REVIEW ARTICLE

Sergei Winogradsky: a founder of modern microbiology and the first microbial ecologist

Martin Dworkin

Department of Microbiology, University of Minnesota, Minneapolis, MN, USA

Correspondence: Martin Dworkin, Department of Microbiology, Medical School, 1460 Mayo Memorial Building, 420 Delaware Ave. S.E. MMC 196, Minneapolis, MN 55455, USA. Tel.: +1 612 624 6190; fax: +1 612 626 0623; e-mail: dworkin@umn.edu

Received 19 May 2011; accepted 4 July 2011. Final version published online 15 August 2011.

DOI: 10.1111/j.1574-6976.2011.00299.x

Editor: David Gutnick

Keywords
Winogradsky; *Beggiatoa*; sulfur oxidation; nitrification; chemolithotrophy; chemoautotrophy.

Abstract

Sergei Winogradsky, was born in Russia in 1856 and was to become a founder of modern microbiology. After his Master's degree work on the nutrition and growth physiology of the yeast *Mycoderma vini* at the University of St. Petersburg, he joined the laboratory of Anton DeBary in Strassburg. There he carried out his studies on the sulfur-oxidizing bacterium *Beggiatoa* which resulted in his formulation of the theory of chemolithotrophy. He then joined the Swiss Polytechnic Institute in Zurich where he did his monumental work on bacterial nitrification. He isolated the first pure cultures of the nitrifying bacteria and confirmed that they carried out the separate steps of the conversion of ammonia to nitrite and of nitrite to nitrate. This led directly to the concept of the cycles of sulfur and nitrogen in Nature. He returned to Russia and there was the first to isolate a free-living dinitrogen-fixing bacterium. In the flush of success, he retired from science and spent 15 years on his familial estate in the Ukraine. The Russian revolution forced him to flee Russia. He joined the Pasteur Institute in Paris where he spent his remaining 24 years initiating and developing the field of microbial ecology. He died in 1953.

Winogradsky's early years

> Those who had the good fortune to come under Winogradsky's influence carried away unforgettable memories of a great mind, of a brilliant personality, of a profound thinker, and of a true experimental scientist. No one ever met him without feeling the presence of a master. He was a Western European in the true sense of the word – a democrat in spirit, although an aristocrat by birth. A man of the land, he was also a scientist, a philosopher, and a brilliant musician.
>
> (Waksman, 1953)

Sergei Winogradsky (Fig. 1) was born in Kiev, the Ukraine on September 1, 1856 and died in Brie, France on February 24, 1953. He grew up surrounded by the ringing bells of the Orthodox church but as an adult 'I simply forgot everything, all mysticism disappeared as I grew older, and childhood tendencies left no trace.' (Waksman, 1953).

The late 1870s saw a profound change in the life and economy of the Ukraine; the cultivation of the sugar beet and the burgeoning commerce of beet sugar transformed the Ukraine and Kiev specifically. Winogradsky's father became the Director of a newly formed bank and the wealth of the Winogradsky family increased immensely. Thus, young Sergei grew up surrounded by wealth and privilege. His early education was in one of the local gymnasia, selected by his father because it taught both Latin and Greek, whereas the other gymnasium taught only Latin. Despite this obvious advantage, young Winogradsky found his classes '…not only uninteresting and unpleasant but depressing, both physically and morally.' (Waksman, 1953). His contempt for his academic experience there was reflected by the fact that immediately upon receiving a gold medal for his scholastic performance he sold it. This was to foreshadow his impatience with the academic and scientific mediocrity he was to encounter often throughout his life and career.

Upon his graduation, he entered the University of Kiev and for 2 years studied Law but predictably found it tedious. He was annoyed and uninterested in the revolutionary activities that were already brewing among the

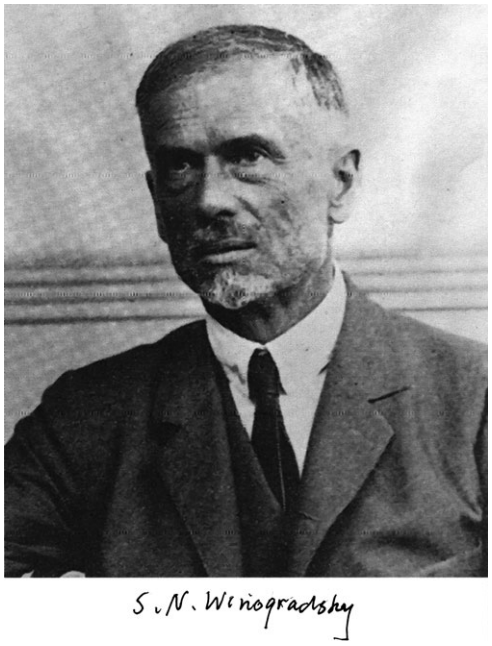

Fig. 1. Autographed photo of S. N. Winogradsky from the L. S. McClung collection at Indiana University.

students. He transferred to the Division of Natural Sciences hoping to find there an outlet for his unformed but powerful creative urges. Once again he was disappointed, this time by the lack of any analytical science and by boring and disorganized classes.

At this point one can almost sense the frustration felt by this brilliant and creative young man as he struggled to establish the trajectory of his life. Abandoning the impulses that seemed to spring from the left side of his brain, young Winogradsky (then 20 years old) shifted to the right side and entered the famed St. Petersburg Academy of Music, to study piano. He came from a musically sympathetic family, had studied piano as a child, and must have been quite talented to have been permitted to study with Theodor Leschetizky, a world class piano teacher and pedagogue. But, alas, after a little more than a year that path too was rejected as he decided 'aesthetic emotions alone, without any activity of the brain were not enough.' (Waksman, 1953). His decision is reminiscent of the career path of another great European biologist, Nobelist Jacques Monod, who also struggled with the decision of whether to follow a musical career as a conductor or to enter science (Judson, 1996). Fortunately both Winogradsky and Monod ended up as microbiologists.

However, St. Petersburg was not Kiev and its university was outstanding. Once again he entered the faculty of the natural sciences but this time was far more fortunate. The faculty included such eminences as Dmitri Mendeleev in chemistry and Elie Metchnikov in biology and Winogradsky was soon convinced that finally he was on the right track. After 3 years of study Winogradsky discovered the laboratory of Andrei Famintsyn, a world famous and charismatic botanist, and chose Plant Physiology as his major. Famintsyn agreed to accept Winogradsky as an undergraduate research student and to serve as Winogradsky's mentor. Upon graduation Winogradsky decided to pursue a Masters degree in Famintsyn's laboratory and, as they say, the rest is history.

Famintsyn had studied with the eminent botanist Anton deBary in Strassburg, a fact that was to become important in Winogradsky's postgraduate work. Upon Famintsyn's return to St. Petersburg from DeBary's laboratory his research focused on the effects of light on the growth of the unicellular alga *Spirogyra* and he used the microscopic and microchemical monitoring of intracellular starch granules as a parameter of algal growth. This experimental approach was later adopted by Winogradsky in his work on *Beggiatoa* in Strassburg, where the appearance and disappearance of intracellular sulfur granules led him to his formulation of the theory of chemolithotrophy and finally of autotrophy.

Although Winogradsky was in a plant physiology laboratory, he was aware of and impressed by the work of such early microbiologists as Ferdinand Cohn and Robert Koch in Germany, but especially by the work of Louis Pasteur in France whose work on fermentation he especially admired. It is fair to say that Pasteur's insistence on factual precision, his incisively logical experimental technique and his ability to draw explicit and persuasive conclusions from his experiments set the standard for all of Winogradsky's career. It is a happy coincidence, or perhaps fated, that the organism Winogradsky was assigned to work on for his Master's degree in Famintsyn's laboratory was *Mycoderma vini. Mycoderma vini* was a plague to the sugar beet industry but also the organism with which Pasteur did the work that culminated in 'Etudes sur la biere', part of the epochal paradigm shift to the concept of a cause and effect relationship between microorganisms and chemical change.

The emphasis of Famintsyn's research was on the relationships between an organism's nutrition and its physiological properties. And the choice of the yeast *M. vini* offered the opportunity for Famintsyn to examine an organism which bridged the gap between plants and animals.

Winogradsky isolated a pure culture of *M. vini* by means of a successive series of dilutions, resulting finally in a single cell. (Robert Koch had only a few years before described his use of solid media to obtain pure cultures. The news had apparently not yet traveled to St. Petersburg). He then proceeded to examine the effects on the

growth of the organisms of various organic and inorganic compounds and of variations in oxygen tension. He did so by means of an ingenious device, used by Pasteur, called a Geissler chamber (Fig. 2) (Pasteur, 1868). The use of these chambers allowed him to maintain the organism for long periods of time as a pure culture, and to add specific nutrients, or to vary the cultural conditions, while observing their effects on the growth and behavior of the yeast. Selman Waksman referred to this work as follows:

> These experiments carried out by the youthful Winogradsky may well be considered as among the first careful investigations ever made on the influence of controlled environment on the growth of microorganisms in pure culture, under well-defined experimental conditions.
>
> (Waksman, 1953).

The work was never published but was presented by Winogradsky at a meeting of the Botanical Section of the University in 1883 and was reported in an abstract in the Botanische Zentralblatt (Winogradsky, 1884). The abstract was written by one of the faculty in attendance, Alexander Borodin, who stated in a footnote 'The writer admired the purity of the cultures exhibited by the speaker in Famintsyn's laboratory.' (It is of interest that Borodin, who was a Professor of Chemistry, was also the composer of the famous Polovotsian Dances).

In 1883, Winogradsky was awarded the degree of Master of Science. He was invited by the faculty to remain at St. Petersburg University to be trained for a Professorship at the University but he declined the invitation. He had never and was never to be impressed with academia. He had other goals in mind and by then had married Zinaida Alexandrovna Tikhotskaya who was to remain his wife and beloved companion until her death 60 years later.

From the beginning, Winogradsky fully accepted Pasteur's conclusion that microorganisms were the cause rather than the effect of chemical changes, and during the early stage of his graduate career he repeated many of Pasteur's experiments. He was impressed by Ferdinand Cohn's careful observations on bacteria and by his insistence that bacteria displayed fixed and stable properties. Robert Koch's demonstration of the techniques and power of pure culture microbiology were *sine qua non*. And Famintsyn's approach of coupling careful microscopic observation with nutritional and physiological experiments became Winogradsky's continuing *modus operandi*. Thus the stage was set for the next and momentous phase of his career.

The climate in St. Petersburg is harsh and was difficult for Zinaida Alexandrovna who was frail. They left St. Petersburg and settled for a while in the sunny Crimea. At the time, Russia was in the throes of reaction against the liberal reforms of Tsar Alexander II who had recently been assassinated. His successor Alexander III was determined to eliminate all revolutionary tendencies, dissenters, Jews, and non-Russian foreigners and to return to a nationalistic slavophilia. This was not an optimal climate for a young iconoclastic scientist. After a year in Crimea, Winogradsky and Zinaida left for more liberal western Europe and in 1885 Winogradsky entered the laboratory of Anton deBary, the famed cryptogamic botanist at the University of Strassburg.

Strassburg, *Beggiatoa* and chemolithotrophy

> Sie haben einen neuen Modus vivendi gefunden
>
> (Anton deBary)

Strassbourg must have been a revelation to the youthful Winogradsky – for its teutonic efficiency, its scientific excellence, personified by deBary, and its role as a scientific center for foreign scientists from all over the world. His stay in Strassburg was the equivalent of today's

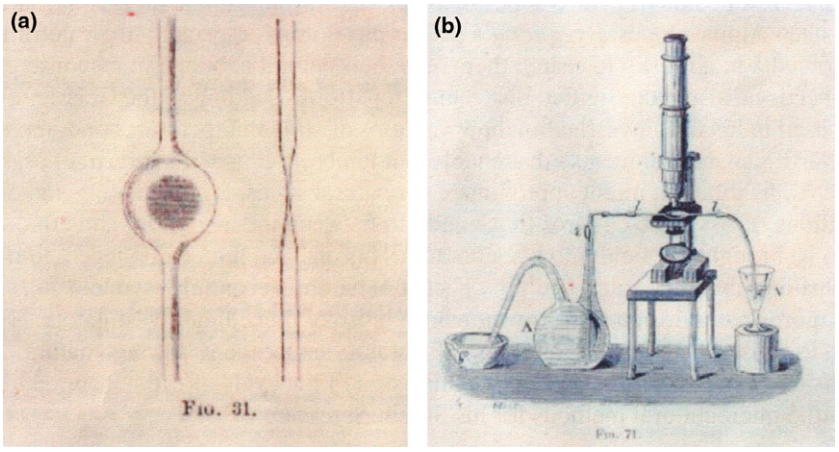

Fig. 2. Geissler chamber (Pasteur, 1868). (a) The chamber is a flattened glass tubing that allows for culture liquids to pass slowly through. (b) Geissler chamber with a microscope.

postdoctoral period, freed as he was from the binding traditions of Russian academia, free of familial obligation, and not burdened with worry, at least for the time being, of having to find an academic position in Russia.

At the time microbiology was struggling to resolve the conflict between the ideas of the monomorphists and the pleomorphists. The classical botanists, totally unfamiliar with the ideosyncracies of bacteria and without a clue as to the extent of bacterial diversity and ubiquity, were bewildered by the kaleidoscopic array of shapes and behaviors present in natural materials. They insisted that there were only a few types of bacteria, which manifested multiple forms determined by a whole series of undetermined variables (e.g. Zopf, 1885). They were, of course, mislead by the common use of mixed cultures.

The monomorphists, led by Ferdinand Cohn, were infatuated with their etiological successes and intrigued by the emerging new discipline of bacterial taxonomy. Any coherent taxonomy depended on stable, fixed forms and the monomorphists were unyielding in their contempt for the chaos of pleomorphism.

DeBary was firmly in the camp of the monomorphists and was eager to have this bright young Russian join his group. Winogradsky was assigned to confirm deBary's position that bacteria manifested fixed, stable, and definable properties and could thus indeed be subjected to a botanical style system of taxonomy. He chose to work on the filamentous bacterium *Beggiatoa* in the hope that a verification of its stable properties would support deBary's position on monomorphism. *Beggiatoa* had already been described and had been shown, when in its natural environment, to accumulate sulfur granules (Cramer, 1870). That interesting and surprising demonstration was confirmed by Ferdinand Cohen in 1875 who also pointed out that similar granules existed in other bacteria whose habitat was sulfur springs and who remarked that this was unique in biology (Cohn, 1875).

Winogradsky could have used any of the existing cultures of *Beggiatoa* in Strassburg but instead, his deep intuitions led him to seek to study the organisms under conditions as close to their natural circumstances as possible. Accordingly, he traveled to sulfur springs in Switzerland and Germany and collected many samples of the white, filamentous masses that grew in the sulfur springs. Upon returning to the laboratory, his attempts to get *Beggiatoa* into pure culture were unsuccessful. That is not surprising, as it was not until the work of Keil in 1912 and Pringsheim (1964) that it was possible to obtain pure cultures of *Beggiatoa*. This led Winogradsky to begin a series of nutritional experiments to determine the optimal growth conditions for *Beggiatoa*. And here his microbiological experience with *Mycoderma* in Famintsyn's laboratory became invaluable. He set up micro cultures similar to the Geissler chambers he had used with *Mycoderma* (Ackert, 2007). The *Beggiatoa* filaments adhered tightly to the sides of the Geissler chambers, allowing him to wash the cells repeatedly with fresh media, thus washing away most of the contaminants. It also allowed him to change the media components while observing the response of the cells microscopically. Winogradsky immediately confirmed that cells freshly retrieved from the sulfur springs were loaded with sulfur granules (Fig. 3) (Winogradsky, 1887; Strohl & Larkin, 1978). This reawakened in him the debate as to the source of the granules and the role of the H_2S in the sulfur springs. This was not the goal that deBary had set for him, but the temptation to understand what role these unusual granules played in the life of *Beggiatoa* must have been irresistible.

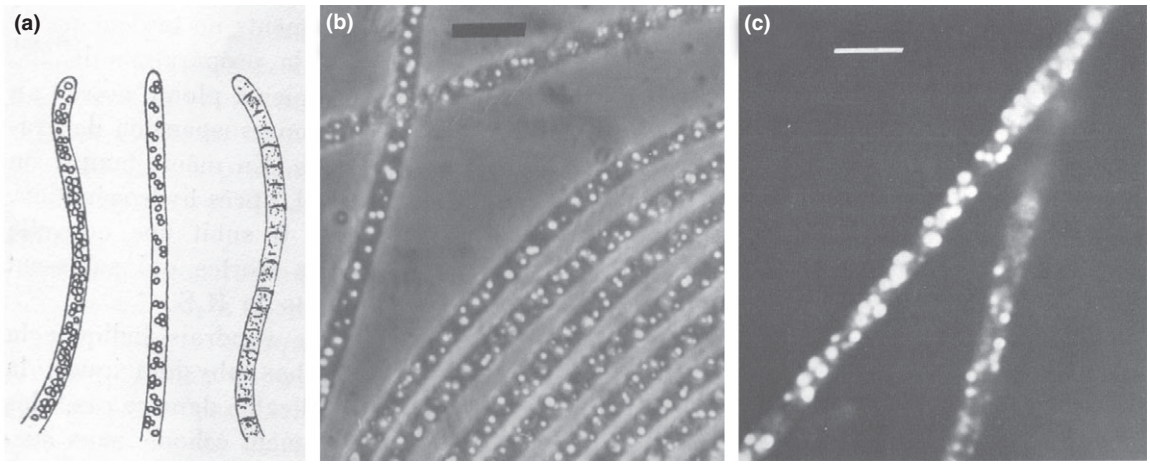

Fig. 3. (a) Winogradsky's drawing of sulfur granules in *Beggiatoa* (Winogradsky, 1887). (b) *Beggiatoa* with sulfur granules by phase contrast. (c) Dark field. Bars: 10 μm (Strohl & Larkin, 1978).

A variety of hypotheses in the literature focused on the fact that the hot springs water contained sulfates. Cohn had pointed out that hot springs water was saturated with H_2S, which was lethal for most other organisms; he suggested that the H_2S was a result of the reduction of sulfates in the springs by sulfur bacteria and that the *Beggiatoa* then detoxified the H_2S by oxidizing it to sulfur (Cohn, 1875). Lothar Meyer (1864) had shown that water from the Landeck hot springs, when incubated for 4 months showed a fourfold increase in H_2S. Plauchud (1882) repeated that experiment and showed that if the water was treated with chloroform or was boiled, H_2S production was essentially eliminated. Thus, it was clear that the process was biological. Etard & Olivier (1882) showed that *Beggiatoa* lost its granules in the absence of sulfates but the granules reappeared when sulfate was reintroduced to the medium. Duclaux (1883) proposed that the sulfur bacteria were indeed reducing the sulfates to H_2S but that some of the sulfate was only partially reduced to sulfur which accumulated in the cells. Alternatively, he preferred the hypothesis that the H_2S was oxidized by the oxygen in the atmosphere and accumulated in the cells.

Thus, the key question that eventually led Winogradsky to his brilliant insight of chemolithotrophy (inorgoxydation) was 'Is the sulfur in the cells of *Beggiatoa* produced by the reduction of sulfate or by the oxidation of H_2S?' (Winogradsky, 1887). He answered the question with a simple, straightforward, and beautiful experiment. He viewed the cells microscopically in the small growth chambers as they were incubated either in spring water containing sulfates or with H_2S. The results were clear. Within 24 h the filaments placed in the presence of H_2S, became stuffed ('vollgestopft') with sulfur granules, while those in the $CaSO_4$ eventually died. Winogradsky concluded 'Development of the sulfur granules occurs only during H_2S oxidation; it is impossible to conclude that the process occurs at one time by the reduction of sulfate and at another by the oxidation of H_2S' (Winogradsky, 1887).

The persistence of hydrogen sulfide in the presence of oxygen, which was required for its oxidation to sulfur was a problem, as the half life of hydrogen sulfide in an aerobic environment at 25 °C is about 1 h (Jorgensen *et al.*, 1978). Thus, *Beggiatoa* must situate itself precisely at the narrow interface where both H_2S and O_2 can coexist. Winogradsky demonstrated this by the following elegantly simple experiment: He used a slide culture with filaments of *Beggiatoa* contained beneath a coverslip. When the medium was devoid of H_2S, the filaments retreated to the center of the coverslip where they clustered in a tight ball. When a dilute solution of H_2S was applied to the edge of the coverslip, the filaments migrated out from the center but stopped about 1 mm from the edge, where they then formed a discrete line of cells. He must have enjoyed doing that experiment as he described almost poetically the oscillating movement of the cells to and fro – 'an exceedingly elegant and animated picture' ('ein äusserst elegantes und belebtes Bild!') (Winogradsky, 1887).

The next question that Winogradsky addressed had to do with the function of the sulfur granules; he mused 'The core of this question was why does *Beggiatoa* need so much sulfur; what meaning does it have for its life processes? … Is it assimilated or excreted? That was for a long time a puzzle for me.' (Winogradsky, 1887). Winogradsky quickly observed that under experimental conditions, healthy cells accumulated sulfur continuously and in their natural environment were filled with it. He then made the key observation that when the cells were deprived of H_2S, within 24 h the granules disappeared. The question then became ' …has the sulfur been dissolved and absorbed and assimilated by the cell or has it been excreted?' He calculated that the volume of sulfur in the cells by far exceeded the volume of cytoplasm and that it was highly unlikely that so much sulfur was needed for the synthesis of the cells' proteins. He then made another wonderful conceptual leap. 'That after the oxidation of H_2S has occurred, the sulfur in the cell is further oxidized to its highest oxidation state, H_2SO_4.' (Winogradsky, 1887).

In his memoirs, in a more informal fashion Winogradsky described the moment that he arrived at this hypothesis. 'Then one day as I was following the Île canal on my way home after a tiring day of chemical work which also involved hydrogen sulfide and sulfur, it suddenly occurred to me that sulfur might be oxidized by *Beggiatoa* to sulfuric acid. I could at once appreciate all the significance and implications of my conjecture, having no doubts that it offered the solution to my problem… The work was humdrum, it dragged on and on sluggishly, and all of a sudden it developed into an interesting result and was finished. All the beating around suddenly made sense, and I matured in my own eyes. Even so, I could not see that my discovery would become an epoch-making discovery, would determine the course of all my future work, and that it would open a new chapter in microbiology and physiology.' (Zavarzin, 1989). What a wonderful description of one of those 'aha!' moments that make scientific research such a unique thrill.

He then proceeded to test this hypothesis by doing a series of careful microchemical determinations on the supernatants of cells that became depleted of their sulfur granules, using the precipitation of barium sulfate as the parameter of the H_2SO_4. The results were conclusive; indeed the cells were excreting sulfuric acid concomitant

with the disappearance of the sulfur granules (Winogradsky, 1887). It is hard to avoid being thrilled by the next sentence. '…the sulfur in these organisms is the sole respiratory source, and in that sense plays the same role as that of carbohydrate in other organisms.' (Winogradsky, 1887). DeBary referred to his student's discovery as follows 'Sie haben einen neuen Modus vivendi gefunden.' (Zavarzin, 1989). And so indeed he had. It was what later became the concept of autotrophy, coming as a result of his work on the nitrifying bacteria; at this moment, however, he had formulated the concept of chemolithotrophy, the ability of bacteria to obtain energy by the oxidation of reduced inorganic compounds such as H_2S, NH_3, Fe^{2+}, and which led eventually to the entire concept of sulfur and nitrogen cycles in Nature.

Zurich and nitrification

> There are few pieces of biological research which can compare with the discovery of the nitrifying bacteria for elegance of method, soundness of reasoning, and daring originality of thought.
>
> (Stanier, 1951)

The year was 1888 and Winogradsky's postgraduate work in deBary's laboratory was essentially finished. It was now time to begin to look for an academic position back in Russia. He traveled to Kiev and to St. Petersburg but was unsuccessful finding a position at either. And upon his return to Strassburg, he discovered that deBary was suffering from a serious cancer of the jaw and was, in fact, to die soon later. It became clear to Winogradsky that the time had come to begin the next phase of his career. This was to focus on the microbiology of the oxidative conversions of reduced nitrogen, *viz.* nitrification.

The story begins with a remarkable French scientist, Jean Jacques Schloesing who is sometimes referred to as one of the founders of soil bacteriology (Doetsch, 1960). In 1868, Schloesing was the director of the École des Tabacs and one of his first papers reported the release of gaseous nitrogen from nitrates in urine and tobacco juice (Schloesing, 1868). Thus began the scientific preoccupation with biological conversions of nitrogen in Nature and in particular the phenomenon of denitrification. Dentrification is the process whereby an anaerobic bacterium can dispose of its metabolically generated protons and electrons by reducing nitrate rather than oxygen, as is the case with aerobic organisms. The products of the reduction, depending on the particular organism, are some form of gaseous nitrogen, such as nitrous oxide or nitrogen gas. In any case, the consequence of this process is the depletion in the soil of valuable nitrate fertilizer. It was obvious that if this reductive process were not counterbalanced by an equivalent oxidative process that returned nitrate to the soil, such a one-way process would eventually be catastrophic. The recognition of the process occurring in the soil created considerable anxiety among agriculturalists – 'A curious reaction known to a few sanitary engineers and fermentation scientists suddenly seemed threatening to the entire agricultural community… The prospect was dismaying.' (Paine, 1981). In addition, nitrate was an indispensable component of gunpowder, a product whose importance had been demonstrated in the recent Napoleonic wars.

In 1861 Louis Pasteur had suggested that the reverse process, that is the oxidative conversion of ammonia to nitrate occurred and was a biological process (Pasteur, 1861). But it remained for Schloesing in 1877, now Professor of agricultural chemistry at the Institut National Agronomique, to obtain experimental proof of this idea in a second paper, whose brevity concealed its profound importance. It was a common practice to use as fertilizer sewer water that had undergone some process of purification. The paper by Schloesing & Muntz (1877) demonstrated conclusively what had been previously suspected; that if the sewer water was allowed to incubate, the organic ammonia in the water was eventually converted to nitrate; and furthermore that the process was biological. Subsequently Gayon and Dupetit (1882) in France presented a series of microbiologically sound laboratory experiments that showed persuasively that the gas-producing, anaerobic reduction of nitrate was catalyzed by bacteria; and here it is important to emphasize the role played by the English chemist Robert Warington whose work set the conceptual and experimental stage for Winogradsky.

Warington had shown that the reverse process, the oxidative conversion of inorganic ammonia was carried out by bacteria. Furthermore, he showed that the conversion was a two-step process comprising the oxidation of ammonia to nitrite and that of nitrite to nitrate. His experiments indicated that these two processes were carried out by two separate organisms but he was unable to isolate them or to demonstrate that persuasively (Warington, 1891). Warington has received short shrift in the history of nitrification, but his work was a precursor to Winogradsky's subsequent research.

Winogradsky chose to pursue his scientific training in Zurich at the Swiss Polytechnic Institute which was famous for its chemistry laboratories. Much of his time there was spent in the laboratory of Ernst Schultz, a noted physical chemist, where Winogradsky learned and perfected the analytical techniques he was to use in his nitrification studies.

He was also eager to refine his techniques for the pure culture isolation and cultivation of bacteria, and spent some time in the laboratory of Otto Roth, a Kochian bacteriologist, for whom Winogradsky had the following words 'The laboratory was as ugly as its boss and it was a branch of the Hygienic Institute. This Roth was running 6-week courses in bacteriology for doctors in the Zurich University, quite similar in program to those arranged in Germany by Robert Koch's school. All he was doing was to repeat his course term after term, with no change, following a syllabus set once and forever. To tell the truth, I have never seen a professor to be such an ass, either before or afterwards.' (Zavarzin, 1989).

Although there is no direct evidence that he chose to go to Zurich with the specific intention of studying nitrification, all of the evidence suggests that that was indeed the case.

And thus began the monumental studies on nitrification that provided clear evidence for the process of bacterial autotrophy and for its role in the cycles of Nature. And in a personal sense, it marked the transition of Winogradsky from a plant physiologist to a microbial ecologist. In fact, Ackert (2004) has pointed out that Winogradsky can be considered not only as the first microbial ecologist, but as the founder of the field.

When Winogradsky began his epochal studies on nitrification (Winogradsky, 1891), he was unaware of Warington's findings that nitrification proceeded in two stages, *viz*., the oxidation of ammonia to nitrite and of nitrite to nitrate, and that these processes were carried out by two separate organisms, or of the paper by Frankland & Frankland (1890) that described having isolated a pure culture of the ammonia oxidizer by a terminal dilution method. He was, however, persuaded that the logic of Koch's postulates required not only establishing a cause and effect relationship between a bacterium and a pathological process, but also, as Pasteur had done with the fermentative yeast, establishing such a relationship between a microbe and a chemical process. At the outset, he was aware of, and agreed with Duclaux (1883) that while the work of Schloesing and Muntz was persuasive, it was necessary that the causative organisms be isolated and shown to be capable in pure culture of carrying out nitrification. Accordingly, he set as his goal the isolation, in pure culture of the nitrifying bacteria, and the demonstration that in pure culture, they could carry out the autotrophic conversions.

He began the first of the historic five papers with a comprehensive review of the literature concerning previous attempts to isolate the nitrifying bacteria (Winogradsky, 1890a), all of which had been unsuccessful. But Winogradsky was confident that he would be successful. 'If there are organisms whose role is exclusively the oxidation of hydrogen sulfide, and others able to oxidize iron salts, one must assume the existence of of special organisms able to oxidize ammonia as a rich source of energy.' (Winogradsky, 1890a). He stated his intention '…to proceed slowly but surely.' (lentement mais sûrement) (Winogradsky, 1890a). His strategy was to optimize conditions for obtaining liquid cultures that could carry out nitrification (with the conversion of ammonium salts to nitrate as the critical parameter), and then to obtain nitrifying colonies on solid media. The final proof would be the ability of isolated colonies when placed back in liquid media to carry out nitrification, i.e. the analogue of Koch's third postulate.

The first part of Winogradsky's studies proceded quite easily. And here, as was the case with his work on *Beggiatoa*, Winogradsky's training in Famintsyn's laboratory, where he was obliged to optimize the nutritional and physiological conditions to grow *M. vini*, was invaluable. He varied the nature and concentration of the ammonium salts, pH, the nature of the inoculum, determined the effect of added organic materials, and finally obtained a medium in which nitrification proceded rapidly. This was, in effect, the invention of the enrichment or elective culture, a technique that has proven to be a powerful tool for the isolation of specific nutritional or physiological types of microorganisms.

However, the next step was not so easy. Repeated attempts to get growth of nitrifying colonies on media solidified with gelatin (the conventional solidifying agent at that time) resulted only in the growth of the organotrophic contaminants, which apparently had found enough organic material in the liquid cultures to survive the repeated subculturing. Winogradsky then rigorously purified his glassware, subjecting them to repeated acid washes, used pure water from Lake Zurich and even incinerated his ammonium salts which, despite the claims of its manufacturer, he found to be contaminated with organic material. Finally, plating on the gelatinized media containing only ammonium salts as the putative source of energy, showed only a single stubborn, persistent colony type (which was not able to carry out nitrification). At this point, Winogradsky's experimental agility kicked in. He performed what he called an inverse elective culture. He had noticed that in the liquid, inorganic salts media, growth took place as a zoogleal mass at the bottom of the flask, where the layer of insoluble magnesium carbonate crystals were coated with a thick layer of bacteria (an early observation of a biofilm). He assumed that these were the sought after nitrifiers. He removed several of these crystals, carefully rinsed them with sterile distilled water and deposited them on the surface of the gelatinized media. (He notes in a footnote that he used the small plates first used by M. Petri). After 6 days of

incubation, he examined the sites on which he had deposited the crystals and picked those sites where there were no colonies growing. These presumably contained the surviving nitrifiers but not the contaminants. These bits of solid media containing no colonies but presumably the nitrifying bacteria were then reinoculated into liquid media whereupon they grew and converted the ammonium salts to nitrate. He then stated triumphantly

> J'avais donc fini par isoler le microbe nitrifiant. ('I have thus finally isolated the nitrifying microbe.')
>
> (Winogradsky, 1890a)

Alas, he had misled himself. As the nitrogen source in the medium was an ammonium salt and as Winogradsky used the diphenylamine test for nitrate as his parameter of nitrification, he undoubtedly did not have a pure culture, but rather one containing both the ammonia and the nitrite oxidizing bacteria. In his retrospective *magnum opus* (Winogradsky, 1949), published 55 years later, he is much more cautious about this matter and does finally refer to all four of Warington's relevant papers which had demonstrated that nitrification proceeded in two steps and that two separate organisms were involved.

But now the work described in the remaining papers in this set of epochal memoires began, rising in a crescendo to the climactic fifth memoire (Winogradsky, 1891), in which Winogradsky finally described the isolation of members of the two separate groups and the demonstration of their ability to carry out specifically ammonia oxidation to nitrite and nitrite oxidation to nitrate.

The second paper of the series (Winogradsky, 1890b) described the organism Winogradsky believed was responsible for the nitrification. At that time, Warington had not yet published his work showing that nitrification proceeded in two steps (that was to happen the following year), so Winogradsky still believed that the nitrifying culture he had obtained consisted of a single nitrifying organism. It was most likely the dominant or perhaps the only microscopically visible organism in the culture, and Winogradsky described it as small, elliptical, and almost spheroid when newly divided. The cells separated after division across the long axis, formed neither filaments nor spores and were motile at some stages of their division cycle. (The Gram stain was not described by Christian Gram until 1894). At the time, any straight, rod-shaped bacterium was named *Bacillus*. But Winogradsky had already introduced the notion of physiological types of bacteria and chose to use that parameter to name and describe the organism rather than its shape. He must have already had an inkling that there were to be many physiological types of bacteria, in contrast to the limited number of possible bacterial shapes. Accordingly, he named it *Nitrosomonas* even though his culture clearly contained both *Nitrosomonas* and what we now classify as *Nitrobacter*. For the rest of the paper. Winogradsky carried out the experiments that allowed him to conclude that the nitrifying bacteria were capable of carrying out what later was to be called autotrophic metabolism. That is, they possessed the ability to grow in the absence of organic carbon and thus were the colorless equivalents of organisms capable of photosynthesis. In view of what we take for granted today, it is hard to imagine what a new paradigm that was. Here was an organism, and perhaps a whole class of organisms, that could substitute the energy derived from oxidizing an inorganic rather than an organic substrate or from the chlorophyll-mediated capture of light energy.

To determine if indeed the organisms were growing and synthesizing their cell material in the absence of added organic material, he proceeded to rigorously exclude any organic material from his media. Accordingly he then used double distilled water, calcined salts and acid-washed glassware. Having done so, he then grew the nitrifying cultures, combusted the total organic material in the culture, collected and measured the CO_2 thus generated, subtracted the amount of CO_2 generated from the uninoculated culture material, and concluded that the data conclusively demonstrated that substantial net organic material had been synthesized. He concluded that a new truth of general physiological importance has been generated, namely '…a complete synthesis of organic material by the action of living organisms has been accomplished on our planet independent of solar energy.' (Winogradsky, 1890b).

The ability of these bacteria to synthesize their cellular components from a medium devoid of any organic carbon had been completely unexpected; the third paper of the series (Winogradsky, 1890c) set out to confirm that conclusion and to refine the measurements. He showed that the production of 1 mg of cellular carbon required the turnover of 35.4 mg of ammonia nitrogen and commented that the slow growth of these organisms must be due to the large amount of substrate that had to be turned over in order for the cell to synthesize its cellular material. In addition, this time, rather than simply measuring the conversion of ammonium to nitrate, he measured both nitrites and nitrates and found unexpectedly that even though nitrate was the end product of nitrification, the amounts of nitrites in the cultures far exceeded the amounts of nitrates. He suggested that this was a result of the fact that some essential component of the process, perhaps oxygen, had been exhausted during the oxidation of ammonia to nitrite. All his experiments designed to test this hypothesis failed to support the notion. At this point he recalled a recent paper of Warington's (1890) which unfortunately he had just

seen, that described nitrite as the end product of his culture of nitrifying bacteria. (Warington was shortly thereafter to report that nitrification proceeded in two steps, ammonia to nitrite and nitrite to nitrate, and that each step was carried out by a different bacterium) (Warington, 1891).

In hindsight, it is obvious that Winogradsky was still working with a bimembered culture making the stoichiometric analysis of the data difficult to interpret accurately. This memoire ends with the comment 'Elles sont profondes et plus compliquées' (Winogradsky, 1890c).

In the fourth memoire Winogradsky sets the stage for the final resolution of the problem of who is doing what in the nitrification process (Winogradsky, 1890d). It seems as if he is now aware that the claim he made in the first memoire that he had isolated a pure culture of the nitrifying bacterium was an oversimplification. He rejects the strategy of dilution as a means of obtaining pure nitrification monocultures (Winogradsky, 1890d); and in reference to his strategy outlined in the first memoire whereby he made transfers back to liquid media from those areas of the gelatin plate that failed to show growth, he now states that such negative evidence does not constitute proof, '… because their inability to grow is not an exclusive property of the nitrobacteria.' (Winogradsky, 1890d). He was convinced that the presence of organic material hindered the growth of the nitrifying bacteria and accordingly selected a method originally described by M. W. Kuhne (1890), namely the use of silicic acid as a gelling agent for preparing solid media. It was a brilliant decision and the results were positive. While Winogradsky observed that non-nitrifying bacterial contaminants also grew on an ammonium-salts medium solidified with the silica gel, they did so as a thin, almost invisible film; the colonies of the putative nitrifiers were identified by picking a small piece of the gel and testing it with diphenylamine, whereupon it immediately gave a positive result. Thus, the same problem emerges as was stated for the first memoire. The medium contained ammonium sulfate as the sole source of nitrogen, not a nitrite salt. How then did a pure colony give rise to nitrate? The material tested must have included both the ammonia and the nitrite oxidizing bacteria.

In the fifth and final memoire of the series (1891), Winogradsky presented convincing evidence that confirmed that nitrification proceeded in two stages, the oxidation of ammonia to nitrite and of nitrite to nitrate. He also showed that the nitrifying bacteria were capable of assimilating carbon in the form of carbonate thus synthesizing cell material in the absence of organic carbon. Thus, to his discovery that the sulfur bacteria were able to derive their energy from the oxidation of inorganic substrates, i.e. lithotrophy, he now added the concept of autotrophy, i.e. the ability of the nitrifying bacteria to synthesize cell material solely from CO_2. And importantly, one sees the beginning of his recognition that what one observes in liquid pure culture in the laboratory needs to be reexamined and reinterpreted if one wishes to understand what is actually happening in soil and in Nature. It is arguably, the beginning of the field of microbial ecology.

He begins the memoire with the comment that chemists consider nitrification as a process that essentially converts ammonia to nitrate. And that in Nature, as in the laboratory one rarely sees the production of nitrite as a part of the process.

In 1890, Winogradsky had set two goals for his work. First, to confirm that the process occurred in two successive steps and was carried out by two separate organisms. Second, to isolate the responsible organisms in pure culture and demonstrate that each was capable of carrying out one and only one of the two conversions.

The first goal was confused by the earlier finding by Warington that, while it was possible to obtain complete conversion of ammonia to nitrate in crude mixed cultures that had been inoculated with soil, repeated subculture eventually resulted in a culture only able to convert ammonia to nitrite. In an indirect way, this was the observation that allowed Warington to conclude that the process occurred in two steps. But it prevented him from obtaining, until later, a culture only able to convert nitrite to nitrate. Winogradsky confirmed this observation but, in his characteristic analytical fashion, extended it by carefully analyzing the dynamics of the appearance of nitrite and nitrate and the disappearance of ammonia as the culture progressed. And he showed that if one was careful, it was possible to demonstrate both processes in culture.

A culture with ammonia as the substrate was inoculated with soil on October 11. By November 3, there were detectable amounts of nitrite. By November 12 the reaction for nitrite was intense and by November 24 had stabilized. By November 20, all the ammonia had disappeared and by December 16, all the nitrite had disappeared and had been replaced by nitrate. Winogradsky concluded that nitrification did indeed involve two steps and that the conversion of nitrite to nitrate did not occur until the original substrate, ammonia, had disappeared.

He then proceeded to streak out from a mixed liquid culture onto silica gel plates and was able to isolate cells that converted ammonia to nitrite. When sterile soil was inoculated with this pure culture, nitrite was produced. Winogradsky then stated cautiously 'We are now at the point of concluding that the causes of the oxidation of ammonia on the one hand and of nitrite on the other are different' (Winogradsky, 1891).

He was able to isolate the nitrite oxidizing bacteria by inoculating an elective culture containing nitrite as the substrate with soil and then carrying out successive subcultures in the same medium. This process excluded the ammonia oxidizer and plating on silica gel plates yielded the nitrite oxidizer which was unable to oxidize ammonia.

In the next section of this fifth memoire, we see the first explicit evidence of Winogradsky as a microbial ecologist, arguably the first ever. He asks whether nitrification in the soil follows the same pattern as nitrification in laboratory cultures with regard to process and products. His experiment was as follows: Two samples of garden soil were prepared, each containing added ammonium sulfate. One was unsterilized and the other sterilized. The sterilized soil was inoculated with the pure culture of the ammonia oxidizing bacterium.

After 10 days of incubation, the unsterilized soil contained considerable nitrate and nitrification was well advanced. Nitrite was barely perceptible. After 3 weeks and then 3 months, there was ample nitrate but no detectible nitrite. In the inoculated soil, there was measurable nitrite at 3 weeks and ample nitrite at 3 months. At no time was there detectable nitrate.

The firm conclusions that emerged from Winogradsky's work were as follows:

(1) Nitrification is indeed a biological process.
(2) It takes place in two steps, the conversion of ammonia to nitrite and of nitrite to nitrate.
(3) Each is carried out by a physiologically specific group of organisms, the exact nature of which may vary from soil to soil.
(4) The dynamics of nitrification and the interactions between the two groups of bacteria differ in soil and in liquid culture. But this is surely a reflection of the physical differences between soil and a laboratory culture.
(5) The process of nitrification is another example of the chemolithotrophy he described for the sulfur bacteria; but in addition, the ability of the organisms to couple the oxidation of an inorganic salt with the fixation of carbon dioxide allows for the growth of the bacteria in a process later to be called autotrophy (Winogradsky, 1891).

Thus ended the first chapter in the story of the biology of nitrification. While Louis Pasteur first suggested that the process was biological, while Schloesing and Muntz first demonstrated experimentally that that was indeed the case and while Warington showed that it consisted of two biochemically separate processes, it remained for Winogradsky to place the phenomenon on a sound scientific basis and, in so doing, to formulate the grand concept of autotrophy and later of cycles of nature. Winogradsky was indeed our first microbial ecologist.

Back to Russia

The idea that there were organisms that contained no chlorophyll but could nevertheless synthesize all their cell material from inorganic components was a new paradigm in biology and swept through the scientific community. Winogradsky named the process 'chemosynthesis' to distinguish it from photosynthesis. It transformed the thinking of agronomists, plant physiologists and microbiologists and resulted in a flood of job offers for Winogradsky. The most attractive of these was personally delivered in 1891 by Elie Metchnikoff as an emissary from Louis Pasteur at the Pasteur Institute. Wingradsky was offered the position of chief of microbiology at the Institute with a laboratory at his disposal. In Winogradsky's own words:

> I was at the point of accepting that offer which was very attractive to me, but another offer reversed my decision. In 1891 another institute in Russia corresponding in its program and goals to the Pasteur Institute was founded by Prince A. d'Oldenbourg and presented by him as the Imperial State Institute of Experimental Medicine at St. Petersburg. I was offered the position of Chief of the General Microbiology Service at that Institute. That presented me with the opportunity to return to my country, whereas to have accepted Pasteur's offer would have obliged me, little by little, to be expatriated, which I did not want. I therefore accepted the offer for St. Petersburg.
>
> (Winogradsky, 1913).

In March 1891, in a characteristic act of courtesy and courtliness, unfortunately no longer common, Winogradsky made a special trip to Paris to notify Pasteur personally of the reasons for his decision. Pasteur repeated the invitation and sought to dissuade Winogradsky who, while impressed by both the spirit and the atmosphere of the Pasteur Institute, would not change his mind.

It is interesting that the Directorship of the Russian Institute had originally been offered to Metchnikov who chose instead to join the Pasteur Institute and who then sought to have Winogradsky join him there. But by then Winogradsky had accepted the position at the Institute in St. Petersburg for which he had been proposed by his old mentor Famintsyn (Zavarzin, 1996).

He remained at the Institute for 15 years, which represented an important second phase in his scientific career. It was characterized by a number of important discoveries, one of which was the first isolation of a free-living nitrogen fixer.

The idea of biological nitrogen fixation was not new. The discovery of biological denitrification, i.e. the

reduction of fixed nitrogen to nitrogen gas, made it clear that there must be an equivalent opposite process that returned nitrogen gas to the soil. The analogous oxidative process had been shown by Winogradsky's work on nitrification. Furthermore in 1888 Hellriegel and Willfarth had described symbiotic nitrogen fixation in leguminous plants and had shown that bacteria were partners in the process (Hellriegel & Willfarth, 1888). Nevertheless, while free-living nitrogen fixation had been suggested by the French chemist Berthelot, it had not been demonstrated experimentally. Winogradsky must have realized that this was an opportunity to take advantage of his increasing expertise at the cultivation of exotic physiological groups of bacteria and proceded to do so. His successful efforts were presented in two short papers to the French Academy of Science (Winogradsky, 1893).

He used the elective culture method that he had invented for isolation of the nitrifying bacteria, consisting of a mineral salts medium, scrupulously devoid of any fixed nitrogen, with glucose as a source of carbon and energy. Thus, obviously, any growth after it was inoculated with soil would require that the microorganisms had used gaseous nitrogen as their nitrogen source. He did indeed obtain considerable growth and identified the dominant organism in the mixed culture as a large, spore-forming rod, accompanied by two other distinctly different species. He determined the amount of nitrogen fixed into the culture and showed that it was correlated with the amount of added glucose and that the organisms produced butyric acid, acetic acid, hydrogen gas, and CO_2.

He recognized the characteristic end products of an anaerobe and used that insight to isolate a pure culture of the nitrogen fixer, which he named *Clostridium pastorianum* (later renamed by others *Clostridium pasteurianum*). He did so by inoculating carrot slices and incubating them under anaerobic conditions.

He then demonstrated that *C. pastorianum* was able to grow as a pure culture in an atmosphere of nitrogen gas but unable to do so under aerobic conditions. And also that the accompanying contaminants were unable to grow in the nitrogen atmosphere but able to do so under aerobic conditions with a source of fixed nitrogen. Then, he wondered why in the initial isolation culture, he had been able to grow both the anaerobic nitrogen fixer and the aerobic contaminants under aerobic conditions. He then placed the *C. pastorianum* in a culture flask with a thin layer of liquid medium and it was unable to grow. He added the two aerobic contaminants and obtained growth of the *C. pastorianum* and the production of the characteristic fermentation products. He concluded that the growth of the aerobes sufficiently reduced the oxygen tension to the point where the *Clostridium* was able to grow anaerobically, which permitted their coexistence in an essentially aerobic soil. One can see the microbial ecologist emerging.

An interesting sidelight: After Winogradsky had presented his first paper, the French chemist Berthelot commented as follows:

> I am happy to have heard the communication by Monsieur Winogradsky; it has not escaped anyone that this presents a great analogy with the methods and results of the memoire which I have read myself at the Academy about 2 months ago. The idea of nitrogen fixation by lower organisms in the soil, an idea that I introduced eight years ago, is developing little by little and the understanding of that mechanism becomes deeper each day.
>
> (Berthelot, 1893).

Obviously priority then was as eagerly sought after as it is today.

That certainly must have rankled Winogradsky, for after it had incubated for over 50 years, Winogradsky responded (1949):

> This claim of priority is characteristic of the manner of the master. Nevertheless, it is difficult to understand on what grounds this argument is based.

He referred to Berthelot's rather vague suggestion that there exist species of bacteria that fix nitrogen, and compared Berthelot's uncritical experiments with his own isolation of a pure culture that demonstrably fixed nitrogen, and concluded:

> It is thus difficult to understand why the discovery of asymbiotic nitrogen fixation is attributed by certain elements in microbiology to Berthelot, even less that it is sufficient to accept peremptorily a result or an idea without valid experimental proof to support it, in order to be recognized as its creator.

While life in St. Petersburg saw the beginning of Winogradsky's more sophisticated ideas on the cycles of Nature and microbial ecology, it was fraught with frustrations and distractions. Prince Oldenbourg had neither the scientific expertise nor the administrative talent to run such an Institute smoothly, a dilemma not uncharacteristic of 19th century Russia, where goals and dreams often outstripped infrastructure and practical reality. And the Russo-Japanese war of 1904–1905 exacerbated the financial problems of the Institute.

Again in Winogradsky's words, a kind of plaint heard often from successful scientists who have been rewarded for their scientific excellence by being removed from it:

> I organized the scientific publications of the Institute and I was Editor and Chief of the Archives of Biological Sciences,

> published in Russian and French. From 1903–1906 I was Director of the Institute. During this period I was not free, as in Strassburg and Zurich to devote my time to scientific research. A large part was always taken by administrative functions, consultations, and other tasks. And outside of the affairs of the Institute I was a member of the Conseil Supérieur Médical de l'Empire, member of the Comité Scientifique du Ministère de l'Agriculture, Président de la Société de Microbiologie, etc., etc.
>
> (Winogradsky, 1913).

In 1898–1899, Winogradsky became ill with nephritis following a bout with influenza. It had already become clear that the severe winters in St. Petersburg were increasingly difficult for both Winogradsky and his wife. In 1899, they spent the winter in the French Riviera. By then, the affairs at the Institute had become more and more burdensome. The opportunity for any personal scientific work became nearly impossible and the bureaucratic atmosphere at the Institute became stifling. Winogradsky had never been adept at political maneuvering and became increasingly uncomfortable with the dominent medical emphasis of the Institute. The Winogradsky family spent more and more time at their estate in Gorodok in the Ukraine and in 1905 Winogradsky resigned as Director of the Institute. He remained, however, as a member of the Institute until 1910 when he left it completely.

Early retirement and back to the farm

Thus, in 1905, Winogradsky then barely 50 years old took early retirement. He returned to his familial estate in Gorodok in the Ukraine, planning to spend the rest of his life as a gentleman farmer or more appropriately as a pomiestchik, a large-scale land owner. He was determined to bring to his estate, and perhaps in a larger sense to Russian agriculture, a progressive, westernized, and scientifically oriented management. Despite the abortive reforms of Tsar Alexander II, Russia was still essentially a feudal society but still struggling with the dreams of Catherine the Great to become a leader among the nations of the world.

He studied the science of forestry and used it to modernize the management of the huge forest on his estate. He planted orchards, started a modern dairy farm, raised horses, and participated in the beet sugar factories and flour mills originally built by his father and still operating on the estate.

He returned to his music and when not working, spent hours playing piano and chamber music duets with his daughter. In the winter, the family would leave for their villa near Clarens in Switzerland. It must have been an idyllic life, but it was rudely changed when war broke out in 1914. For Russia, the war was largely a series of defeats that led to the first revolution of March 1917, when Russia's participation in the war was exchanged for a brutal series of internal struggles between the Bolsheviks on the one hand, and the White Russians, the Germans, the Poles and the Western Allies on the other, culminating in the triumph of the Bolsheviks in the second revolution in November 1917. The land owners, the nobility and many on the losing side, Winogradsky among them, were forced to flee. In 1921 in Odessa, Winogradsky boarded a French warship bound for Marseilles and thence to his villa in Switzerland. It now seemed clear to Winogradsky that his future lay in resuming his career in science and he found his way to Belgrade where the Agricultural Institute of the University was delighted to be able to appoint him to a Professorship. He had received an invitation while he was in Russia but until now had not viewed a move to Yugoslavia enthusiastically.

Upon his arrival in Belgrade, he found to his dismay that the Institute lacked even the most rudimentary facilities that would have allowed him to resume his scientific career. There was no available laboratory or scientific equipment nor even a library containing books and journals. As a result, he was not only unable to resume his experiments but also unable to deliver the series of lectures he had planned to give. Fortunately, however, a copy of the Centralblatt für Bakteriologie 2. Abteilung turned up and Winogradsky was able to review what had happened in the field of bacteriology while he was in Gorodok.

Winogradsky and his wife Zinaida had been separated when he had been forced to flee Russia. By means of a harrowing and difficult trip, she had been spirited out of Russia into Poland and subsequently joined him in Belgrade. Their stay in Belgrade was brief, for fortunately, in February of 1922 a gracious letter arrived from Emil Roux, then Director of the Pasteur Institute.

> My colleagues and I will be very grateful to you if you will come and establish yourself at the Pasteur Institute. You will bring to it your scientific fame and you will be able to pursue there, without being troubled by teaching duties, your magnificent investigations. After Metchnikov, we shall be proud to number Winogradsky among our own. You will be our leader in matters that concern the bacteriology of the soil….
>
> (Winogradsky, 1949).

The Pasteur Institute was then a center of microbiology in Europe and the invitation must have appeared to Winogradsky as a blessing. But also as an ironic postlude to the invitation 30 years ago that he had chosen to reject. Thus began the final period of Winogradsky's life and career, which was to be spent formulating the ideas and precepts of what has come to be known as microbial ecology.

The Pasteur Institute and microbial ecology

Winogradsky's opening salvo came in a paper written in 1923, while he still languished in Belgrade (Winogradsky, 1923). He pointed out that while Pasteur's germ theory of fermentation 50 years ago had had considerable success in the fermentation industry and Koch's work had resulted in giant accomplishments in microbial pathogenesis, soil science had yet to reap its equivalent harvest. Many soil microorganisms had been isolated and grown in pure culture, but neither their actual role in Nature nor their collaborative nor competitive interactions had been studied. He insisted on the notion that '…conditions of pure culture in an artificial environment is never comparable to that in a natural environment', and also that '…one cannot challenge the notion that a microbe cultivated sheltered from any living competitors and luxuriously fed becomes a hot-house culture, and is induced to become in a short period of time a new race that could not be identified with its prototype without special study.' (Winogradsky, 1949).

The laboratory which Winogradsky inhabited for the rest of his life and scientific career was part of the Pasteur Institute located in the small village of Brie-Comte-Robert, about 20 miles from the city of Paris. It was part of an estate of about 12 acres that had been donated to the Institute and comprised a small house that served as Winogradsky's laboratory and a larger house that was the residence for Winogradsky and his family (Fig. 4).

From 1924 until his death in 1953 Winogradsky's efforts focused on microbial ecology.

He was emphatic in his distinction between what he referred to as 'general microbiology' and 'soil microbiology' which he considered as a distinct subdivision of microbiology. He acknowleged the power of pure culture microbiology for understanding the physiological nature and possibilities of bacteria but insisted on its limitations when studying the actualities of their activities in natural environments. An understanding of the actuality of an organism's role in Nature demanded that the organism be studied under conditions as close as possible to its natural environment (Winogradsky, 1931).

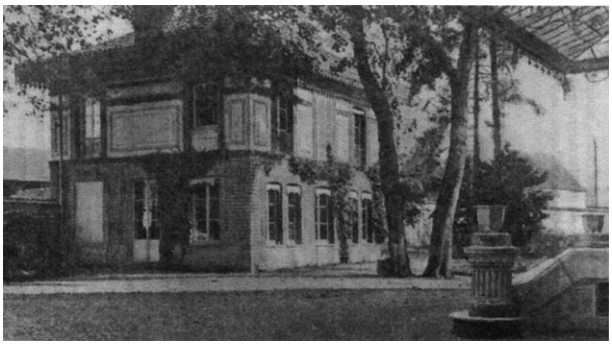

Fig. 4. The laboratory of agricultural microbiology at Brie-Compte-Robert (Ackert, 2007).

Winogradsky developed what he called the 'direct method' for studying the microbiology of the soil. Its basic components were as follows:

(1) Avoid on principle working with stock cultures. Use for experiments strains freshly isolated from the soil by a method as short and direct as possible.
(2) Supply them with nutrients that can be supposed to be utilized by them in the soil.
(3) Make a special point of studying the reactions of the soil population as a whole, as the competition between its components is the principal determinant of their individual functions.
(4) Where solid media was to be used to study the soil population, the use of silica gel media was recommended, to be inoculated with fine particles of soil (Winogradsky, 1923).

Winogradsky used the direct method more as a technique for assessing the agronomic health and potential of a soil, rather than examining the nature of the complex microbial processes occurring in the soil. This involved developing and refining techniques for determining the ability of microorganisms to enhance the various beneficial aspects of the nitrogen cycle. In the early 30s, he began promoting the idea of viewing the soil not as a mass of dead organic and inorganic debris but rather as '…a living environment as a collective entity that posesssed the characteristic functions of a living organism' (Winogradsky, 1931). He viewed the soil as an entity that respired, transformed organic and inorganic molecules and kept the components of the soil in a dynamically healthy balance. Zavarzin (1996) has pointed out how this point of view anticipated the Gaia hypothesis of Lovelock (1989) that 'The biosphere is a huge organism'.

While it was Louis Pasteur who introduced the notion of microorganisms as agents of chemical change, it was Winogradsky who directed that view to the soil. From his discovery of the ability of *Beggiatoa* to oxidatively convert hydrogen sulfide to elemental sulfur and thence to sulfate, and the ability of bacteria to reductively return sulfate to H_2S, the sulfur cycle was born. Pasteur first suggested that nitrification was a biological process and the work of Schloesing and Muntz, Warington, and finally Winogradsky showed that that was indeed the case. The reductive assimilation of dinitrogen gas via nitrogen fixation made it clear that there was a nitrogen cycle. It was thus inevitable that Winogradsky began to see the goals of soil microbiology not merely as the isolation and examination of pure cultures from the soil,

but as a science struggling to deal with the questions of what is actually happening in the complex biological world of the soil itself. His 'direct method' was an attempt to approach that problem. Thus was born microbial ecology.

Wingradsky's legacy to modern microbiology was not only his astonishing discovery of lithotrophy, nor his brilliant clarification of the microbiology of nitrification, nor his isolation of the first free-living nitrogen-fixing bacterium, but in a much larger sense his recognition that if we are to understand the role of the microbe in catalyzing chemical change in complex natural populations, we must study those populations *in situ* or as close as possible to the natural environment, replete as it is with symbiotic and antibiotic interactions.

In an even larger sense, Ackert (2004, 2007) has pointed out that it is important to acknowlege the role that Winogradsky's work played, not only in the birth of the new discipline of microbial ecology, but in the transformation of ecology in general. His work with bacteria helped to insert a rigorous experimental methodology into what had hitherto been a strictly historical, observational, speculative, and hypothetical one.

His elucidation of the role of microorganisms in the natural conversions of nitrogen and sulfur resulted in recognition of the idea that biological materials, both organic and inorganic, underwent cycles of oxidation and reduction. This led inexorably to the notion of cycles of life and to an appreciation of the role of microorganisms in those conversions. Winogradsky's ideas led to the very idea of an 'ecosystem'. It was an intellectual tectonic shift; what Kuhn later called 'revolutionary science' (Kuhn, 1970). (Most beginning microbiology laboratory courses include construction of a 'Winogradsky column' which is, in effect, a microbial ecosystem in a column. (http://ecosystems.mbl.edu/SES/MicrobialMethods/MicrobialBiogeochemistry2010.pdf).

In addition, Winogradsky's work with bacteria inserted an experimental methodology into what had hitherto been a strictly historical one. These new paradigms not only brought a new discipline into biology, but also gave soil biologists a powerful new approach for dealing with the practical aspects of agronomy and soil science.

His work on the direct microscopic examination of soil in a sense was a forerunner of the use of metagenomics to detect and study complex natural populations. He would be delighted if he could see today the attempts to understand the dynamics of the interactions of complex microbial populations by means of increasingly sophisticated systems analysis.

Winogradsky was 100 years ahead of his time. He would be thrilled to see microbiology and microbial ecology today.

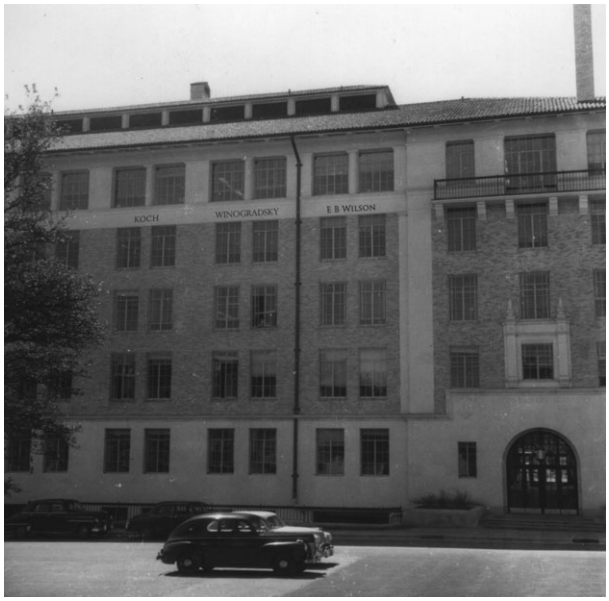

Fig. 5. Experimental Sciences Building, University of Texas, Austin, TX, 1952. Photograph by Martin Dworkin.

Table 1. Curriculum vitae for Sergei N. Winogradsky (Kindly provided by Professor Georgi A. Zavarzin)

1881 – Diplôme d'agrégé from the Université de Saint-Petersbourg
1884 – Doctorat ès sciences botaniques from the Université de Saint-Petersbourg
1891 – Chef de service for Microbiologie générale, Institut impérial de Médicine Expérimentale in St. Petersburg
1892 – Doctorat ès sciences botaniques 'honoris causa', awarded by the Université de Kharkoff
1902 – Director of the Institut impérial de Médicine Expérimentale in St. Petersburg
1922 – Chef de Service of the Laboratoire de Microbiologie Agricole de l'Institut Pasteur at Brie-Comte-Robert (Seine-et-Marne)
1894 – Membre correspondant de l'Academie des Sciences de Russie
1902 – Correspondant de l'Académie des Sciences de Paris
1903 – Membre correspondant de la Société Nationale d'Agronomie de Paris
1924 – Membre d'honneur de l'Académie des Sciences de Russie
1924 – Membre de l'Institut de France, with the title of Associé étranger de l'Académie des Sciences

Postlude

In 1951, The University of Texas was in the final stages of planning for construction of its new Experimental Sciences building. A prominent feature of the building was to be its entablature along the top of the building containing the names of the scientific greats of the various disciplines. A call went out to the faculty asking for nominations. It was required, however, that the names

be of heroes no longer alive. Professor Jackson Foster of the Bacteriology Department proposed the name of Sergei Winogradsky, as one of the giants of microbiology. The suggestion was rejected, as Winogradsky was still alive. Foster countered by pointing out that Winogradsky was at that time 95 years old and would certainly be dead by the time the building was finally completed. It was agreed that this was reasonable; the building went up in 1952 with Winogradsky's name on it. (Fig. 5). Winogradsky was still alive and thanked Foster in his characteristically gracious and humble fashion:

> I can hardly find words to express to you how deeply touched I am by the honour you have done me by including my name in the galaxy of gret(sic) names adorning the cornice of your new building. I will thus have the feeling of presiding in spirit over your work and giving it my scientific blessing.
>
> (Winogradsky, 1952a).

Winogradsky's last paper (Winogradsky, 1952b) appeared in 1952. He died February 24, 1953 (Table 1).

References

Ackert L (2004) *From the Thermodynamics of Life to Ecological Microbiology: Sergei Vinogradskii and the Cycle of Life, 1850–1950*, Chapter 4, PhD Thesis. Johns Hopkins University, Baltimore, MD.

Ackert L (2007) The "Cycle of Life" in Ecology: Sergei Vinogradskii's Soil Microbiology: 1885–1940. *J Hist Biol* **40**: 109–145.

Berthelot M (1893) Comment after Winogradsky's presentation. *C R Acad Sci* **116**: 1388.

Cohn F (1875) Untersuchungen über Bacterien II. *Beiträge zur Biologie der Pflanzen* **1**: 141–207.

Cramer A (1870) Dr. Ch. Müller Chem. Phys. Beschreibung der Thermen von Baden in der Schweiz.

Doetsch RM (1960) *Historical Contribution from 1776 to 1908 by Spallanzani, Schwann, Pasteur, Cohn, Tyndall, Koch, Lister, Schloesing, Burrill, Ehrlich, Winogradsky, Warrington, Beijerinck, Smith, Orla-Jensen*. Rutgers University Press, New Brunswick, NJ, p. 103

Duclaux E (1883) *Chimie Biologique (Microbiologie), Encyclopédie Chimique IX*, p. 719–720, 908 pp.

Etard A & Olivier L (1882) De la réduction des sulfates par les êtres vivants. *C R Acad Sci* **95**: 846–849.

Frankland PF & Frankland GC (1890) The nitrifying process and its specific ferment Part 1. *Phil Trans Royal Soc B* **181**: 107–128.

Gayon V & Dupetit G (1882) Sur la fermentation des nitrates. *C R Acad Sci* **95**: 644–646.

Hellriegel H & Willfarth H (1888) Beilageheft zu der Zeitschrift des Vereins für die Rübenzucker-Industrie des Deutschen Reiches. Nov. 1888, 1–234.

Jorgensen BB, Kuenen JG & Cohen Y (1978) Microbial transformation of sulfur compounds in a stratified lake (Solar Lake, Sinai). *Limnol Oceanogr* **24**: 799–822.

Judson H (1996) *The Eighth Day of Creation. Extended Edition*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Keil F (1912) Beiträge zur Physiologie der farblosen Schwefelbakterien. *Beiträge zur Biologie der Pflanzen (Breslau)* **11**: 335–372.

Kuhn T (1970) *The Structure of Scientific Revolutions*, 2nd edn. Chicago University Press, Chicago, IL.

Kuhne MW (1890) Kieselsäure als Nährboden für Organismen. *Z Biol* **9**: 172–179.

Lovelock JE (1989) Geophysiology, the science of Gaia. *Rev Geophys* **27**: 215–222.

Meyer L (1864) Chemische Untersuchungen der Thermen zu Landeck in der Grafschaft Glatz. *Journal für Praktische Chemie* **91**: 1–14.

Paine W (1981) *Denitrification*, John Wiley and Sons, N.Y.

Pasteur L (1861) Études sur les mycodermes. Rôle de ces plantes dans la fermentation acétique. *C R Acad Sci* **54**: 265–270.

Pasteur L (1868) *Études sur le vinaigre: sa fabrication, ses maladies, moyens de les prevenir: nouvelles observations sur la conservation des vins par la chaleur*. Gauthier-Villars V. Masson et Fils, Paris.

Plauchud ME (1882) Sur la réduction par les sulfuraires et sur la formation des sulfures métalliques naturels. *C R Acad Sci* **95**: 1363–1365.

Pringsheim EG (1964) Heterotrophism and species concept in *Beggiatoa*. *Am J Bot* **51**: 898–913.

Schloesing JJ (1868) Sur la décomposition des nitrates pendant les fermentations. *C R Acad Sci* **66**: 237–239.

Schloesing JJ & Muntz A (1877) Sur la nitrification pas les ferments organisés. *C R Acad Sci* **84**: 301–303.

Stanier RY (1951) The life work of a founder of bacteriology. *Q Rev Biol* **26**: 35–37.

Strohl WR & Larkin JM (1978) Enumeration, isolation and characterization of *Beggiatoa* from freshwater sediments. *Appl Env Microbiol* **36**: 755–770.

Waksman S (1953) *Sergei N. Winogradsky: His Life and Work*. Rutgers University Press, New Brunswick, NJ.

Warington R (1890) Note on the isolation of the nitrifying organism. *Chem News* **51**: 135–136, No. 1582 March 21.

Warington R (1891) On nitrification. Part IV. *J Chem Soc* **59**: 484–529.

Winogradsky S (1884) Ueber die Wirkung äusserer Einflüsse auf die Entwicklung von *Mycoderma vini*. *Bot Zentralbl* **20**: 165–167.

Winogradsky S (1887) Ueber Schwefelbacterien. *Bot Zeit* **45**: 489–610.

Winogradsky S (1890a) Recherches sur les organisms de la nitrification. *Ann Inst Pasteur* **1**: 213–231.

Winogradsky S (1890b) Recherches sur les organisms de la nitrification. *Ann Inst Pasteur* **2**: 257–275.

Winogradsky S (1890c) Recherches sur les organisms de la nitrification. *Ann Inst Pasteur* **3**: 760–777.
Winogradsky S (1890d) Recherches sur les organisms de la nitrification. *Ann Inst Pasteur* **4**: 92–100.
Winogradsky S (1891) Recherches sur les organisms de la nitrification. *Ann Inst Pasteur* **5**: 577–616.
Winogradsky S (1893) Sur l'assimilation de l'azote gazeux de l'atmosphère par les microbes. *C R Acad Sci* **116**: 1385–1388 & **118**: 353–355.
Winogradsky S (1913) Letter from Winogradsky to Hope Sherman, U. of Chicago, Nov.18, 1913; from the Winogradsky Honorary Member File, ASM Archives.
Winogradsky S (1923) Sur la méthode directe dans l'étude microbiologique du sol. *C R Acad Sci* **177**: 1001–1004.
Winogradsky S (1931) *"La biologie du sol" Le Mans*. Imprimerie Monnoyer, Paris.
Winogradsky S (1949) *Microbiologie du Sol. Problèmes et Méthodes*. Masson, Paris.
Winogradsky S (1952a) Letter to Jackson Foster April 27, 1952. Winogradsky Honorary Member File, ASM Archives.
Winogradsky S (1952b) Sur la classification des bactéries. *Ann Inst Pasteur* **82**: 125–131.
Zavarzin GA (1989) Sergei N. Winogradsky and the discovery of chemosynthesis. *Autotrophic Bacteria* (Schlegel GH & Bowien B, eds), p. 23. Science Tech Publishers, Madison, WI & Springer-Verlag, Berlin.
Zavarzin GA (1996) The biosphere: a huge organism. *Herald Russ Acad Sci* **66**: 497–498.
Zopf W (1885) *Die Spaltpilze nach dem neuesten Standpunkte bearbeitet*. Verlag von Eduard Trevendt, Breslau.