the entire area is very uniform and monotonous, according to P. H. Nienhuis' surveys. Consisting of green and blue-green algae it did not change much this year. As this algal vegetation had been a simple one even during the time previous to the construction of the dam in the mouth of the Haringvliet, data from Nienhuis' files were used by Dr. A. G. Vlasblom to construct a dendrogram according to the method of Orloci and thus compare the results of the classical way of classification into various groups according to the methods of plant sociology, with the results of modern mathematical calculation. The results of both methods were found to agree quite well.

Along the Haringvliet halophytes have almost disappeared from the meadows, but for the lowest spots, and have been replaced by trivial grasses. Even the species characteristic for a brackish tidal environment are still deteriorating. Research on this vegetation will be curtailed in the future. This year again various *Taraxacum* taxa were collected along the main rivers further inland and forwarded to Prof. Dr. J. L. van Soest (Leiden) for his studies. This work will not be continued. With the inventory of higher plants along these rivers, to be continued in 1974, the survey of the Delta region will be finished and it is intended to publish all results in an Atlas.

Littoral fauna did not changed either. The western part is still the poorer one and in the Hollands Diep *Pseudamnicola confusa* disappeared. Fresh water sponges were observed for the first time. The fauna of the

Biesbosch is developing towards a stagnant water association. *Acroloxus lacustris* was observed for the first time in the Biesbosch as well as Gammarids in the Haringvliet. *Neomysis integer* is the only opossum shrimp in this water.

Data on the soft bottom fauna are being worked out by Dr. W. J. Wolff. The number of species is increasing slowly, Oligochaetes still predominate.

On two trips a freshwater fishfauna could be sampled containing many more species than in the river further inland, roach and perch being the most frequent species. Two more interesting hybrids were captured by P. de Koeijer: *Bliccopsis erythrophthalmoides* Jäckel and *Alburnus dolabratus* (Holandre).

3. Grevelingen

The activities of various departments in this area have been coördinated by an intramural research group and, since last year, the ultimate construction of the carbon balance of the Grevelingen was formulated as a joint aim. Primary production is being studied by direct methods, secondary production of various major groups of biota will be derived from assessments of biomass.

Some chemical parameters, measured by F. Vegter are given below.

Salinity:	15.8–16.8 ^{0/00} Cl'.
Orthophosphate:	1.3 μgat PO ⁴ -P/l end May – 10 μgat in autumn.
Ammonia:	14 μgat NH ₃ -N/l February – undetectable in summer – 3 μgat in winter.
Nitrite:	7 μgat NO ₂ -N/l winter – undetectable in summer and autumn.
Nitrate:	4 μgat NO ₃ -N/l February – undetectable in summer and autumn.
Silicate:	35 μgat SiO ⁴ -Si/l January – minimum 3 μgat end of spring bloom.
pH:	8.3 spring – 8.0 summer – 7.9 autumn.

Primary production

(monthly measurements): 3 stations: 144–165 and 174 g C/m²/year.

The production data are comparable to Vegter's figures for 1972.

Peelen collected seventy phyto- and zooplankton samples on a single day in March and again in May. After counting and measuring the data were converted to mg C/m² and total amount of C for the whole Grevelingen, with the following results:

Phytoplankton			Zooplankton	
22/3	630	69.3 × 10 ³	235	25.9 × 10 ³
17/5	217	23.9 × 10 ³	76	8.3 × 10 ³
	mg C/m ²	Total kg C	mg C/m ²	Total kg C

In the 52 m deep pit at Scharendijke a thermostratification was again established, this time in medio May. In July H₂S could be detected in a 2 m deep layer over the bottom, with a concentration of 2.6 mg/l, notably less than in previous year. The reason why this phenomenon was less pronounced than in the year before, might be found in the reduced runoff from the polders, and this may also have resulted in the absence of the sudden destruction of phytoplankton witnessed in 1972. In the zooplankton *Harpacticus chelifera* and *Podon polyphemoides* developed so well this year that the larvae of *Polydora* could no longer dominate the picture as they have done heretofore.

The impressive vegetation of macrophytobenthos—no doubt a major constituent in the carbon cycle of the lake—was quantitatively investigated this year by a team including three divers, under supervision of Nienhuis. Divers surveyed and mapped the submerged vegetation and took samples from representative squares. The wet weight was measured as soon as possible in the laboratory and from a series of samples the dry weight and the carbon content were established. The vegetation was split up in 6 groups, together responsible for 95% of the total vegetation. The more than 200 samples, on the average taken about 600–700 m from one another, cover the entire surface of the lake and all vertical planes down to 6 m. The results can be summarized as follows:

	1	2	3
<i>Zostera marina</i> + <i>Z. noltii</i>	13.9	64.9	9.0
Ectocarpales (Brown algae)	5.0	35.1	1.8
Red algae (<i>Polysiphonia violacea</i>)	19.7	2.7	0.6
<i>Chaetomorpha linum</i> , <i>Ch. aerea</i> + <i>Ch. crassa</i>	2.9	13.7	0.4
<i>Ulva lactuca</i> + <i>U. rigida</i>	3.2	32.1	1.0
<i>Codium fragile</i>	<0.1	95.5	0.1

1=percentage of total surface occupied
 2=g/m²C of plant in its territory
 3=g/m²C of plant for entire Grevelingen

It follows that sea grasses are the principle elements of the subaquatic vegetation. They grow in summer in large areas in the eastern part and east of Veermansplaat. Next year some further details will be taken care of in order to refine this preliminary estimation.

In the top layer of the mud flats at Herkingen and Battenoord, drained after the closure of the Grevelingen in 1971, extremely high salinities were once more found. In the dry summer months of this year values as high as 200–300‰ Cl⁻ were measured, against 20‰ in winter. Halophytes are rare in these surroundings. On and in the sand Chrysophyceae and Cyanophyceae (*Schizothrix calcicola*) grow, giving the bottom a brown-

green colour for many hectares. Their gelatinous envelopes glue the sand grains together and thus counteract wind erosion.

On the stony slopes of the sea-walls, where the tidal algae had died immediately after the closure, higher plants rooting between the stones can now be found, with among those many marine lichens (*Xanthoria parietina*, *Caloplaca marina*).

According to Beeftink's surveys of permanent sample plots, still many changes are taking place in the vegetation of the Springersgors. Some halophytes, such as *Halimione portulacoides*, decreased, others—*Glaux maritima*—increased in numbers, as did non-halophytes growing in soils rich in nutrients. Using "false-color" aerial photographs, provided by the Division for Environmental Research of the Department of Roads and Waterways, Beeftink's assistant, W. de Munck, constructed a map 1 : 2000 of the vegetation. Owing to the intrusion of tall herbs (*Epilobium* spp. *Urtica dioica*) the pattern of the vegetation became slightly more coarse.

On the embanked salt marshes along the coast of Flakkee most halophytes persisted, some even increased. On the unembanked salt marsh *Spartina townsendii* almost disappeared and the preliminary outburst of *Suaeda maritima* is nearly finished. The migration of halophytes to lower plots and the deterioration of those that stayed, is accompanied by an intrusion of grasses and tall herbs (*Agrostis stolonifera*, *Cirsium* spp).

In the littoral fauna Mrs. C. H. Borghouts found changes to be of a more quantitative than a qualitative character, this year. *Crepidula fornicata* became more frequent. *Ligia oceanica* and *Orchestia gammarella* have left the plains and salt marshes and can only be found along the shore. *Hydrobia ulvae* persisted until medio 1973 in a severed former creek, filled with almost fresh water and sometimes totally dry. Its reproduction however, had already stopped in 1972. The opossum shrimp *Praunus flexuosus* increased in numbers, contrary to *Neomysis integer*. This shrimp decreased, even in places where fresh water enters. *Mesopodopsis slabberi* also increased. The constant and high salinity of the lake may be the cause of these developments.

Mrs. Borghouts also studied migration of the mysid *Praunus flexuosus* in the lake. During daytime it was only caught near the bottom, between January and March during the night at all depths, from April till the end of May near the bottom only and from June till October again at all depths. No explanation can yet be offered for this behaviour. A significant difference could be found between a population of *Praunus* living near the shore and another one living in deeper water and sampled monthly at the same time. The studies on migration and population dynamics are being continued.

Wolff continued his measurements of the secondary production of the soft-bottom fauna, sampling 6 times 4 representative stations. This work will be continued next year. With the assistance of Miss S. Tyzack, a student from New Castle (U.K.), the role of *Asterias rubens* as a predator

of mussels was studied. The relevant samples had been collected by our divers in June and September. The student A. M. van Haperen (Utrecht) studied feeding of birds—herring gull, black-headed gull, sandwich tern and shelduck—in order to assess their role in the carbon cycle. A. J. J. Sandee studied monthly bottom samples taken since the closure with the object of evaluating changes in diversity during the course of the succession of the fauna. These investigations are still in progress.

Dr. K. F. Vaas took monthly samples of the benthic fishfauna. The population of plaice still survives in spite of heavy fishing by sportsmen. Several times ripe eggs of plaice, flounder and dab were collected and hatched in the laboratory by W. Rozing and by P. van Boven in the aquarium. They succeeded in growing them until a length of 3.4 mm. In spite of all efforts to offer suitable food the larvae could not be kept alive after that size had been reached. The guts of the adult plaice were always crammed with worms, small molluscs and crustaceans. In the colder months shrimps (*Crangon crangon*) and small gobies (*Pomatoschistus minutus* and *P. microps*) were eaten as well.

4. Keeten, Krammer, Volkerak

Since 1971 a constant flow of river water from the Hollands Diep enters the Krammer via the sluices in the Volkerak. This arrangement was chosen in order to counteract the intrusion of saline water via the sluices into the waters further north. The rise in the salinity, which took place in 1969 when the sluice began to operate, is arrested, notably in the region near the sluices. Various river phytoplankters, such as *Asterionella formosa* and *Scenedesmus quadricauda*, are now common in this area. Marine diatoms—*Coscinodiscus concinnus*—brought in from the south are found here now. Marine zooplankton in this area is represented by *Euterpina acutifrons*, larvae of *Polydora* and balanids.

The reactions of the higher plants show that the vegetation on the salt marshes had been able to cope with an increase in tidal water level of 25–30 cm. At the moment a development towards a stable situation can be observed. However, an increase over 50 cm and the concomittant increase of salinity in the soil, proved to have been a most severe shock. On the Weipolderse Gors, for instance, hardly any new vegetation developed after the destruction of the original one.

The reaction of the soft bottom fauna on the increase in salinity in 1969 and 1970 was the subject of an article by Wolff (no. 103, list of publications).

5. Oosterschelde

The annual fluctuations of metabolic elements in the water did not differ from that of previous years.

Owing to a development of the diatom *Coscinodiscus concinnus*, the spring bloom of the phytoplankton was very strong this year and lasted

the whole of April. According to C. Bakker, the reasons were the following. In the first place the average wind velocity during the latter part of March was lower than usual and therefore sedimentation of detritus and suspended inorganic material was very pronounced, creating high values for visibility of the Secchi-disc. The transparency of 5 m measured in this period, was not reached afterwards. In the second place insolation during this critical period was very high. The combination of these two factors caused the diatoms to bloom excessively, as was the case in the North Sea, according to Giekes' studies. The summer bloom was heavy as well, reaching chlorophyll values about as high as those from the spring bloom. Its summit was reached in August and consisted of various diatoms together with the dinoflagellate *Ceratium fusus*. This caused Secchi-disc values to remain below the 3 m level. The zooplankton reacted strongly on the increase of phytoplankton in spring, notably the copepod *Temora longicornis*.

Under supervision of Nienhuis, two students from Leiden University, Miss L. A. van der Lelie and R. P. Marchand, studied the aut- and syn-ecology of two red algae growing in eu- and polyhaline tidal biotopes: *Bostrychia scorpiodes* and *Catenella repens*. Miss van der Lelie's work on *Bostrychia* is finished and the report is in progress, Marchand will continue his work on *Catenella* next year.

Study of the changes in the higher vegetation along the Oosterschelde revealed that the influence of the closure of the Volkerak in 1969—absence of irregular riverwater inflow—is still to be felt. Species such as *Puccinellia maritima* and *Halimione portulacoides* still extend their territory, others—*Armeria maritima* species—lose ground. Noteworthy is the sudden and totally inexplicable decline of *Spartina maritima*. About half or one third of previous year's vegetation is still alive.

Littoral molluscs were sampled on the salt marshes. In order to facilitate sorting the shells a special set of sieves was constructed to be used in a shaker.

The results of several bird census, carried out in previous years, were published by Wolff (no 107, list of publications).

Benthic fishes were collected twice. Interesting finds were: the wrasse *Labrus turdus*, the shanny *Blennius pholis* and the goby *Pomatoschistus pictus*. The latter is a denizen of the coastal seawater and is very seldom encountered in estuaries.

6. Veerse Meer

This year salinity reached the highest values ever recorded since the lake was formed. Although the annual average did not differ much from that of the previous years, fluctuations were larger. These ranged from 12.2 to 14.3⁰/₀₀ Cl' in the north-western part and from 11.3 to 14.8⁰/₀₀ in the eastern part. During spring and early summer oxygen supersaturation rose to 140% at the surface.

The spring bloom of the phytoplankton consisted of diatoms—among which group *Detonula confervacea* deserves special mention—, Cryptophyceae and μ -algae. It was qualitatively richer than in 1972, so it can be concluded that the tendency to increase in number of species along with an increase in salinity, still holds. Owing to the outburst of *Coscinodiscus concinnus* in the Oosterschelde, this diatom was introduced in such large numbers that it was able to maintain itself throughout the whole length of the lake.

The zooplankton showed similar phenomena. Nauplii of the marine copepod *Temora longicornis*, as well as the ciliate *Tintinopsis ventricosa* and *Noctiluca scintillans* could be found now all over the lake. Among the rotifers the poly-till euhalobe species *Synchaeta vorax* superseded *Synchaeta littoralis*, the dominant species at salinities below 10⁰/₀₀ Cl'.

In collaboration with N. de Pauw (Ghent, Belgium) Bakker compared composition, abundance and seasonal periodicity of the phytoplankton of the lake with that of a traject of the Westerschelde with similar salinity, thus separating the influence of salinity per sé from that of other factors such as turbulence and currents. This analysis was the subject of a lecture delivered at the Redeke Symposium of the Hydrobiological Society and will be published in the Bulletin of that society. Bakker and his assistant W. J. Phaff finished their study of *Tintinoidea* and are working on a publication.

Beeftink continued his research on the development of vegetation on the—now permanently dry—Middelplaten. Mrs. I. van Noordwijk, a student from Utrecht University, is analysing the data with computer methods in Utrecht. Spacial variation is also studied and a vegetation map is being constructed with the aid of "false-color" aerial photographs kindly provided by the Division for Environmental Research of the Department of Roads and Waterways.

Miss H. M. Beinema (Utrecht) studied the impact of recreation, cattle grazing, shore erosion and protection, on the shore vegetation.

The barnacle *Balanus amphitrite* extended its territory. Continuing her studies on shipworms, Mrs Borghouts hung wooden blocks in the water in spring. If settling of larvae in the blocks does occur, the adults will mature and reproduction can be expected during spring in 1974. It is her intention to establish the time of reproduction. There has been a fairly heavy settling this year.

Data gathered in previous years on the difference between the population of *Neomysis integer* and that of the same mysid in the inland pool Den Inkel have been analysed, with the result that some—yet unknown—factors are inhibiting the growth of the population at Den Inkel, but are not effective in the Veerse Meer.

The benthic fishfauna did not change much. The black goby, *Gobius niger*, increased in numbers. At the moment five generations are present. With one final observation in January 1974, Vaas hopes to conclude his

analysis of the population of this new addition to the Dutch fishfauna. A publication describing two years of growth and a statistical treatment of the differences in growth in length and weight between the sexes—to be carried out by Vlasblom—is in preparation.

7. *Westerschelde*

The conduit-pipe, flushing the sewage of western Brabant into the Westerschelde at Waarde at the rate of the equivalent of 350.000 inhabitants, became effective in autumn.

Shortly afterwards an extensive sampling program was carried out, organised by A.G.A. Merks. During a tidal cycle chemical parameters, such as visibility, BOD₅, Coli titer, and various nutrients, were measured. The dye Rhomadine B was added to the outflowing sewage and the extension and tidal movement of the coloured sewage was closely followed. An internal report on the results is in preparation, but it can preliminarily be stated that the sewage is quickly diluted by the water of the estuary and dispersed over a large surface. The surrounding shores—and notably those selected for study some years ago—will be influenced by the sewage, specially east of the outlet.

In the brackish traject of the estuary phytoplankton—*Coscinodiscus jonesianus* var. *commutata* and *Biddulphia* spp.—was clearly less well developed in July than in the same month in 1972. When temperature as well as salinity moved up the copepode *Acartia tonsa* came to the fore. Bakker holds grazing by zooplankton responsible for the drop in the development of the phytoplankton, as the numbers of *Acartia* found in 1972 had been definitely lower. The analysis of the data from the past two years once more clearly shows how strongly plankton cycles may differ owing to natural causes, which fact necessitates the use of a series of annual cycles before exogenic influences—pollution—can be safely evaluated.

According to M. C. Daane, plots on the Verdrongen Zwarte Polder where the ground water regime depends on tidal influence, show the lowest ground water levels in June, other plots, under the influence of rain water, do so in August.

In these this level dropped 20 cm below the level of 1972. Extremely high salinities were measured in plots flooded during storms, in low dunes pH values below 7 were sometimes found.

The salt march near Rilland, damaged by an oil spill in May 1972, was practically normal again towards the end of the year.

Mysids were present throughout the whole length of the estuary, except near the Belgian border. In the eastern part numbers were rather low, *Gastrosaccus spinifer* proved to be quite abundant in spite of a rather low percentage of oxygen saturation in the deeper water layers (11–15%).

Littoral fauna in the environment of the sewage conduit did not show any reaction yet, neither did the benthic fish fauna.

8. *Coastal waters and terrains outside the dykes*

When in May a good deal of riverwater was flushed through the sluices in the Haringvliet, many fresh water diatoms, such as *Asterionella formosa*, were found in the coastal water at the Punt van Voorne. The large bloom of *Coscinodiscus concinnus* in the mouth of the Ooster- and Westerschelde, which caused excessive formation of foam at the surface, was less conspicuous in the north. The zooplankton consisted of *Tintinopsis cylindrica*, *Favella serrata* and various harpacticideids.

On the grounds outside the Haringvliet dyke, at the Kwade Hoek, the soil gradually became more silty, because the flow of river water is blocked and floods have become higher. The grounds are longer submerged and less suitable for cattle grazing.

9. *Inland waters and terrestrial vegetation*

Investigations in these waters are carried out by a team supervised by Mrs Borghouts and Dr. S. Parma. The object is to collect and analyse data on future nuclei of dispersion of fresh and brackish water biota and also to know which inland waters merit conservation. Mrs. Borghouts studies the dispersion of mysids in some of these waters.

In cooperation with the Forestry Department and with Vegter, the team started a two weeks sampling program in the creeks of Westkapelle and Canisvliet. The creek at Westkapelle proved to be heavily burdened by sewage and showed thermostratification as well as salt stratification in summer. Both disappeared in autumn. Previously raw sewage entered the Canisvlietse Kreek in Zeeuws Vlaanderen, but in May the sewage was diverted and drained away elsewhere. In September a special investigation was started. The results are being worked out. In a creek near Rammekenshoek a situation was found where a water layer of $2\frac{1}{2}$ -3 meters with a chlorinity of 12⁰/₀₀ floats on a lower layer of 17⁰/₀₀ Cl'. This stable stratification lasts the whole year and sometimes shows an inverse thermostratification. The bottom layers are anaerobic the year round with H₂S-concentrations as high as 600 mg/l.

The students C. M. P. and K. V. Sykora (Nijmegen) finished the report on their investigation of the vegetation of the inland sea-walls on Zuid-Beveland. They found that natural influences such as exposition of the slope and composition of the soil are less important for the development of the vegetation than anthropogenous influences, such as burning, hay making, use of herbicides and deposition of sewage. Also under Beeftink's supervision the students Miss C. J. Smits (Leiden) and Miss A. v. d. Vijver (Utrecht) studied the vegetation of the polders. They finished their field work this year and are working on the reports. Miss van de Vijver will make an effort to construct a botanical evaluation of the entire polder complex on the strength of all work carried out at present. The student R. Kemmers (Utrecht) is engaged in an investigation of the "inlagen" along the borders of the Oosterschelde. "Inlagen", plural of "inlaag",

are brackish, low-lying lands or water basins, excavated in order to build a retired embankment as a second line of defense against the sea. A biological evaluation of these "inlagen" is most useful in view of the decision between damming of the Oosterschelde and reinforcement of the sea-walls (vide introduction).

In his search after saline soils with a low pH, Daane found some in "inlagen" on Schouwen and Zuid-Beveland, usually in spots where seepage from the Oosterschelde was present. The lowest values, mainly found at the depth of 20–30 cm, were situated around pH 3.7 at a salinity of 15‰ NaCl.

Studying the dispersion of chironomids, Mrs Borghouts and Parma together with their assistants, sampled about 40 inland waters. Larvae of the *halophilus* and the *salinarius*-type were common in a broad salinity range, but *salinarius* larvae may be somewhat more tolerant against high salinities. At salinities of a few promilles *plumosus* and *barbipes*-type larvae are also found. The larvae were reared in the laboratory, identification of the adults is in progress.

To study population dynamics of chironomids a creek on Noord-Beveland was selected. In autumn 7000 larvae per m² could be counted, mainly belonging to the *halophilus*-type. Until medio October puppae were found and more than 70% of the larvae belonged to the second and third stage. In spring *Procladius* larvae were seen at a density of 100–200/m², mainly along the shallow borders, being absent in the centre at a depth of 110 cm. *Halophilus*-larvae showed such a wide range of morphological characters that it may be possible that some *thummi*-type larvae are among them.

III. Experimental research

1. Bacteriological research

In continuous culture experiments at low substrate concentrations of various amino acids as sole source of carbon, nitrogen and energy, A. B. J. Sepers isolated various bacterial strains. Substrate specificity and relation between substrate concentration and specific growth rate of these isolates will be investigated. Relevant analytical techniques have been tested. The student J. Rijstenbil (Wageningen) surveyed the literature on quantitative analysis of amino acids. The chemist Ch. Wessels was temporarily attached to the bacteriological department and given the task to investigate quantitatively the mineralisation rate of organic substances in natural waters.

2. Research on plankton

Supervised by Peelen, Rijstenbil also followed the plankton cycle in the Brielse Meer in 10 open plastic containers of 70 l placed on the roof of the institute. His intention was to simulate the large-scale experiment,

carried out by the Division of Environmental Research of the Department of Roads and Waterways in the lake with the object of lowering the phosphate load of the water by flocculation methods. Containers with and without mud from the lake and with various concentrations of phosphate in the water, were used. Thread algae growing attached to the walls, were regularly removed, their phosphate content established and that of the water brought back to its original level by means of addition of phosphate solution. A report is in progress.

The student A. van Noordwijk (Utrecht) carried out cluster- and variance analysis of the data of plankton analyses of the Grevelingen, collected during the past five years. The scattering of the plankton data obtained from repeated sampling on a single station was also calculated. He also gathered some experience with the cultivation of plankton according to the method of Prof. G. Persoone (Ghent). His reports are in progress.

3. *Research on euryhaline algae*

This work, carried out by B. H. H. de Bree under supervision of Nienhuis, was mainly concerned with the green filamentous alga *Rhizoclonium riparium*. The aut- and synecology of this alga is the subject of a thesis by Nienhuis, who finished the manuscript this year.

It was found that clinal variation in morphology, observed in *Rhizoclonium* growing along an environmental gradient from low to high in the littoral zone, can be regarded as modification and can be changed completely in cultures. Culturing totally different *Rhizoclonium* samples the same morphological form could be obtained under certain combinations of environmental factors: 5 or 15° C, liquid or solid culture medium and different combinations of light and dark. At the higher temperature growth is most rapid and morphological characteristics are apt to change fast. Morphology changes faster in liquid media than in agar media and the light/dark rhythm is relatively unimportant.

The efforts to make chromosome portraits of *Rhizoclonium* did not yet meet with complete success, although staining with orcein after treatment with HCl gave some results. The work is being continued. Some culture experiments were carried out in order to establish the tolerance against combinations of salinities in the range of 0.002–100‰ Cl' and acidities of pH 4 to 10. A pH tolerance between 5 and 9 and a chlorinity tolerance between 0.002 and 50‰ Cl' could be established at 15° C, 8 h. light. Beyond these limits the algal filaments died within 20 days.

4. *Influence of various cultural practices on higher vegetation*

Beeftink's experiments on the Stroodorpepolder reached their second growing season this year. It was found that mowing was beneficial to *Trigochin maritima* and harmful to *Puccinellia maritima*. After spraying recolonisation by *Puccinellia* followed very rapidly. Next year a similar

set of experiments will be carried out on the Middelplaten in the Veerse Meer, preparations are carried out now. Here special attention will be paid to nitrogen—and phosphorus balance in the soil.

5. *Influence of temperature and salinity on metabolism of animals*

Vlasblom and his assistants have measured respiration of *Idotea chelipes* in closed vessels in previous years. These measurements were repeated this year with the Gilson respirometer for combinations of 5 and 10° C with chlorinities between 2 and 12‰ Cl'. With *Praunus flexuosus* tolerance experiments were carried out, also at 5 and 10° C with chlorinities between 0 and 20‰ Cl.

The measurements of the osmotic value of the blood of plaice from North Sea, Grevelingen and Veerse Meer, carried out by W. Rozing under supervision of Vlasblom and Vaas, have by now yielded sufficient data as regards the second and third biotope. In 1974 some additional samples from North Sea plaice will be collected with the collaboration of the State Institute for Fisheries Research at IJmuiden. The analysis of the data will begin after this.

6. *Ecology of Chironomid larvae*

Supervised by Mrs Borghouts and Parma the student A. A. Middeldorp (Amsterdam) started an investigation of the repercussions of the existence of a salinity gradient in the mud-water interface on the dispersion of chironomid larvae. He followed the species composition of six selected gradient situations in the field. In the laboratory he observed the burying behaviour and the construction of tubes of the larvae by means of transparent agar and silica substrates. The animals seem to live in water from the mud surface circulating in the tubes owing to the ventilation movements of the larva.

Ch. halophilus fourth instar larvae were reared into adults at 17‰ Cl. These investigations are being continued.

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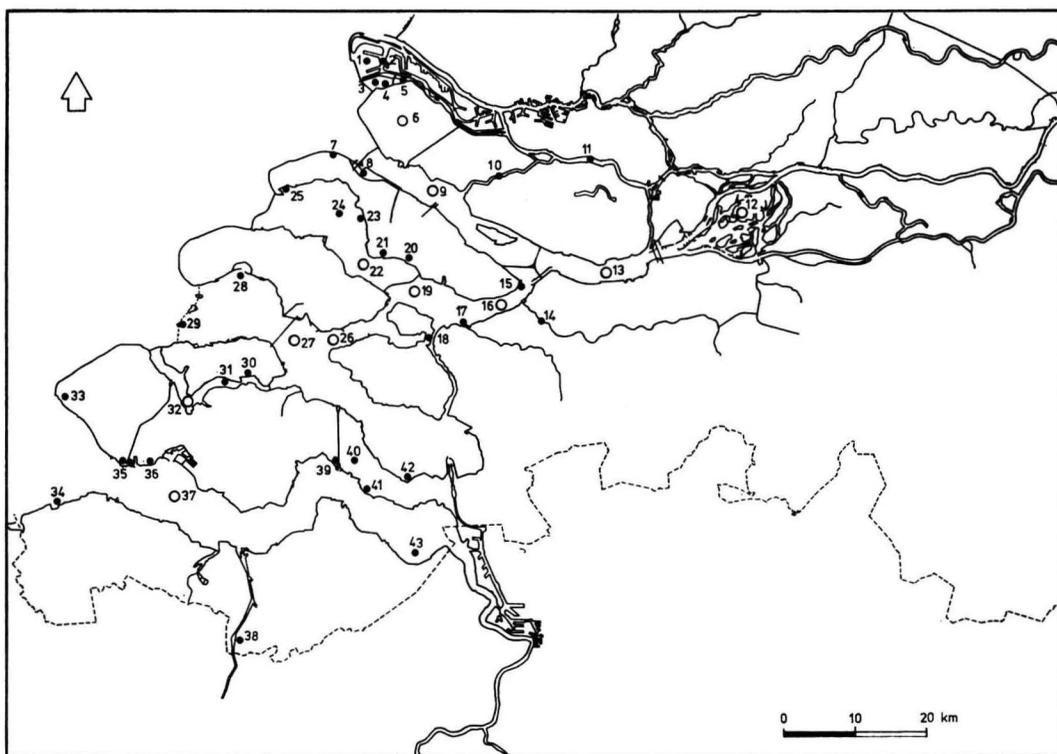
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